The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse

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We generated mice carrying a loss-of-function mutation in Brn-2, a gene encoding a nervous system specific POU transcription factor, by gene targeting in embryonic stem cells. In homozygous mutant embryos, migratory precursor cells for neurons of the paraventricular nuclei (PVN) and the supraoptic nuclei (SO) of the hypothalamus die at ~E12.5. All homozygous mutants suffered mortality within 10 days after birth, possibly because of a complete deficiency of these neurons in the hypothalamus. Although neither developmental nor histological abnormalities were observed in heterozygous mice, the levels of expression of vasopressin and oxytocin in the hypothalamus of these animals were half those of wild-type mice. These results strongly suggest that Brn-2 plays an essential role in the determination and development of the PVN and SO neuronal lineages in the hypothalamus.

[Key Words: POU transcription factor, Brn-2, neuronal development, magnocellular neurons, paraventricular nucleus, supraoptic nucleus]

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Structural analysis of three mammalian transcription factors, Pit-1, Oct-1, and Oct-2, as well as the nematode developmental regulatory gene unc-86, has led to the identification of a conserved DNA-binding motif, termed the POU domain (Bodner et al. 1988; Finney et al. 1988; Ingraham et al. 1988; Müller et al. 1988; Staudt et al. 1988; Sturm et al. 1988b). The POU domain is ~150 amino acid residues long and consists of two highly conserved regions separated by a 15- to 20-amino-acid residue linker region. The amino-terminal conserved region contains ~70 amino acid residues and is called the POU-specific domain. The carboxy-terminal 60 amino acids are also highly conserved, and this region is called the POU homeo domain because its primary structure is highly homologous to the homeo domain of homeotic gene products. The POU domain function is assumed to mediate specific interactions of POU transcription factors with certain DNA motifs, and both the POU-specific and POU homeo domains have been shown to be essential for this binding (Sturm and Herr 1988a; Ingham et al. 1990). Pit-1 encodes a POU transcription factor whose function in mammalian development has been analyzed extensively [Voss and Rosenfeld 1992]. It was cloned initially as a factor that activates the expression of growth hormone in somatotroph cells in the anterior lobe of the pituitary gland (Bodner et al. 1988; Ingraham et al. 1988). Further analysis has shown that the Pit-1 protein is also essential for the expression of prolactin and the growth hormone-releasing factor (GRF) receptor [Nelson et al. 1988; Lin et al. 1992]. Mutations of Pit-1/GRF-1 have been detected in patients suffering from dwarfism and in recessive mutant dwarf mouse strains (Li et al. 1990; Radovick et al. 1992). Interestingly, most of these mutations cause atrophy of the anterior lobe of the pituitary gland (Roux et al. 1982). This observation suggests that Pit-1 regulates the growth as well as the differentiation of committed cells in the developing anterior lobe of the pituitary gland.

More than 20 POU transcription factors have been identified and cloned from several species, including mammals, as a result of extensive screening for novel members. The varied degree of similarities among these factors has made it possible to sort them into six subclasses (Treacy and Rosenfeld 1992, Wegner et al. 1993). In mammals, four closely related POU domain factors, named Brn-1, Brn-2, Brn-4, and Tst-1/Oct-6/SCIP, have been identified and categorized as class III POU domain

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genes (He et al. 1989; Hara et al. 1992). Although the POU domain is the most conserved region of each protein, conservation is observed throughout their primary structures (Hara et al. 1992, Wegner et al. 1993). Among >10 mammalian POU domain factor-encoding genes, only class III genes have no introns in their genomic structure. This suggests that all class III POU domain genes of mammals originated from one ancestral gene. This set of genes is expressed predominantly in the central nervous system (CNS), being widely expressed in the neural tube early in development and subsequently restricted to distinct regions of the brain. In adults, their expression is observed in certain regions of the CNS, including the cortex, thalamus, hypothalamus, brain stem, and cerebellum (He et al. 1989). Furthermore, co-expression of a subset of these genes has been observed in additional discrete regions.

**Brn-2** is expressed in specific regions of the mouse brain including the paraventricular nuclei (PVN) of the hypothalamus, which synthesize the neuropeptide corticotropin-releasing factor (CRF) (He et al. 1989). Brn-2 may bind to and activate the CRF promoter in vitro (Li et al. 1993). It has also been shown that **Brn-2** is required for the in vitro differentiation of P19 embryonal carcinoma cells to neuronal lineages (Fuji and Hamada 1993). Although these data suggest a novel aspect of the Brn-2 gene function, it is clearly impossible to elucidate the whole picture only from indirect evidence such as the pattern of expression, binding activity to some DNA motifs in vitro, or gene activation incultured cells.

In this paper we have generated mice that carry a loss-of-function mutation in **Brn-2** by targeted mutagenesis. Homozygous mutants show a recessive lethal phenotype in the postnatal period with complete penetrance. Homozygous for this mutation were totally deficient for homeostasis in mammals. Heterozygous breeding was set up for FI animals derived from these two clones were crossed to C57BL/6 females, ~50% of F1 offspring with agouti color were phenotypically normal and fertile.

