Role of the Brn-3 Family of POU-domain Genes in the Development of the Auditory/Vestibular, Somatosensory, and Visual Systems

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Understanding the genetic regulatory networks that specify neuronal identity is one of the central challenges in developmental neurobiology. A number of transcription factors have been implicated in decisions related to neuronal versus nonneuronal cell fate, regional specification in the nervous system, or determination of the terminally differentiated phenotype. For example, basic helix-loop-helix (bHLH) factors such as neuroD and the achaete-scute family control neural versus ectodermal cell fates (Jan and Jan 1993; Lee et al. 1995), and Hox genes control regional specification along the neuraxis (Keynes and Krumlauf 1994). Among the transcriptional regulators that control neuronal development in both vertebrates and invertebrates are members of the POU-domain family. This family was initially defined by the mammalian pituitary-specific transcription factor Pit-1/GHF-1, the octamer-binding proteins Oct-1 and Oct-2, and the Caenorhabditis elegans gene unc-86 (Herr et al. 1988). The POU-domain functions as a bipartite DNA-binding domain that contains an approximately 70-amino-acid POU-specific domain and an approximately 60-amino-acid POU-homeodomain, joined by a variable linker. 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 (Wegner et al. 1993).

Genetic studies in mice and humans show that many POU-domain genes function in the terminal stages of nervous system development (Fig. 1). SCIP/Tst-1/Oct-6 controls the differentiation of Schwann cells (Weinstein et al. 1995; Bermingham et al. 1996; Jaegle et al. 1996); Pit-1/GHF-1 is required for the normal development of the anterior pituitary (Li et al. 1990); Brn-4/RHS2/POU3F4 is required for the normal development of the inner and middle ear (Bitner-Glindzicz et al. 1995; de Kok et al. 1995); and Brn-2 is required for the specification of subsets of neurons in the hypothalamus (Nakai et al. 1995; Schonemann et al. 1995).

The POU-domain family has been divided into six classes on the basis of primary sequence similarities in the POU-domain (Wegner et al. 1993). The class IV POU-domain group is defined by the unc-86 gene (Finney et al. 1988), the Drosophila I-POU gene (Treacy et al. 1991, 1992), and the three vertebrate Brn-3 genes (Gerrero et al. 1993; Theil et al. 1993, 1994, 1995). The Unc-86 protein is found exclusively within a subset of neurons and neuroblasts, and unc-86 loss-of-function mutations affect some of these cells by causing a daughter cell to assume the fate of its mother or by altering cell phenotypes postmitotically (Chalfie et al. 1981; Desai et al. 1988; Finney and Ruvkin 1990). The Drosophila I-POU gene encodes two isoforms that are generated by alternative splicing (Treacy et al. 1991, 1992; Turner 1996). In mammals, there are three highly homologous class IV POU-domain genes, Brn-3a, Brn-3b, and Brn-3c (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, inner ear, and dorsal root and trigeminal ganglia (Gerrero et al. 1993; Ninkina et al. 1993; Xiang et al. 1993, 1995, 1997; Turner et al. 1994; Fedtsova and Turner 1996). In this paper, we review our work on the Brn-3 family and the role of these genes in sensory system development (Xiang et al. 1993, 1995, 1996, 1997; Gan et al. 1996).

METHODS


RESULTS

Identification and Characterization of the Brn-3 Subfamily of POU-domain Genes

The first member of the Brn-3 family was identified by He et al. (1989) as a polymerase chain reaction (PCR) product encoding a novel POU-domain. By degenerate PCR and low-stringency DNA hybridization, we and others subsequently identified three genes with POU-domains that are identical or highly homologous to that re-
POU domain genes

- mouse Brn-1
- rat Brn-2
- Drosophila Cf1A
- rat RHS2/Brn-4
- rat Tst-1/SCIP/Oct-6
- mouse Oct-2
- rat Oct-1
- rat Skn-1a/Oct-11
- Drosophila pdm-1/Nubbin
- human Pit-1/GHF-1
- mouse Brn-3c/Brn-3.1
- mouse Brn3b/Brn-3.2
- mouse Brn-3a/Brn-3.0
- Drosophila I-POU
- nematode UNC-86
- rat Emb/Brn 5
- human RPF-1
- rat Sprm-1

