TARGETED DELETION OF THE MOUSE POU DOMAIN GENE Brn-3a CAUSES A SELECTIVE LOSS OF NEURONS IN THE BRAINSTEM AND TRIGEMINAL GANGLION, UNCOORDINATED LIMB MOVEMENT, AND IMPAIRED SUCKLING

MENGQING XIANG*†‡§, LIN GAN§, LIJUAN ZHOU*†, WILLIAM H. KLEIN¶, AND JEREMY NATHANS*†||*††

Departments of *Molecular Biology and Genetics, **Neuroscience, and ††Ophthalmology, †Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ‡Department of Biochemistry and Molecular Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Contributed by Jeremy Nathans, July 30, 1996

ABSTRACT The Brn-3 subfamily of POU domain genes are expressed in sensory neurons and in select brainstem nuclei. Earlier work has shown that targeted deletion of the Brn-3b and Brn-3c genes produce, respectively, defects in the retina and in the inner ear. We show herein that targeted deletion of the Brn-3a gene results in defective suckling and in uncoordinated limb and trunk movements, leading to early postnatal death. Brn-3a (--/-) mice show a loss of neurons in the trigeminal ganglia, the medial habenula, the red nucleus, and the caudal region of the inferior olivary nucleus but not in the retina and dorsal root ganglia. In the trigeminal and dorsal root ganglia, but not in the retina, there is a marked decrease in the frequency of neurons expressing Brn-3b and Brn-3c, suggesting that Brn-3a positively regulates Brn-3b and Brn-3c expression in somatosensory neurons. Thus, Brn-3a exerts its major developmental effects in somatosensory neurons and in brainstem nuclei involved in motor control. The phenotypes of Brn-3a, Brn-3b, and Brn-3c mutant mice indicate that individual Brn-3 genes have evolved to control development in the auditory, visual, or somatosensory systems and that despite differences between these systems in transduction mechanisms, sensory organ structures, and central information processing, there may be fundamental homologies in the genetic regulatory events that control their development.

A central issue in neural development is the manner in which genetic regulatory circuits govern the development of vast numbers of distinct neuronal types. Among the transcriptional regulators implicated in both invertebrate and vertebrate neural development are members of the POU domain family. This family was initially defined by the mammalian pituitary-specific factor Pit-1, the octamer binding factors Oct-1 and Oct-2, and the Caenorhabditis elegans gene Unc-86 (1). The POU domain functions as a bipartite DNA binding domain that contains a ~70-amino acid POU-specific domain and a ~60-amino acid POU-homeo domain, joined by a variable linker. The POU domain family has been divided into six classes based on primary sequence similarities in the POU domain (2). Many members of this gene family have distinctive patterns of expression in the developing and adult nervous systems, consistent with a role for these factors in neural development (2). Genetic and biochemical studies in C. elegans, Drosophila, mice, and humans have begun to elucidate the roles of several POU domain factors in neurogenesis and neuronal specification. For example, in the neuroendocrine system Pit-1 binds to and activates the growth hormone gene (3, 4), and mutations in Pit-1 result in dwarfism in both mice and humans secondary to a loss of the growth hormone secreting somatotrophs in the anterior pituitary (5–8). The class III POU domain gene Brn-4/RHS2 is expressed in the otic vesicle and in other regions in the central nervous system in the developing rat (9, 10), and in humans, mutations in this gene result in stapes fixation and progressive sensorineural deafness (11). Another class III gene, Brn-2, is essential for the specification of certain neuronal precursors in the hypothalamus (12, 13).