**Results**

**Targeted disruption of Brn-2 and germ-line transmission of the mutated allele**

Genomic clones for mouse genomic **Brn-2** were obtained from a λ phage library constructed from 129/Sv mouse DNA using mouse **Brn-2** cDNA as a probe. Genomic DNA fragments from λ phage clones were used for the construction of a targeting vector. The genomic structure of mouse **Brn-2** is illustrated in Figure 1A. The gene is small and contains only one exon. The targeting vector contained a neo cassette from the plasmid pGEM7[Kjl]R (Rudnicki et al. 1992) for positive selection, and a herpes virus thymidine kinase expression cassette (pGK-TK) for negative selection against random integration of the targeting vector (Mansour et al. 1993). The targeting vector contained 7.5 kb of homologous sequence 5’ and 2.2 kb of homologous sequence 3’ to the neo cassette [Fig. 1A]. This construct deleted a 1.0-kb Ncol-BamHI fragment that contains the open reading frame (ORF) encoding both the POU-specific and POU homeo domains of mouse **Brn-2**. Because this targeting vector deletes ~60% of the carboxy-terminal portion of the **Brn-2** protein, we refer to this mutant allele as **Brn-2**

I1 embryonic stem (ES) cells (Li et al. 1992) were electroporated with the targeting vector linearized at a unique NotI site and subjected to positive−negative selection (Mansour et al. 1993) using G418 and FIAU. Two hundred thirty-eight double resistant ES clones were subcloned and five independently targeted clones were identified after HindIII digestion by Southern blot analysis using a DNA fragment isolated from the 3’ side of homologous sequence in the targeting vector as a probe (probe A; Fig. 1B). Mutant clones were injected into C57BL/6 recipient blastocysts, and male chimeras were crossed to C57BL/6 female. Germ-line transmission of **Brn-2** was obtained for two independently targeted clones, 44 and 225. When founder chimeras obtained from these two clones were crossed with C57BL/6 females, ~50% of F1 offspring with agouti color were found to have the **Brn-2** allele. Southern blot analysis confirmed that the **Brn-2** alleles, which had passed through the germ line, contained the expected mutation as a result of the planned homologous recombination event (Fig. 1C). F1 mice heterozygous for **Brn-2** were phenotypically normal and fertile.

**Brn-2** does not cause embryonic lethality

Heterozygous breeding was set up for **Fi** animals derived from clones 44 and 225 to analyze the effect of **Brn-2** on homozygous mutants. The resulting litters were of normal size, and all the animals appeared normal at birth. Systemic genotyping of **Fi** mice at birth showed a ratio of **Brn-2** genotypes close to the expected 1:2:1 for Mendelian inheritance in offspring derived from clone 44 or 225 [Table 1]. This indicated that **Brn-2** did not cause embryonic lethality. We performed electrophoresis mobility-shift assays (EMSA) to confirm that the **Brn-2** allele did not produce any functional products. Because **Brn-2** is expressed specifically in the CNS, brain homogenates from wild-type, heterozygous, and homozygous mutant mice just after birth were incubated with classic octamer-labeled oligonucleotide probes (Okamoto et al. 1990) to test for the presence of octamer-binding proteins [Fig. 1D]. The results showed that **Brn-2** protein was not present in homogenates obtained from homozygous mutants, and heterozygotes expressed approximately half the amount of **Brn-2** as wild-type mice. The **Brn-2** protein may bind to and activate the 5’ cis-
actin DNA elements of the CRF gene [Li et al. 1993].

EMSA using the oligonucleotides containing binding sequences identified in the 5’ region of CRF gene produced the same results [data not shown]. Therefore, the $Brn-2^{-AC}$ allele did not express any products with octamer-binding activity.

The EMSA revealed an additional interesting finding. In newborn brain homogenates, a $Brn-1$ protein complex

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**Figure 1.** Targeted inactivation of $Brn-2$ in mouse ES cells and generation of mutant mice. **A** Schematic diagram of the strategy used to target $Brn-2$. The wild-type allele of $Brn-2$ (middle) contains only one exon. The protein-coding region ($Brn-2$ ORF) is shown as a stippled box and the regions coding for POU-specific and POU homeo domains are shown as solid boxes. The replacement-type targeting vector (top) was used for electroporation. A neo expression cassette [PGK-neo-pA], including the phosphoglycerate kinase promoter and polyadenylation signals, was inserted into the partially deleted coding region of $Brn-2$. The ES cell line J1 was electroporated with the targeting vector, followed by selection with G418. Resistant clones were expanded and analyzed by Southern blot analysis. Homologous recombination should generate the mutant allele shown at the bottom. Positions as well as orientations of primers used for genotyping wild and mutant alleles by PCR, are shown by arrows (primers 1-3). **B1** Identification of homologous recombinant ES cell clones by Southern blot analysis. Genomic DNA extracted from four positive clones [clones 44, 148, 151, and 225] as well as one negative clone (47) were digested by HindIII and analyzed by Southern blot analysis with a probe obtained from outside of the 3’ homologous region [probe A]. The wild-type allele gives a band of 9.2 kb, whereas the mutated allele yields a band of 3.2 kb. **C** Southern blot analysis of the $Brn-2^{-AC}$ allele transmitted to F1 offspring, obtained by double heterozygous breeding of F1 mice derived from a pair of targeted ES clones [clones 44 and 225]. Genomic DNA samples extracted from skin of newborn mice were digested with HindIII and analyzed by Southern blot analysis. In addition to probe A described in B, a neo probe and probe B that was obtained from the 5’ region of $Brn-2$, were used for hybridization. The wild-type allele gives a band of 9.2 kb, whereas the mutated allele yields bands of 6.0 kb or 3.2 kb. Positive bands with expected sizes are observed in lanes of wild-type (+/ +), heterozygous (+/ -), and homozygous (-/ -) mutants. **D** Expression of octamer binding proteins in the brains of $Brn-2^{-AC}$ mutant mice. Brain homogenates of newborn $Brn-2^{-AC}$ mutants and controls were analyzed by EMSA with a probe containing a classical octamer-binding site. Littermates, obtained from double heterozygous breeding, were sacrificed just after birth and their brains were processed for analysis. Subsequent genotyping showed that there are three homozygous mutants (-/-, 1-3), two heterozygotes (+/-, 4, 5), and two wild-type mice (+/+ , 6, 7) in this litter. As a control for $Brn-2$ protein, a cell lysate obtained from P19 cells treated with retinoic acid was used [P19RA]. A lysate prepared from NIH-3T3 cells transfected with $Brn-1$ expression plasmid, was used as a control for $Brn-1$ protein [3T3-Brn-1]. Positions of free probes as well as the probes forming complexes with Oct-1, Brn-1, and Brn-2, are indicated with arrows. Positions of the bands containing Oct-6 or Brn-4 are indicated with arrowheads on the left (top, Oct-6; bottom, Brn-4).
that migrated slower than the Brn-2 protein complex was also detected, with octamer oligonucleotides and CRF oligonucleotides (Fig. 1D). This complex was identified as Brn-1, as it comigrated with the band generated by NIH-3T3 cells ectopically expressing Brn-1 protein (Fig. 1D). NIH-3T3 cells expressing Brn-4 or Oct-6 generated the bands that migrated faster than that of Brn-2 [data not shown]. The amount of Brn-1 was constant in wild-type mice and heterozygotes of the Brn-2 mutant; however, it is increased by 30% in homozygous mutants. This increase in the level of Brn-1 protein may be a physiological response to the loss of Brn-2 protein in the homozygous mutants and the brown adipose tissues in subcutaneous adipose tissues were totally absent in homozygous mutants (Fig. 2H). These histological findings suggest that surviving homozygous mutants suffer from severe malnutrition. No additional apparent abnormalities were detected on histological analysis of major organs, including the heart, kidneys, spleen, thymus, lungs, intestinal tract, and adrenal glands.