Cells or tissues affected by mutation

- hypothalamic neurons
- middle ear
- Schwann cells; neurons controlling respiration
- B-lymphocytes
- neuroblasts; wing
- anterior pituitary
- auditory and vestibular hair cells
- retinal ganglion cells
- primary somatosensory neurons; brainstem sensory-motor nuclei
- multiple neuronal lineages

Figure 1. Dendrogram of POU-domains (left) and the phenotypes associated with mutation of the corresponding genes (right). The dendrogram was constructed by aligning POU-domain amino acid sequences and determining percent amino acid identity beginning with the first amino acid of the POU-specific domain (in Oct-1: EEPS...) and ending with the 58th amino acid of the POU-homeodomain (in Oct-1: KEKR) as defined in Klemm et al. (1994). Loss-of-function phenotypes are described in the following references: Brn-2 (Nakai et al. 1995; Schonemann et al. 1995), RHS2/Brn-4 (de Kok et al. 1995), Tst-1/SCIP/Oct-6 (Weinstein et al. 1995; Bermingham et al. 1996; Jaegle et al. 1996), Oct-2 (Corcoran et al. 1993), pdm-1/Nubbin (Ng et al. 1995; Yeo et al. 1995), Pit-1/GHF-1 (Li et al. 1990), Brn-3a (McEvilly et al. 1996; Xiang et al. 1996), Brn-3b (Erkman et al. 1996; Gan et al. 1996), Brn-3c (Erkman et al. 1996; Xiang et al. 1997), and UNC-86 (Chalfie et al. 1981; Desai et al. 1988; Finney and Ruvkin 1990).

Supported by He et al. (1989). These genes, identified in humans, mice, and rats, are referred to as Brn-3a, Brn-3b, and Brn-3c. They reside on distinct autosomes and encode proteins of 423, 410, and 338 amino acids in length, respectively. The intron-exon structures of the three Brn-3 genes are identical, consisting of two coding exons interrupted by a small intron. In pairwise comparisons, the Brn-3 proteins share approximately 95% amino acid identity within the POU-domain, and approximately 70% identity outside of the POU-domain. A comparison of POU-domain sequences shows that the Brn-3 family is most closely related to the Drosophila I-POU and the C. elegans unc-86 genes (Fig. 1).

We have localized the expression of Brn-3a, Brn-3b, and Brn-3c by RNA-blot hybridization, RT-PCR, and immunostaining with affinity-purified antibodies raised against the most divergent regions of the Brn-3 proteins. Each of the anti-Brn-3 antibodies specifically recognizes the Brn-3 family member against which it was raised and does not cross-react with other Brn-3 proteins as determined by (1) Western blotting against recombinant fusion proteins produced in Escherichia coli, (2) the distinctive patterns of tissue staining obtained with each antibody, and (3) the selective elimination of immunostaining in mice lacking the corresponding gene (Xiang et al. 1995, 1996, 1997; Gan et al. 1996). During development and in the adult, expression of the Brn-3 family is found principally within the central nervous system (CNS). Each Brn-3 gene is expressed in a subset of retinal ganglion cells, somatosensory neurons in the trigeminal and dorsal root ganglia, and scattered cells within a small number of brainstem nuclei. Brn-3a and Brn-3b are expressed in many neurons within the spiral and vestibular ganglia, and Brn-3c is expressed in auditory and vestibular hair cells (Fig. 2). Other regions of the adult CNS, including the cortex and cerebellum, do not express the Brn-3 genes. Figure 3 shows Brn-3a, Brn-3b, and Brn-3c immunolocalization in the adult mouse retina (Fig. 3A–F) and Brn-3b immunolocalization in the developing mouse retina (Fig. 3G–J). In all mammalian retinas tested to date (mouse, rabbit, cat, and macaque), Brn-3 immunoreactivity is confined to ganglion cells. In cat and macaque retinas, the expression pattern of individual Brn-3 family members correlates with previously defined morphologic and functional classes of retinal ganglion cells.
Figure 2. Brn-3c in the developing and adult mouse inner ear. Immunostaining of sections of the inner ear with anti-Brn-3c antibodies at e17.5 (A–C) and P5 (D–F). Staining is present in the nuclei of developing hair cells at all stages shown and is absent from other cell types. (G–I) Brn-3c in the adult organ of Corti. One turn of a wild-type organ of Corti adjacent to the apex is shown. The cochlea was immunostained as a whole mount with affinity-purified anti-Brn-3c antibodies, and the dissected organ of Corti was then incubated with DAPI. (G) DAPI staining reveals nuclei of supporting cells that are not immunostained. (H,I) Anti-Brn-3c immunoreactivity is found exclusively within the single row of inner hair cell nuclei and the three rows of outer hair cell nuclei. All hair cell nuclei are immunostained. Note that the precipitate formed by reaction of 3-amino-9-ethylcarbazole, the immunoperoxidase substrate, quenches DAPI fluorescence. (Cri) Crista; (Co) cochlea; (Oto) otolith organ; (IHC) inner hair cells; (OHC) outer hair cells. Bar in F: (A) 50 μm; (B,C,E,F) 25 μm; (D) 100 μm. Bar in H: (G,H) 100 μm. Bar in I: 50 μm. (Reprinted, with permission, from Xiang et al. 1997.)