The class IV POU domain group is defined by the C. elegans Unc-86 gene, the Drosophila I-POU gene, and the three vertebrate Brn-3 genes (14–21). The Unc-86 protein is found exclusively within neurons and neuroblasts, and Unc-86 loss-of-function mutations alter the fates of a subset of these cells (22, 23). The Drosophila I-POU gene encodes both a neuron-specific inhibitor (I-POU) and an activator (I1-POU) that are generated by alternative splicing (15, 16). In mammals, there are three highly homologous class IV POU domain genes, Brn-3a, Brn-3b, and Brn-3c (refs. 17–21; also referred to as Brn-3.0, Brn-3.2, and Brn-3.1, respectively). Each Brn-3 gene is expressed in a distinct pattern in the developing and adult brainstem, retina, and dorsal root and trigeminal ganglia (17, 19–21, 24, 25). Brn-3c is unique among Brn-3 family members in being expressed in cochlear and vestibular hair cells (ref. 26; M.X. and J.N., unpublished work). The recent production of targeted mutations in Brn-3b and Brn-3c have revealed a high degree of functional specificity in vivo. Targeted deletion of the Brn-3b gene results in a selective absence of ~70% of retinal ganglion cells with little or no effect on other neurons (26, 27), and targeted deletion of Brn-3c leads to defective inner ear function as a result of a failure to produce sensory hair cells (26).

During mouse development, Brn-3a is expressed in most neurons in the dorsal root and trigeminal ganglia, medial habenula, red nucleus, and inferior olivary nucleus; expression is also seen in scattered neurons in the superior colliculus and periaqueductal grey. In the medial habenula and red nucleus, Brn-3a is the only Brn-3 family member expressed (19–21, 24, 25). In this paper we report the behavioral and neuroanatomic phenotype of targeted deletion of the Brn-3a gene.

MATERIALS AND METHODS

Targeted Disruption of the Brn-3a Gene. Mouse Brn-3a genomic sequences were isolated from a genomic DNA library (strain 129/J) constructed in bacteriophage λ using human Brn-3a, Brn-3b, and Brn-3c POU domain segments as probes (17, 21, 27). To generate the gene targeting construct, the KpnI and NotI sites of the 3.0-kb Brn-3a KpnI–NotI fragment 5’ to the Brn-3a gene were converted to NotI and SalI sites, respectively, by blunting the ends and adding NotI and SalI linkers.

Abbreviations: ES, embryonic stem; e, embryonic day; P0, birth.
†Present address: Center for Advanced Biotechnology and Medicine, Department of Pediatrics, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854.
‡M.X. and L.G. contributed equally to this work.
§To whom reprint requests should be addressed.
The 
embryonic day (e) 20: (+/+); (−/−)), 1.19 ± 0.11 g, n = 12; (+/−), 1.19 ± 0.10 g, n = 19; (−/−), 1.18 ± 0.09 g, n = 3. Body weight at birth (P0): (+/+/+), 1.36 ± 0.14 g, n = 3; (+/−), 1.38 ± 0.05 g, n = 6; (−/−), 1.35 ± 0.11 g, n = 6. Postnatally, Brn-3a (+/+) mice are indistinguishable from the wild type in growth and behavior, and breeding of Brn-3a heterozygotes gave a ratio for

FIG. 2. Behavioral differences between Brn-3a (+/+) and Brn-3a (−/−) siblings at P0. (A and B) The Brn-3a (+/+) mouse (right) has nursed as seen by the presence of milk in its stomach (arrowhead); the Brn-3a (−/−) mouse (left) has not. Both animals were placed on their backs and photographed over the ensuing several minutes. The Brn-3a (+/+) mouse uses a forelimb to right itself (arrow in B); the Brn-3a (−/−) animal extends its limbs, head, and trunk but fails to right itself.
indistinguishable from the wild type. (ii) *Brn-3a* (-/-) mice show a deficiency in righting due to a lack of coordinated limb and trunk movements. When a *Brn-3a* (-/-) neonate is placed on its back or side, it typically moves and stretches its limbs ineffectually and displays extended postures not observed in the wild type (Fig. 2). By contrast, *Brn-3a* (+/+) and (+/-) neonates invariably succeed in righting themselves, typically by using the forelimb closest to the ground [Fig. 2B; successful righting: (+/+), 100%, n = 17; (+/-), 100%, n = 35; (-/-), 0%, n = 23]. Failure to feed does not appear to be the cause of this righting defect because wild-type siblings that are not allowed to feed retain the ability to right themselves.