Posterior lobe of the pituitary gland is hypoplastic in homozygous mutants

Because Brn-2 is expressed specifically in the brain, precise histological analyses of the CNS of newborn mice were performed to determine any abnormality specific to homozygous mutants. The most remarkable changes in their brains were observed in the posterior lobe of the pituitary gland (Fig. 2H). The posterior lobe of the pituitary gland, also called the neurohypophysis, is composed predominantly of nonmyelinated nerve fibers of mature neurons. The cell bodies of these neurons reside in the hypothalamus, and their processes extend into the posterior lobe. Those neurites contain the so-called neurohypophyseal peptide hormones, VP and OT.

In homozygous mutants, the posterior lobe of the pituitary gland was found to be extremely hypoplastic, whereas anterior and intermediate lobes were indistinguishable from those of wild-type mice or heterozygotes. Histological analysis of the pituitary glands of 10 homozygous mutants was carried out by sequential sectioning in the coronal or sagittal planes. Results showed hypoplastic posterior lobes in all homozygous mutants but not in five heterozygotes and six wild-type mice examined. The anterior and intermediate lobes of any homozygous mutants showed normal size and morphology. Representative results are shown in Figure 2, G and H. Immunohistochemical analysis of the posterior lobes of any homozygous mutants did not detect any nerve fibers containing VP, which were observed throughout the posterior lobes of wild-type mice in the neonatal period (Fig. 3E,F). The posterior lobe of the pituitary gland also contained some nucleated components, pituicytes, assumed to be supportive cells of a glial nature, and these cells were also present in the hypoplastic posterior lobes of homozygous mutants (Fig. 2H). These observations suggest that the development of neural components in the

Mice homozygous for Brn-2<sup>−/−</sup> die within 10 days after birth

Mice homozygous for Brn-2<sup>−/−</sup> did not show any morphological abnormalities at birth. Their movement was normal and they suckled normally. However, they began to die within several hours after birth and all homozygous mutants died within 10 days after birth. As a result, no homozygous mutant was identified when F<sub>2</sub> offspring were genotyped at 3 weeks of age [Table 1]. More than half of the homozygous mutants died within 2 days after birth, but it was difficult to distinguish them from their littersmates in this period. Homozygous mutants that survived for >2 days showed remarkably retarded growth. Their body weight was about half that of their littersmates at day 4 after birth, but they still moved and suckled normally. Histological analysis showed that subcutaneous adipose tissues were totally absent in homozygous mutants and the brown adipose tissues in their back did not contain any fatty deposition [data not shown]. Although fatty liver was commonly observed in newborn mice of all genotypes, only homozygous mutants retained this feature after 1 week [data not shown]. These histological findings suggest that surviving homozygous mutants derived from the mutant ES cell clone 44.

| Table 1. Genotypes of F<sub>2</sub> offspring obtained by double heterozygous breeding |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Clone | Genotype | | | |
| At birth | wild type | heterozygous | homozygous | Total |
| 44<sup>a</sup> | 55 | 114 | 63 | 232 |
| 225<sup>b</sup> | 11 | 31 | 16 | 58 |
| 3 weeks old | | | | |
| 44<sup>a</sup> | 44 | 104 | 0 | 148 |
| 225<sup>b</sup> | 18 | 29 | 0 | 47 |

<sup>a</sup>Mutant mouse line derived from the mutant ES cell clone 44.
<sup>b</sup>Mutant mouse line derived from the mutant ES cell clone 225.
these neurons was impaired or whether the extension and innervation of neurites into the posterior lobe were affected specifically in homozygous mutants. The cell bodies of these neurons reside mainly in the PVN and SO of the hypothalamus, which are located in the subependymal zone of anterolateral third ventricle and above the proximal optic tract, respectively (Fig. 2C,E). In situ hybridization studies indicated that the PVN and SO express Brn-2 specifically (He et al. 1989). The neurons that produce VP or OT in the PVN and SO have relatively large cell bodies with characteristic features such as eccentric nuclei, prominent nucleoli, and abundant cytoplasm and are termed magnocellular neurons. These neurons extend their neurites through the median eminence of the hypothalamus into the posterior pituitary gland.

Histological analysis showed both the PVN and SO to be missing in the hypothalamus of homozygous mutants (Fig. 2). All neurons that form the PVN and SO, including magnocellular neurons, were lost, and the median eminence of homozygous mutants were remarkably hypoplastic. The anuclear light region development of the median eminence was impaired specifically in homozy-

posterior lobe of the pituitary gland is impaired specifically in Brn-2AC homozygotes.