DNA-binding Properties of Brn-3 Proteins

An optimal DNA-binding site for the Brn-3 proteins was identified using iterative cycles of in vitro binding and PCR amplification (the selex method; Thiesen and Bach 1990). In these experiments, we selected from a pool of random sequences that subset which bound to a fusion protein containing the POU domain of Brn-3b fused to glutathione S-transferase (GST). Following several cycles of enrichment, the selected double-stranded DNA segments were cloned, sequenced, and individually tested for their ability to bind the GST-POU domain fusion protein. Of 33 cloned segments, representing 30 different DNA sequences, 31 were found to bind the Brn-3b POU-domain and each of the 31 segments contained the consensus (A/G)TTAATGAG(C/T) or a close derivative of it. (In the original description of this experiment [Xiang et al. 1995], we refer to the complementary strand of this sequence.) To test the DNA-binding properties of the intact Brn-3b protein, whole-cell protein extracts were
Figure 3. Anti-Brn-3a, -Brn-3b, and -Brn-3c immunoreactivity in the mouse retina. To localize Brn-3 proteins and visualize nuclei simultaneously, mouse retina sections were double-stained with anti-Brn-3a (A) and DAPI (B), anti-Brn-3b (C) and DAPI (D), or anti-Brn-3c (E) and DAPI (F). Note that the purple HRP product of the immunostaining reaction partially quenches DAPI fluorescence when both are present in the same nucleus. The three layers of nuclei are (from top to bottom): outer nuclear layer, inner nuclear layer, and ganglion cell layer. (G–J) Brn-3b appears early in ganglion cell development. Sections through the developing mouse retina at e12.5 (G), e13.5 (H), e16.5 (I), and P1.5 (J) immunostained with anti-Brn-3b antibodies. Brn-3b is initially present in both the developing ganglion cell layer and the proximal part of the mitotic zone. As development proceeds, Brn-3b is increasingly confined to cells within the developing ganglion cell layer. The localized expression of Brn-3b within the central retina at e12.5 reflects the earlier development of this region. In all micrographs of cross sections, the inner retina is at the bottom. Bar in J: (H–J) 25 μm; (G) 50 μm. (Reprinted, with permission, from Gan et al. 1996.)
prepared from untransfected human embryonic kidney cells (293) or from 293 cells that had been transiently transfected with a Brn-3b expression construct or an Oct-2 expression construct (Fig. 4A). Brn-3b produced in 293 cells showed strong binding to the selected Brn-3 consensus site (C) and no detectable binding to a canonical octamer site (Oct; Singh et al. 1986). In contrast, endogenous Oct-1 from 293 cells and expressed Oct-2 bound well to the octamer site but poorly or not at all to the selected Brn-3 consensus site.