In wild-type mice, *Brn-3a* is expressed during development and in the adult in most, if not all, neurons in the trigeminal and dorsal root ganglia, in ~70% of retinal ganglion cells, and in several brainstem nuclei (19–21, 24, 25). As described below, we have examined the *Brn-3a* (-/-) nervous system in the late gestational and neonatal periods (e20 and P0, respectively) using a combination of histochemistry and immunohistochemistry. This analysis has revealed developmental anomalies in several central nervous system and peripheral nervous system structures that plausibly account for the behavioral defects.

**Dorsal Root Ganglia, Trigeminal Ganglia, and Retina.** As an initial step in the analysis of the retina and sensory ganglia in *Brn-3a* (-/-) mice, dorsal root ganglia were examined by cresyl violet staining of four *Brn-3a* (+/+) or (+/-) and four *Brn-3a* (-/-) e20 embryos and one pair of *Brn-3a* (+/-) and (-/-) neonates (Fig. 3). Retinas from three *Brn-3a* (+/+) or
(+/-) and three Brn-3a (-/-) e20 embryos and from one pair of Brn-3a (+/-) and (-/-) neocortices, and trigeminal ganglia from three Brn-3a (+/+) or (+/-) and three Brn-3a (-/-) e20 embryos were similarly examined (Figs. 4 and 5). While the sizes of the developing retina and dorsal root ganglia, the number of cells within them, and their appearance in the light microscope show no significant differences between Brn-3a (+/+) or (+/-) and Brn-3a (-/-) mice, the volumes of the trigeminal ganglia of Brn-3a (-/-) mice at e20 are approximately one-half that of Brn3a (+/+) or (+/-) mice (indistinguishable rostrocaudal lengths and a ratio of coronal cross-sectional areas of 0.58 ± 0.12, n = 4; Fig. 4 A and B). Moreover, Brn-3a (-/-) trigeminal ganglia show a significant decrease in the density of large neurons with an accompanying increase in the density of smaller cells compared with Brn-3a (+/+) and (+/-) trigeminal ganglia (Fig. 4 C–F).

The dorsal root and trigeminal ganglia were further characterized by immunostaining of e20 embryos with a series of antibodies specific for neurons or neuronal subsets. Antibodies against the Olf-1 transcription factor (anti-Olf-1; ref. 28), and the neuron-specific class III β-tubulin (mAb TuJ1; ref. 29) immunolabel the vast majority of neurons in both the dorsal root and trigeminal ganglia of e20 Brn-3a (+/+) embryos (Figs. 3 G and I and 4M and data not shown), while anti-neurofilament 160 (anti-NF160) antibodies stain only a minority of neurons in these two ganglia (Fig. 3K and data not shown). Consistent with the cresyl violet staining, there is no significant difference between Brn-3a (+/+) and (-/-) animals in the appearance or number of dorsal root ganglion cells that immunostain with anti-Olf1, TuJ1, or anti-NF160 (Fig. 3 G–L). Immunostaining for substance P, galanin, neuropeptide Y, and calcitonin gene-related peptide also reveals equal numbers of immunoreactive neurons in Brn-3a (+/+) and Brn-3a (-/-) dorsal root ganglia (data not shown). In the trigeminal ganglia, the density of neurons that are Olf-1 immunoreactive show a modest decrease in e20 Brn-3a (-/-) embryos as compared with the wild type (Fig. 4 M and N). Visualization of encapsulated nerve endings in the skin and at the base of hair follicles using nonspecific choline esterase histochemistry (30, 31) did not reveal an alteration in the density of this class of mecanoreceptors in Brn-3a (-/-) mice in either the face or the trunk (data not shown).

The other two members of the Brn-3 family, Brn-3b and Brn-3c, are expressed in subsets of neurons in the dorsal root and trigeminal ganglia and in subsets of ganglion cells in the retina. Within each of these populations, Brn-3b is expressed in a majority of neurons and Brn-3c in a minority of neurons (Figs. 3 C and E and 4 I and K). Interestingly, Brn-3b-immunoreactive cells are nearly absent in the trigeminal and dorsal root ganglia of Brn-3a (-/-) mice (Figs. 3 C and D and 4 I and J), and Brn-3c-immunoreactive cells are diminished in number by 5- to 10-fold (Figs. 3 E and F and 4 K and L). By contrast, the number and appearance of Brn-3b- and Brn-3c-immunoreactive cells in the e20 retina are unaffected by loss of Brn-3a (Fig. 5 C and D; and data not shown).