Magnocellular neurons in the PVN and SO of the hypothalamus are absent in Brn-2AC homozygotes

The neural components of the posterior lobe of the pituitary gland consist predominantly of the neurites of hypothalamic neurons. Further histological analysis was performed to determine whether the development of Figure 2. Pituitary gland and hypothalamic phenotypes of neonatal Brn-2AC mutants. Coronal sections through the anterior region of the hypothalamus (A,B) as well as the pituitary gland (G,H) of a 4-day-old heterozygote (+/-) and a homozygous mutant (-/-) stained with hematoxylin and eosin are shown. The region around PVN, which is marked with box 1 in A and B, is also shown at higher magnification (C,D). The region around the SO nuclei, which is marked with box 2 in A and B, is also shown at higher magnification (E,F). Hypoplasia of the posterior lobe of the pituitary gland (PL) is clearly evident in the homozygous mutant (H). PVN and SO nuclei in the anterior region of the hypothalamus, easily recognized in the heterozygote (A,C,E) and marked with arrows, are missing in the homozygous mutant (B,D,F). H were shot at the same magnification, (A,G) bar, 300 μm; (C,E) bar, 200 μm. (AHA) Anterior hypotalamic area; (SCN) suprachiasmatic nuclei; (V) third ventricle; (OC) optic chiasma; (AL) anterior lobe of the pituitary gland; (PL) posterior lobe of the pituitary gland.

Figure 3. VP-immunoreactive neurons in the hypothalami of Brn-2AC mutants. Coronal sections through the brain of a 4-day-old heterozygote (+/-) and a homozygous mutant (-/-) labeled with antibody to VP. Staining of the PVN (A,B) and the SO (C,D) of hypothalamus, as well as the pituitary gland (E,F) are shown. In the PVN and SO areas of the homozygous mutant (B,D), there are clearly no neurons positive for VP. The posterior lobe of pituitary gland of the homozygous mutant is also negative (F). A,B,E, and F were shot at the same magnification, scale bars in A and E indicate 200 μm. C and D were at the same magnification, scale bar in C indicates 100 μm. (V) Third ventricle; (OC) optic chiasma; (AL) anterior lobe of the pituitary gland; (PL) posterior lobe of the pituitary gland.
Mammalian mutants. This region of median eminence consists predominantly of neurites of hypothalamic neurons including magnocellular neurons.

Magnocellular neurons of newborn mice do not have the characteristic shape found in adults. Immunohistochemical analysis was performed to confirm that there were no magnocellular neurons in the anterior hypothalamic region of homozygous mutants. Analysis of sequential sections with anti-VP or anti-OT antibodies did not detect any positive cells in the PVN and SO regions or positive neurites in the median eminence of homozygous mutants (Fig. 3B,D). The OT-positive cells were not detected in the PVN and SO nuclei of wild-type newborn mice by immunohistochemistry (data not shown). This result suggested that the amount of OT at this stage of development is below the detection limit of immunohistochemistry, which correlates with previous reports (Sinding et al. 1980). Therefore, the expression of the OT gene was analyzed by RNase protection assay. Total RNA was extracted from whole brain of 25 F2 offspring [7 wild-type mice, 12 heterozygotes, and 6 homozygous mutants], and the expression of OT and VP was analyzed by RNase protection assay. A representative result is shown in Figure 4A. Although the expression level of OT was lower than that of VP, as shown by the additional bands of OT probes protected by accidental cross-hybridization (Fig. 4A, arrowheads), the OT gene transcripts were always detected in the brain of wild-type and heterozygous newborn mice. In contrast, we could not detect any expression of OT gene in homozygous mutants even with extended periods of exposure. The data, taken together with those from the analysis on posterior lobe of pituitary gland, indicate that the development of magnocellular neurons of hypothalamus was totally im-

![Figure 4](genesdev.cshlp.org)
paired in homozygotes of the Brn-2AC mutant. Therefore, the Brn-2 gene product is essential for the development of magnocellular neurons in the mouse.

Neuronal precursors die in Brn-2AC homozygotes during migration to the PVN and SO

Homozygous embryos were analyzed further to determine the stage at which the development of the neurons of the PVN and SO was disrupted. Magnocellular neuronal precursors are generated from neuroepithelial cells at embryonic day 10.5 (E10.5) to E12.5, leaving the ventricular region to migrate in a lateral direction. At E14.5, although some cells are still migrating, VP synthesis is initiated. They finally reach their destination at E14.5–E16.5, where they form the PVN and SO [Karim and Sloper 1980; Okamura et al. 1983]. The anterior hypothalamic region of embryos at E14.5 was sectioned, and VP-positive cells migrating in hypothalamus of wild-type embryos were identified at E14.5. However, no neurons positive for VP were detected in the hypothalamus of homozygous embryos at E14.5 [data not shown]. This result suggested that the development of PVN and SO was impaired before E14.5.

Because neuropeptides are not expressed in migrating neurons before E14.5, spot 35 was used as an alternative marker for the neurons of the PVN and SO in development. Spot 35 is a member of the calbindin family, has calcium-binding activity and is closely related to calbindin-D-28k, both structurally and in cellular localization [Yamakuni et al. 1987]. Neurons of the PVN and SO stain positive with anti-calbindin-D-28k antibody [Enderlin et al. 1987]. The pattern of distribution of spot 35-positive cells in the hypothalamus of wild-type embryos at various stages of development was determined. Cells reactive with anti-spot 35 antibody were first detected in the anterior hypothalamus at E11.5 [data not shown]. These formed a cluster of cells that was separated from the ventricular surface. At E12.5, positive cells separated into two groups; one remained medial near the third ventricle and the other moved laterally toward the pial surface [Fig. 5A]. By E14.5, most cells with strong spot 35-immunoreactivity settled in the PVN and SO, although some immunoreactive cells were still moving [Fig. 5E]. Therefore, anti-spot 35 antibody was a good marker for the neurons of PVN and SO through development.