The regions of the selected Brn-3 consensus site that are essential for binding were delineated using a series of derivative sites containing base substitutions in or around the consensus region (M1-M6). These segments were tested for binding by full-length Brn-3b or GST-POU-domain fusion proteins derived from Brn-3a, Brn-3b, Brn-3c, and Oct-1 (Fig. 4B,C and data not shown). Two other segments of identical length, containing either a canonical octamer site or an Unc-86 site (M7; Xue et al. 1992), were also tested. The selected Brn-3 consensus site binds to full-length Brn-3b and to each of the Brn-3 GST-POU-domain fusion proteins with high specificity, and mutating pairs of nucleotides within the selected consensus sequence TTAATGAG dramatically reduces DNA binding (mutant sites M2-M5). Surprisingly, the GST-POU-domain fusions derived from Brn-3a and Brn-3c bind the Unc-86 consensus site (M7) poorly, and the isolated Brn-3b POU-domain binds this site with a reduced affinity relative to the selected Brn-3 consensus site (Xiang et al. 1995). However, full-length Brn-3b binds to the Unc-86-binding site and to the selected Brn-3 consensus site with similar affinities.

These in-vitro-binding experiments show that the Brn-3 POU-domains can bind to a selected consensus site (TTAATGAG) distinct from the site shown previously to bind Unc-86 (CAT(N)3TAAT) and that full-length Brn-3b can bind to both sites. The numerous differences between the two sites suggest that the Brn-3 POU-domains may contact the selected DNA site by rearranging the positions and/or orientations of the POU-specific domain and the POU homeodomain relative to that observed in the Oct-1-DNA crystal structure (Klemm et al. 1994), as postulated by Li et al. (1993) for the interaction between Brn-2 and its DNA target in the promoter of the corticotropin-releasing hormone gene (Fig. 4D). Unlike most other POU-domain proteins described thus far, the Brn-3

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**Figure 4.** DNA-binding specificity of Brn-3b. (A) Comparison of the binding of Brn-3b and Oct-2 to the selected Brn-3 consensus site (C) and the canonical octamer site (Oct). Whole-cell protein extracts from 293 cells (293), and 293 cells transiently transfected with a Brn-3b expression construct (Brn-3b) or an Oct-2 expression construct (Oct-2) were utilized for gel mobility shift assays. (B) DNA-binding affinity of Brn-3b for the selected Brn-3 consensus site (C), mutant sites (M1-6), the Unc-86-binding site (M7), and the octamer site (Oct). Whole-cell protein extracts from 293 cells transiently transfected with the Brn-3b expression construct were used for gel mobility shift assays. Mobility-shifted complexes with Bm-3b, Oct-1, and Oct-2 are indicated. Note that all of the 293 cell extracts contain endogenous Oct-1. (F) Free probe. (C) Nucleotide sequences of one strand of the double-stranded DNA segments used for mobility shift assays in A and B. M1-M6 each differ by two nucleotides from the consensus obtained by the selex method. (In Xiang et al. [1995], the opposite strands of sequences C and M1-M6 were shown.) (D) Possible geometries of Brn-3 POU-domain-DNA interactions, based on the protein-DNA contacts observed in the Oct-1-DNA crystal structure (Klemm et al. 1994). The amino-terminal POU-specific domain (POUSD) and the carboxy-terminal POU-homeodomain (POUHD) are represented by trapezoids connected by a space; the labels POUSD and POUHD at the top of the figure refer to the Oct-1-DNA complex. In the model of Brn-3b binding to the Unc-86 consensus site (M7), the POUSD is positioned to the left of the POUHD, and in the two models of Brn-3b binding to the selex consensus site (C), the POUSD is to the right of the POUHD. (N) Amino terminus; (C) carboxyl terminus. (Reprinted, with permission, from Xiang et al. 1995.)
proteins bind poorly to the canonical octamer site, and, conversely, Oct-1 and Oct-2 bind poorly to the selected Brn-3 consensus site. These observations suggest that the Brn-3 proteins act on target genes in vivo that are distinct from those of previously described POU-domain proteins.

Brn-3a Is Required for the Development of Primary Somatosensory Neurons and Select Brainstem Sensory and Motor Nuclei

To determine the role of the Brn-3 family in vivo, mice lacking either Brn-3a, Brn-3b, or Brn-3c were generated by homologous recombination in embryonic stem cells. The phenotypes associated with the three targeted deletions are described in Gan et al. (1996) and Xiang et al. (1996, 1997) and are summarized below. All of the phenotypes that we have been able to identify are recessive with complete penetrance. Similar results have been obtained by Erkman et al. (1996) and McEvilly et al. (1996).