Brainstem. Loss of Brn-3a affects neither the superficial appearance nor the size of the brain at e20 or P0. To search for defects in the Brn-3a (-/-) brain, a complete series of 20-μm coronal sections was prepared from four pairs of e20 Brn-3a (+/+) or (+/-) and Brn-3a (-/-) brains, and one pair of P0 Brn-3a (+/-) and Brn-3a (-/-) brains.

In the rostral brainstem, Brn-3a expression has been localized by in situ hybridization to the habenula in the developing and adult rat brain, and Brn-3a is the only Brn-3 family member expressed in this nucleus (20). At e20 in the mouse, Brn-3a immunoreactivity is present in most neurons in the medial

![Fig. 5. Retinal structure in Brn-3a (-/-) mice at e20. The photographs are aligned with the pigment epithelium at the top and the vitreal surface at the bottom. At this stage the retina consists of a broad zone of mitotic cells and developing neuroblasts in the outer two-thirds of the retina and a ganglion cell layer in the inner one-third. In Brn-3a (+/+) retinas, Brn-3a (A) and Brn-3b (C) immunoreactivity are found only in ganglion cells as reported previously (17, 19–21). In Brn-3a (-/-) retinas, Brn-3a immunoreactivity is absent as expected (B), and Brn-3b immunoreactivity is unaffected (D). In Brn-3a (-/-) mice, no differences in retinal structure are discernible by cresyl violet staining (E and F). (Bars = 25 μm.)](image)

![Fig. 6. Habenula in Brn-3a (-/-) mice at e20. The coronal sections chosen show the medial habenula at the point of its greatest lateral extent. The dorsal third ventricle is seen at the center of each section. In Brn-3a (+/) mice, Brn-3a-immunoreactive neurons are present at high density in the medial habenula and at lower density in the lateral habenula (A). In Brn-3a (-/-) mice, Brn-3a immunoreactivity is absent as expected (B). Cresyl violet staining shows a loss of neurons in the medial habenula in Brn-3a (-/-) mice (C and D). MH, medial habenula; LH, lateral habenula; Hi, hippocampus. (Bars = 100 μm.)](image)
In the midbrain, Brn-3a is expressed during development and in the adult in the superior colliculus, dorsal central gray, red nucleus, and interpeduncular nucleus (Fig. 7A). With the exception of the red nucleus, Brn-3b is also expressed in each of these regions (Fig. 7C). No differences are observed between Brn-3a (+/+) or (+/−) and Brn-3a (−/−) mice at e20 in the number of neurons or in the numbers and locations of Brn-3b-immunoreactive neurons within the superior colliculus, dorsal central gray, or interpeduncular nucleus (Fig. 7C–F and data not shown). By contrast, the large neurons of the red nucleus that are clearly seen in wild-type mice as two symmetric clusters by cresyl violet staining are not seen in Brn-3a (−/−) mice at e20 and P0 (Fig. 7E and F).

In the inferior olivary nucleus, both Brn-3a and Brn-3b are expressed during development, but only Brn-3a expression persists in the adult (17, 20, 25). At e20, strong Brn-3a immunoreactivity is uniformly present in many neurons in the inferior olivary nucleus along its entire rostrocaudal extent (Fig. 8A and C). At this stage, strong Brn-3b immunoreactivity is also present in many neurons in the rostral region of the inferior olivary nucleus, but in the caudal region strong Brn-3b immunoreactivity is confined to the ventral part of the nucleus (Fig. 8E and G). Little or no difference is seen between Brn-3a (+/+) and Brn-3a (−/−) mice in the density or location of cells that are stained by anti-Brn-3b antibodies or by cresyl violet in the rostral region of the inferior olivary nucleus (compare Fig. 8E and F with I and J). However, cresyl violet staining reveals a reproducible diminution in the number of cells in the caudal region of the inferior olivary nucleus in Brn-3a (−/−) mice (Fig. 8K and L) with little accompanying change in the number of cells that are strongly immunoreactive for Brn-3b (Fig. 8G and H).