Spot 35-positive cells were observed in the anterior hypothalamus of homozygous mutants at E12.5, but localization was restricted to a region between the PVN and SO, and the number of cells was remarkably decreased compared to wild type [Fig. 5B]. At higher magnification, immunoreactive cells in mutant animals appeared shrunken and lacked bipolar processes typical of wild-type cells [Fig. 5D]. At E14.5, strongly immunoreactive cells were no longer seen in the anterior hypothalamus [Fig. 5F]. Nissl-stained sections further revealed the loss of neuronal mass in the regions corresponding to the PVN and SO at E14.5 [data not shown]. These observations indicate that neuronal precursors for the PVN and SO die at around E12.5 during migration.

Brn-2AC heterozygotes have reduced VP and OT expression

Loss of VP and OT expression correlated with the loss of all magnocellular neurons in homozygous mutants. Heterozygous mutants showed no morphological differences in the hypothalamus to wild-type mice. However, close examination of the RNase protection data in Figure 4 indicates that expression levels of VP and OT in heterozygotes were reduced relative to wild-type animals. To confirm these results, the expression of VP and OT was determined by RNase protection assay in wild-type and heterozygous mice. Expression levels were calculated as ratios to β-actin gene expression, and the results varied from mouse to mouse within each group, as summarized in Figure 4B. This might have been attributable to the outbred background of the F2 mice or subtle differences in physiological conditions such as dehydration when they were sacrificed. Nevertheless, the results show clearly that the levels of VP and OT mRNA in heterozygotes were reduced by less than half the levels in wild-type mice. These results correlate with the reduction in expression levels of Brn-2 gene product in the heterozygous mice relative to wild type detected by EMSA [see Fig. 1D]. Brn-2 may also function to regulate VP and OT expression in the magnocellular neurons of adult mice.

Brn-2AC homozygotes lack parvocellular neurons of the PVN

Although the SO of the hypothalamus consists predominantly of magnocellular neurons, the PVN also contains parvocellular neurons that synthesize and secrete CRF and thyrotropin-releasing hormone (TRH). The neurites of parvocellular neurons have their axon terminals in the median eminence and release CRF and TRH into perivascular space there. In turn, these regulate the synthesis and release of adrenocorticotropic hormone [ACTH] and thyroid stimulating hormone [TSH] in the anterior lobe of the pituitary gland.

Histology suggested that the parvocellular neurons are also absent in homozygous mutants [see Fig. 2D]. To confirm this, an immunohistochemical analysis of the median eminence was performed with anti-CRF and anti-TRH antibodies [Fig. 6]. Axons stained positive for CRF and TRH in wild-type animals, although TRH staining was very weak at this stage of development as indicated by arrowheads in Figure 6E. In homozygous mutants, in turn, no neurites in the median eminence were positive for CRF or TRH [Fig. 6D,F]. The development of parvocellular neurons that produce CRF or TRH in the PVN was also disturbed in homozygotes of the Brn-2AC mutants, indicating that the Brn-2 gene product regulates the development of the PVN of hypothalamus.
Figure 5. spot 35 immunoreactive neurons in the hypothalami of Brn-2AC mutant embryos. Coronal sections through the hypothalami of heterozygous (+/-) and homozygous (-/-) embryos at E12.5 (A,B) or E14.5 (E,F) were labeled with anti-spot 35 antibody. The regions boxed in A and B are also shown at higher magnification in C and D, respectively. In the E12.5 embryo heterozygous for Brn-2AC mutation, migrating spot 35-positive cells, which are in a pair of cell clusters, are observed (arrowheads in A). The cells have a bipolar shape with two processes extending from their poles as shown at higher magnification (C). Migrating spot 35-positive cells are also identified in the homozygous mutant embryo at E12.5 (arrowhead in B). However, their number is clearly smaller than in the heterozygote and they appear shrunken and the bipolar processes are not clear. In the heterozygote at E14.5, cells positive for spot 35 are already close to the PVN and SO regions (E). On the other hand, spot 35-positive cells have disappeared in the homozygous mutant at E14.5 (F).

Somatostatin-producing cells in the periventricular region of the hypothalamus are absent in Brn-2AC homozygotes

In situ hybridization studies indicated that Brn-2 is also expressed in the periventricular (PEV) region of the hypothalamus of normal mice (data not shown). The PEV region contains parvocellular neurons that express somatostatin (SS). Immunohistochemistry using antisera against SS revealed that axons positive for SS, present in wild-type newborn mice, were absent in homozygous mutants [Fig. 6G,H]. In addition, Bnn-2AC homozygous mutants lacked somatostatinergic neurons in the PEV.

Immunohistochemistry using anti-tyrosine-hydroxylase (TH) antibodies showed that the dopamine-producing neurons in the PEV had developed normally in homozygous mutants [data not shown]. The development of SS-producing neurons was impaired specifically in the PEV region of homozygous Brn-2AC mutants.

Parvocellular neurons innervating the median eminence and secreting distinct neuropeptides, such as GRF or luteinizing hormone-releasing hormone [LHRH], were found to develop normally in Brn-2AC homozygous mutants by application of respective antibodies [data not shown]. The effects of the Brn-2AC mutation on the development of hypothalamic neurons are summarized in Figure 7. Brn-2 is essential for the development of the neurons that compose the PVN and SO, as well as somatostatinergic parvocellular neurons in the PEV. This phenotype correlates well with the expression pattern of Brn-2 in the developing CNS, which was restricted to the PVN, SO, and PEV in the anterior hypothalamic region of mice [Alvarez-Bolado et al. 1995].