Brn-3a (−/−) mice exhibit grossly normal prenatal development but do not survive beyond 24 hours after birth. Brn-3a (−/−) neonates display two overt behavioral defects: They lack a suckle reflex (and therefore do not nurse), and they lack the coordinated limb and trunk movements required to right themselves. When a Brn-3a (−/−) neonate is placed on its back or side, it typically stretches its limbs ineffectually and displays extended postures not observed in the wild type (Fig. 5A). Anatomically and histologically, Brn-3a (−/−) mice at e20 and P0 show a twofold decrease in the volume of the trigeminal ganglion and a severalfold decrease in the density of trigeminal neurons relative to the wild type (Fig. 5B–E). Although there is little or no difference between Brn-3a (+/+) and (−/−) animals in the appearance or number of dorsal root ganglion cells, Brn-3b immunoreactive cells are nearly absent in the trigeminal and dorsal root ganglia of Brn-3a (−/−) mice, and Brn-3c immunoreactive cells are diminished in number by five- to tenfold (Fig. 5F–I). Three sites of Brn-3a expression in the brainstem are also affected in Brn-3a (−/−) mice: The medial habenula and the caudal region of the inferior olivary nucleus show a modest diminution in cell number, and the large neurons of the red nucleus, which are clearly seen in wild-type mice as two symmetric clusters, are not seen in Brn-3a (−/−) mice (Fig. 5J–O). Other parts of the CNS, including those regions of the brainstem and retina 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. Although 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 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 rubro-spinal tract, and the inferior olivary nucleus integrates sensory information and sends its output to cerebellar Purkinje cells, defects in these two nuclei could plausibly impair limb and trunk coordination or posture (Paxinos 1995). Functional alterations in the dorsal root ganglia related to the loss of Brn-3b and Brn-3c expression may also be relevant.

Brn-3b Is Required for Retinal Ganglion Cell Development

Brn-3b (−/−) and (+/+) mice are indistinguishable from their wild-type littermates in viability, growth rate, fertility, gait, and response to handling and loud sounds. These characteristics suggest that in Brn-3b (−/−) and (+/+) mice, the somatosensory, motor, and auditory/vestibular systems are grossly intact. Visual system function has not been assessed behaviorally, but it is likely to be at least partially functional as determined by recording light responses from retinal ganglion and/or amacrine cells with an array electrode (E. Soucy et al., unpubl.).

The only gross anatomic defects in Brn-3b (−/−) mice are associated with the eye: Brn-3b (−/−) mice show, on average, a fivefold reduction in the cross-sectional area of the optic nerve and a corresponding reduction in ganglion cell number. Whereas the overall structure of the Brn-3b (−/−) retina resembles that of the wild type, retinas from Brn-3b (−/−) mice are on average 20% thinner, due primarily to a decrease in the thickness of the inner plexiform, ganglion cell, and nerve fiber layers (Fig. 6A–D). The number of nuclei in the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer is reduced by 30%, 15%, and 10%, respectively (Fig. 6A–F).

In the ganglion cell layer of both mouse and rat retinas, displaced amacrine cells and ganglion cells are equally abundant. To assess independently these cell types, retinas were immunostained for Thy-1, which is present in the axons and dendrites of all ganglion cells, for phosphorylated neurofilaments (monoclonal antibody SMI-32), which is found predominantly in the axons of large ganglion cells, and for the amacrine cell markers tyrosine hydroxylase and glutamic acid decarboxylase. As seen in Figure 6, G–J, Brn-3b (−/−) mice show a large reduction in both anti-Thy-1 and SMI-32 immunoreactive processes, suggesting a significant loss of retinal ganglion cells. Ganglion cells expressing Brn-3a and Brn-3c are reduced in number approximately sixfold (Fig. 6K,L,O,P). In contrast, the density of dopaminergic and GABAergic amacrine cells does not differ between Brn-3b (+/+) and (−/−) retinas. Other parts of the CNS, including those regions where Brn-3b is expressed, appear to be unaffected.