**DISCUSSION**

**Specificity of Brn-3a Function.** In this paper we report the behavioral and anatomic phenotypes associated with targeted deletion of the Brn-3a gene. Newborn Brn-3a (−/−) mice exhibit aberrant limb and trunk movements and impaired suckling, and they die within 1 day. Brn-3a (−/−) mice have a decrease in the number of neurons in the trigeminal ganglion, medial habenula, red nucleus, and inferior olivary nucleus and a decrease in the fraction of neurons in the trigeminal and dorsal root ganglia that express Brn-3b and Brn-3c. Other regions within the nervous system, including those that normally express Brn-3a, are not detectably affected. The behavioral defects seen in Brn-3a (−/−) mice could arise from somatosensory dysfunction, motor dysfunction, or a combination of the two. While the observed anatomic defects do not unequivocally define the sites responsible for each behavioral defect, they suggest plausible correlations between the two. In particular, a decrease in the number of trigeminal ganglion neurons could produce sensory defects in the face

**Fig. 7.** Midbrain at the level of the red nucleus in Brn-3a (−/−) mice at e20. In Brn-3a (+/+) mice, Brn-3a-immunoreactive neurons are present in the superior colliculus, dorsal central grey, red nucleus, and the interpeduncular nucleus (A); Brn-3b-immunoreactive neurons are present in the superior colliculus, dorsal central grey, and the interpeduncular nucleus (C). In Brn-3a (−/−) mice, Brn-3a immunoreactivity is absent as expected (B), and Brn-3b immunoreactivity is unchanged (D). By cresyl violet staining, the large neurons of the red nucleus are absent (E and F) (arrows in F indicate the expected location of the red nucleus). AQ, aquaduct; DCG, dorsal central grey; IP, interpeduncular nucleus; RN, red nucleus; SC, superior colliculus. (Bars: A–D, 200 μm; E and F, 400 μm.)

habenula and in scattered neurons in the lateral habenula (Fig. 6A). At this stage, cresyl violet staining shows a reduction of approximately 2-fold in the number of cells in the median habenula in Brn-3a (−/−) mice in comparison with the wild type (Fig. 6C and D).

**Fig. 8.** Inferior olivary nucleus in Brn-3a (−/−) mice at e20. For each staining condition, pairs of sections are shown from the rostral and caudal regions of the inferior olivary nucleus. In Brn-3a (+/+) mice, the patterns of Brn-3a (A and C) and Brn-3b (E and G) immunoreactivity are similar in both rostral and caudal regions of the inferior olivary nucleus, except that Brn-3b immunoreactivity is weak or absent in the dorsal division in the caudal region (vertical arrows in C and G). In Brn-3a (−/−) mice, Brn-3a immunoreactivity is absent as expected (B and D), and Brn-3b immunoreactivity shows little change (F and H). Cresyl violet staining shows a diminution in the number of cells in the caudal region of the inferior olivary nucleus (I–L). (Bars = 100 μm.)
and mouth that impair the suckling response. With respect to coordination of limb and trunk movement, the observed phenotype could arise from defects in one or more intrinsic spinal reflexes or in any of the pathways that integrate ascending information from the dorsal root ganglia or descending information from the motor cortex or cerebellum. As the red nucleus conveys information from the cerebellum and the cerebral cortex to the spinal cord via the rubrospinal tract and the inferior olivary nucleus integrates sensory information and sends its output to the cerebellar Purkinje cells, defects in these two nuclei could plausibly impair limb and trunk coordination or posture (32). Functional alterations in the dorsal root ganglia related to the loss of Brn-3b and Brn-3c expression could also play a role in this phenotype.