Discussion

The present study has demonstrated that Brn-2 is essential for the development and the mature function of specific hypothalamic neurons in mice. Mice homozygous for a mutation in the Brn-2 gene die within 10 days after birth. In these mice, the development of magnocellular as well as parvocellular neurons of the PVN and SO is disturbed at ~E12.5 during migration, resulting in mice
assumed that the gene product may support the survival of migrating neurons by activating or inactivating the expression of target genes in those neuronal precursors. Although results have shown that Brn-2 may regulate VP and OT expression, it is unlikely that the expression of these genes is essential for the survival of neuronal precursors at E12.5. As shown previously, neither VP nor OT is expressed in the neuronal precursors of wild-type mice at E12.5. Other potential target genes of Brn-2, which are essential for the survival of migrating neurons, might include genes encoding a receptor for neurotrophic factors or a protein with anti-apoptotic activity.

During the development of neurons in the PVN and SO, Brn-2 expression can be detected first in the neuroepithelial cells at E10.5 and it is observed continuously in migrating neuronal precursors as well as in mature neurons in these sites by in situ hybridization [Alvarez-Bolado et al. 1995]. However, our data suggest that the Brn-2 gene product first becomes essential at E12.5 for the development of neurons. This discrepancy may be explained by assuming some degree of functional redundancy between the class III POU factors. Among four members of class III POU factors, three of them (Brn-1, Brn-2, and Brn-4) show very similar patterns of expression in development and in adults [Treacy and Rosenfeld 1992]. Importantly, the expression pattern of Brn-1 is almost identical to that of Brn-2. DNA-binding specificity of these factors is probably determined by the structure of the POU domains that shows only one amino acid substitution between Brn-1 and Brn-2 [Hara et al. 1992]. Potentially, Brn-1 may compensate for the loss of the Brn-2 gene product in neuroepithelial cells of homozygous mutant embryos at E10.5–E12.5 by activating

![Figure 6. Immunoreactivity for hypothalamic neuropeptides in the median eminence of newborn Brtn-2ac mutant mice. Sections through the median eminence of a 4-day-old heterozygote (+/−) and a homozygous mutant (−/−) were labeled with anti-VP antibody (A,B), anti-rat CRF antibody (C,D), anti-TRH antibody (E,F), and anti-SS antibody (G,H). The specificities of the anti-rat CRF, anti-TRH, and anti-SS antibodies have been described previously [Kawano et al. 1982; Daikoku et al. 1984; Hisano et al. 1993]. Vasopressinergic magnocellular neurons in the PVN and SO extend their axons through the median eminence into the posterior pituitary gland. Parvocellular neurons synthesizing CRF and TRH in the PEV and those synthesizing SS in the PEV innervate the median eminence. The median eminence of the heterozygote is strongly positive for VP, CRF, TRH, and SS (A,C,E,G). On the other hand, no signals for VP, CRF, TRH, and SS are visible in the median eminence of homozygous mutant (B,D,F,H). (A–H) Same magnification; scale bar in A indicates 100 μm.

![Figure 7. Developmental defects of magnocellular and parvocellular neurons in the hypothalamus of Brtn-2ac homozygous mutants. Schematic view of the magnocellular and parvocellular neurons of the hypothalamus. The locations of cell bodies and axons of those neurons are shown in the plane that may appear in sagittal sections through the hypothalamus and pituitary gland of mice. The neurons indicated with an open-circle cell body and a dashed line axon are those whose development was found impaired in Brtn-2ac mutants. Those with a solid-circle cell body and a continuous line axon are neurons whose development was not affected by the Brtn-2ac mutation. (p and m) Parvocellular and magnocellular regions of PVN, (POA) preoptic area, (ARN) arcuate nucleus, (OC) optic chiasma, (ME) median eminence, (AL, IL, and PL) anterior, intermediate, and posterior lobe of the pituitary gland, (LHRH) luteinizing hormone-releasing hormone, (DA) dopamine.

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common target genes. However, the results show clearly that there is no functional redundancy between Brn-1 and Brn-2 in migrating neurons from E12.5. Specificity may be mediated by cofactors that interact only with the Brn-2 or the Brn-1 gene products. A cofactor for the POU protein Oct-2 has been shown to be essential for the development of B cells [Strubin et al. 1995]. The result of in vitro experiments, which showed an important role for Brn-2 in neuronal differentiation, might also suggest the presence of a cofactor for the Brn-2 gene product. In vitro, the expression of Brn-2 is induced strongly during neuronal differentiation of P19 cells treated with retinoic acid and this differentiation is not observed when the induction of Brn-2 is blocked by the expression of antisense RNA. Therefore, it was concluded that expression of Brn-2 is an essential factor in this system [Fujii and Hamada 1993]. However, neuronal differentiation cannot be achieved by transfection of P19 cells with Brn-2 expression vector alone. Expression of Brn-2 may not be enough to drive neuronal differentiation, a process that may require some other genes also activated by retinoic acid treatment and necessary for Brn-2 specificity.

The present findings in heterozygous mutants, regarding the regulation of VP and OT expression at the transcriptional level, are interesting in view of the close link between these two genes in all mammalian species examined [Ivell and Richter 1984; Sausville et al. 1985; Hara et al. 1990]. In the mouse, the VP and OT genes are linked tail to tail, separated by a short 3.5-kb intergenic sequence and share a similar three-exon structure [Hara et al. 1990]. Expression analysis in the rat showed both to be induced by osmotic challenge in vivo [Majzoub et al. 1983; Van et al. 1987], suggesting the presence of a common mechanism of transcriptional regulation that might involve Brn-2. Of course, as VP and OT are expressed generally in mutually exclusive sets of neurons in the PVN and SO [Mohr et al. 1988], there must also be distinct mechanisms that regulate the expression of each gene in a specific manner.

A specific cause for the postnatal lethality of the Brn-2AC mutation is not apparent. Disruption of the specific cell lineages described in this report may disturb both the hypothalamo–hypophysial neuroendocrine system (loss of magnocellular neurons) and the functional regulation of the anterior lobe of the pituitary gland (loss of the neuropeptide-secreting parvocellular neurons of the PVN and PEV). Mutant mice lacking a CRF gene have been established by targeted mutagenesis [Muglia et al. 1995] and although an impaired, sexually dimorphic adrenal response to stress was observed, homozygous mutants for CRF gene are healthy and exhibit no obvious phenotype during development. A rat mutant, named the Brattleboro, which carries a mutation in the VP gene and exhibits a phenotype of hypothalamic diabetes insipidus, remains viable when homozygous [Schmale and Richter 1984]. Postnatal death in Brn-2AC homozygotes may be attributable to a combination of defects observed, or alternatively, may arise from as yet unidentified subtle deficiency.

Therefore, the Brn-2 may play an essential role in the determination and development of specific cell lineages of hypothalamic nuclei, and in addition, may regulate cell-specific expression of the VP and OT genes in mature neurons. Interbreeding of mice with targeted mutations in the class III POU genes may reveal a new level of regulation of cell fate and development in the brain from interactions between Brn-2 and other POU genes.

Materials and methods

Targeting vector construction

To construct the targeting vector, we used λ phage clones obtained from a 129/Sv mouse genomic library. The genomic organization of the mouse Brn-2 gene, determined by nucleotide sequencing, agreed with the results reported by another group after the initiation of these studies [Hara et al. 1992]. The plasmid pGKneo–NotI was constructed by inserting a NotI linker into an AatII site of the plasmid pGEM7(KI)R [Rudnicki et al. 1992]. The 7.5-kb HindIII–NotI fragment from the 5' region of Brn-2 was subcloned into a unique EcoRI site of pGKneo–NotI and this construct was named p5-neo–Brn-2. The 2.2-kb BamHI–SacI fragment from the 3' region of Brn-2 was subcloned into a Xhol site of pGK-TK [Rudnicki et al. 1992] and this construct was named p3-TK–Brn-2. In the process of this subcloning, the Xhol–BamHI fragment from multicloning sites of pBluescript II KS(+) (+), which contained HindIII and EcoRI sites, was added to the 5' end of the 2.2-kb fragment. Then a Xhol–SalI fragment of p3-TK–Brn-2, which contained the 3' homologous region and the TK cassette, was isolated and introduced into a SalI site of p5-neo–Brn-2, which is located at the 3' end of the neo cassette. The construction of the targeting vector was thus completed. For electroporation, the construct was linearized at a NotI site located at the 5' end of the 5' homologous fragment.

Electroporation, selection, and screening of ES cells

11 ES cells [Li et al. 1992] at passages 7–8 were electroporated with 20 μg of linearized targeting vector DNA using a Bio-Rad Gene Pulser (25 μF, 400 V). The electroporated cells then were cultured on feeder cells, which were prepared from G418 resistant primary embryonic fibroblasts as described earlier [Rudnicki et al. 1992], and selected for resistance to G418 (200 μg/ml) for 7 to 10 days. FIAU (200 nm) was also added to the selection medium for the initial 2 days of selection. Double-resistant ES colonies were picked up and subcultured in a well of 24-well plates. When ES subclones became semiconfluent in the well, two thirds of each were frozen as stock and one-third passed for Southern blot analysis. DNA samples were obtained by the method described previously [Laird et al. 1991]. For screening of homologous recombinants, DNA samples [15 μg] were digested with HindIII, fractionated on a 0.7% agarose gel, transferred to Duralon-UV membrane (Stratagene), and hybridized with probe A.

Blastocyst injection, animal breeding, and genotyping

Frozen stocks of ES cell clones, identified as positive by Southern blot analysis, were thawed and expanded on primary embryonic fibroblast feeder cells for injection and DNA isolation. Mutant ES cells were trypsinized, centrifuged, and resuspended in injection medium (Dulbecco's modified Eagle medium containing 10 mM HEPES buffer and 10% fetal bovine serum). ES cells [14–17] were injected into blastocoeal cavities of 3.5-day
postcoitum (dpc) blastocysts from C57BL/6J mice. Injected blastocysts were surgically transferred into the uteri of pseudopregnant MCH1ICR) recipients at 2.5 dpc. Male chimeras with extensive ES cell contributions to their coats were bred with C57BL/6J female mice and germ-line transmission of the dominant agouti coat color marker was observed. Germ-line transmission of the Brn-2 allele was screened by Southern blot analysis and heterozygous F1 animals were intercrossed. Genotyping of embryos and postnatal animals (P0—P12) was performed by the PCR method. Two primers, primer 1 and 2 in Figure 1, were synthesized and used for amplification of wild-type allele of Brn-2. Nucleotide sequences of primers 1 and 2, respectively, correspond to nucleotides 2304–2328 and nucleotides 2684–2660 of the Brn-2 sequence in GenBank (accession no. M88300). The primer 1 is in the sense orientation and primer 2, whose sequence is not present in the mutated allele, is in the anti-sense orientation. The primer 3 (5'-GCTAAAGCGCATGCTCCAGACTGCCTTG-3') in Figure 1, which anneals to the enhancer region of PGK gene, was also synthesized and used for amplification of the Brn-2* allele in combination with primer 1.