In the normal mouse retina, trigeminal ganglion, and dorsal root ganglion, the cells that express Brn-3b and/or Brn-3c form a subset or largely coincide with the cells that express Brn-3a (21). In Brn-3b (−/−) mice, Brn-3a and Brn-3c expression is unaltered in the dorsal root and trigeminal ganglia but is diminished in the retina as a consequence of the ≈70% decrease in retinal ganglion cell number (26, 27). By contrast, in Brn-3a (−/−) mice, expression of Brn-3b and Brn-3c is unaffected in the retina but is dramatically reduced in the dorsal root and trigeminal ganglia. We infer, therefore, that in the course of normal development Brn-3a expression directly or indirectly leads to the activation of Brn-3b and Brn-3c in subsets of dorsal root and trigeminal ganglion neurons but does not play a role in their activation in the retina.

In the developing central nervous system, both Brn-3a and Brn-3b are expressed in subsets of cells in the superior and inferior olivary, dorsal central gray, and interpeduncular nucleus (Fig. 7). Neither Brn-3a (−/−) nor Brn-3b (−/−) mutants show obvious defects in the number of neurons in these regions, suggesting that Brn-3a and Brn-3b play no role in the development of these neurons, that they alter cellular characteristics other than cell number, or that they function redundantly.

**Functional Diversification of the Unc-86/Brn-3 Gene Family.** The high degree of sequence similarity between the POU domains of Unc-86 and the Brn-3 family and the conserved Brn-3 gene structures suggest that the Brn-3 genes arose by duplication and divergence of an ancestral Unc-86-like gene. As discussed below, the Unc-86 and Brn-3 genes also bear intriguing functional similarities as seen in their expression in subsets of neurons and in the phenotypic consequences of their mutation.

In *C. elegans*, Unc-86 is expressed exclusively in neurons and neuroblasts. Of the 302 neurons in the adult, 57 express Unc-86, including sensory neurons, motor neurons, and interneurons (23), and the Unc-86 phenotype includes defects in mechanosensation, chemotaxis, and egg laying (22, 33). Analysis of Unc-86 mutants at the single cell level reveals defects both during and following the period of cell proliferation leading to subtle changes in neuronal phenotype, to neuronal loss, and to the generation of supernumerary neurons. Thus, Unc-86 appears to be involved in a variety of developmental decisions that differ depending on the cellular context. Each member of the Brn-3 family is expressed in a small subset of neurons in the brainstem and in the auditory (Brn-3c), somatosensory (Brn-3a, Brn-3b, and Brn-3c), or visual systems (Brn-3a, Brn-3b, and Brn-3c). Expression in each sensory system is confined to cells close to or at the site of sensory transduction, cochlear hair cells, primary somatosensory neurons, and retinal ganglion cells. Interestingly, the phenotypes associated with mutation in each Brn-3 gene reveal a greater degree of functional specialization than the partially overlapping zones of expression would suggest: the only anatomic defect thus far identified in Brn-3b (−/−) mice is a decrease of 70% in retinal ganglion cell number (26, 27); and the only defects apparent in Brn-3c (−/−) mice is a loss of vestibular and cochlear hair cells and a secondary degeneration of their associated sensory ganglia (26). As described above, Brn-3a (−/−) mice show a decrease in the number of neurons in the trigeminal ganglia but do not show any abnormalities in the retina. This functional diversification of Brn-3 family members indicates that the expansion and evolution of specialized sensory systems in more complex organisms has been accompanied by a parallel expansion and evolution of genetic regulatory proteins. Thus, these observations suggest that despite the great differences in transduction mechanisms, sensory organ structures, and central information processing of the auditory, somatosensory, and visual systems, there may be fundamental homologies in the genetic regulatory events that control their development.

We thank Drs. Janine Davis, Randall Reed, and Anthony Frankfurter for antibodies and Drs. Randall Reed and King-wai Yau for helpful comments on the manuscript. This work was supported by the National Eye Institute and the Howard Hughes Medical Institute (J.N.) and by the National Institutes of Health and the Robert Welch Foundation (W.H.K.)