Histology and immunocytochemistry

For histology, anesthetized animals were perfused with Bouin's solution. After fixation for 3 days at 4°C, organs were removed, processed for wax embedding, sectioned at 8 μm (brain) or at 4 μm (other tissues), and stained with hematoxylin and cosin. For immunocytochemistry the heads of embryos and newborn animals were fixed in ice-cold fixative consisting of 4% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (pH 7.4). For immunohistochemistry of TRH, the brain was fixed with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde. The tissue blocks were cryoprotected in 20% sucrose until they sank, and frozen with crushed dry ice. Coronal cryostat sections through the hypothalamus were cut serially at 10-μm thickness, air dried, and stored in a refrigerator. The sections were pretreated with 3% hydrogen peroxide for 15 min at room temperature, rinsed in phosphate-buffered saline (PBS) and incubated at 4°C overnight with primary antibodies diluted with 0.5% nonfat milk. After rinsing in PBS, sections were incubated with the horseradish peroxidase-labeled Fab' fragment of anti-rabbit IgG (1:100) at 37°C for 1 hr. Visualization of immunoreactions was performed in 0.05 M Tris buffer (pH 7.6), containing 0.1% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide at 37°C for 5–15 min. Antibodies used and their respective dilutions were as follows: rabbit anti-VP (1:5000), Hisano et al. 1985), rabbit anti-rat CRF (1:5000, Daikoku et al. 1984), rabbit anti-TRH (1:5000, Hisano et al. 1993), rabbit anti-SS (1:2000, Kawano et al. 1982), rabbit anti-LHRH (1:1000, Daikoku et al. 1978), rabbit anti-bovine TH (1:2000, Nagatsu 1983), rabbit anti-spot 35 (1:1000, Yamakuni et al. 1984).

EMSA

To prepare samples for EMSA, whole brains of newborn animals were surgically removed, rinsed with ice-cold PBS, and sonicated in 100 μl of Schreiber's buffer C [400 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 1 mM DTT] containing proteinase inhibitors [1 mM PMSF, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 1 μg/ml of aprotinin (Schreiber et al. 1989)]. Sonication was carried out using a Branson sonicator with micropipette twice (10 sec each). Samples were then centrifuged at 15,000 rpm for 10 min and the supernatants were used for binding reactions. For positive controls, extracts of cultured cells were also prepared in the same manner. To obtain cells expressing Brn-2 predominantly, cultured P19 cells were treated with 0.3 μM retinoic acid for 3 days on bacterial petri dishes and processed for samples. To express Brn-1 in cultured cells, mouse Brn-1 cDNA was cloned and introduced into the plasmid pCAGGS (Niwa et al. 1991), which contains a cytomegalovirus enhancer, a chicken β-actin promoter, and a poly(A) addition signal. Twenty micrograms of this construct, named pCAGGS–Brn-1, were transfected into NIH-3T3 cells by the calcium phosphate coprecipitation method and cells were collected after 48 hr. Two double-stranded oligonucleotides (26-mers) were synthesized, annealed by denaturation and renaturation, labeled by end-filling with [α-32P]dCTP, and used as probes for EMSA. The OCTA26 probe (GGTCAGACTATTTGTTAAGA) contained classic octamer-binding sequences (Okamoto et al. 1990). The CRH26 probe contained Brn-2-binding sequences, which were first identified in the promoter region of the rat CRF gene (Mathis et al. 1992). Binding reactions and electrophoresis were performed as described previously (Schöler et al. 1989). In brief, 10 μg of extract and 0.1 ng of labeled probe were added to 17 μl of binding buffer [20 mM HEPES (pH 7.9), 0.3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 10% glycerol, 2 μg of poly[dI-dC]-cl] and incubated at room temperature for 20 min. After incubation, 10 μl of each sample were loaded onto 4% nondenaturing polyacrylamide gels and electrophoresed in low salt 1× TAE [6.7 mM Tris HC1 (pH 7.9), 3.3 mM NaOAc, 1 mM EDTA] at 250 V for 2 hr at room temperature. Gels were dried and processed for autoradiography.

RNase protection analysis

Levels of VP and OT mRNA were measured by RNase protection assay essentially as described earlier (Sambrook et al. 1989). Whole brains of newborn mice or regions containing the basal ganglia, thalamus, and hypothalamus of 10-week-old male mice were removed surgically and total cellular RNA was extracted by the lithium chloride–urea method (Auffray and Rougeon 1980). A mouse VP cDNA fragment (335 bp) corresponding to nucleotides 2793–3331 of the genomic sequence (GenBank accession no. M88334) and an OT cDNA fragment (332 bp) corresponding to nucleotides 1142–1817 of the genomic sequence (GenBank accession no. M88335) were amplified by RT–PCR, subcloned into an EcoRV site of plasmid vector (Stratagene) and nucleotide sequences were confirmed by dideoxy sequencing. As a control, we used Unp gene expression for newborn mice and β-actin gene expression for 10-week-old mice. A plasmid containing a mouse Unp cDNA of 246 bp was obtained from D. Gray. A Kpn1–Xba1 fragment of mouse β-actin cDNA (250 bp) was also subcloned and used for cRNA synthesis (Alonso et al. 1986). These plasmid DNAs were linearized and radiolabeled antisense riboprobes were transcribed in vitro from the T7 promoter using a Promega riboprobe kit according to the manufacturer's instructions. Because the optimal temperatures varied with the probes, the protection reaction for each was carried out independently. The consistency of results was confirmed by repeating experiments at least three times and representative results are shown. Total RNA (20 μg) was hybridized with 1 × 107 cpm of radiolabeled probe and incubated overnight. Incubation temperatures were 55°C for VP, 55°C for OT, 47°C for Unp, and 45°C for β-actin. Digestion was carried out with RNase A (3 μg) and RNase T1 (20 units) for 30 min at 37°C. Yeast tRNA was used as a control. Protected fragments were analyzed by electrophoresis through a 5% denaturing gel with reference to a 1-kb ladder. Dried gels were analyzed by Image Analyzer (Fuji Film) and the radioactivities of protected bands were counted.
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