In the mouse, [3H]thymidine-labeling studies show that between embryonic days 11 and 18 (e11 and e18), most
Figure 5. Phenotypes of Brn-3a (−/−) mice. (A) Behavioral differences between Brn-3a (+/+ ) and Brn-3a (−/−) siblings at P0. The Brn-3a (+/+ ) mouse (right) has nursed as seen by the presence of milk in its stomach; 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; the Brn-3a (−/−) animal extends its limbs, head, and trunk but fails to right itself. Cresyl violet staining (B−E,N,O) and immunostaining with anti-Brn-3a antibodies (F,G,I,K) or anti-Brn-3b antibodies (H,I,L,M) of sections from e20 Brn-3a (+/+ ) mice (B,D,F,H,I,L,N; i.e., the left member of each pair of matched panels) or Brn-3a (−/−) mice (C,E,G,I,K,M,O; i.e., the right member of each pair of matched panels). (B−E) Trigeminal ganglia in Brn-3a (−/−) mice at e20. (B−E) Cresyl violet staining shows a twofold reduction in size of the Brn-3a (−/−) trigeminal ganglia (C) relative to the Brn-3a (+/+ ) trigeminal ganglion (B) and a selective loss of large cells (compare D and E; large vertical arrows, large cells; arrowheads, small cells). In Brn-3a (+/+ ) trigeminal ganglia, Brn-3a immunoreactivity is present in most neurons (F); in Brn-3a (−/−) trigeminal ganglia, Brn-3a immunoreactivity is absent as expected (G). In Brn-3a (−/−) trigeminal ganglia, the density of Brn-3b immunoreactive cells is greatly reduced (compare H and I; rare Brn-3b immunoreactive cells are indicated by small arrows). (J−O) The midbrain at the level of the red nucleus 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 (J); in the Brn-3a (−/−) midbrain, Brn-3a immunoreactivity is absent as expected (K). Brn-3b immunoreactive neurons are present in the superior colliculus, dorsal central grey, and the interpeduncular nucleus regardless of Brn-3a genotype (L,M). By cresyl violet staining, the large neurons of the red nucleus are readily visualized in Brn-3a (+/+ ) mice (N) but are absent in Brn-3a (−/−) mice (O; arrows indicate the expected location of the red nucleus). (TGG) Trigeminal ganglion; (Br) brain; (Aq) aqueduct; (DCG) dorsal central grey; (IP) interpeduncular nucleus; (RN) red nucleus; (SC) superior colliculus. Bars: (B,C) 200 μm; (D,E) 25 μm; (F−I) 50 μm; (J−M) 200 μm; (N,O) 400 μm. (Reprinted, with permission, from Xiang et al. 1996.)
Figure 6. Reduction in the number of retinal ganglion cells in Brn-3b (−/−) mice. For each pair of micrographs, a Brn-3b (+/++) retina is on the left (A, C, E, G, I, K, M, O) and a Brn-3b (−/−) retina is on the right (B, D, F, H, J, L, N, P). The paired samples were obtained from adult littermates. In all micrographs of cross sections, the inner retina is at the bottom. (A, B) 1-μm thick plastic sections of retina stained with toluidine blue. (C, D) DAPI staining to visualize nuclei in 10-μm sections of retina. (E, F) Retinal whole mounts in which nuclei are visualized by staining with SYTOX, the region shown is in the vicinity of the optic disc and the plane of focus is in the ganglion cell layer. (G, H) 10-μm sections immunostained with anti-Thy-1 monoclonal antibody, a marker for ganglion cells. (J, L) Retinal whole mounts immunostained with mAb SMI-32, a marker for large ganglion cells and their axons. (K–P) Retinal whole mounts immunostained with affinity-purified anti-Brd3a (K, L), anti-Brd3b (M, N), or anti-Brd3c (O, P). In Brn-3b (−/−) mice, the number of Brd-3a and Brd-3c immunoreactive nuclei is reduced approximately fivefold; Brd-3b immunoreactivity is absent as expected. As described previously (Xiang et al. 1993 1995), the abundance of the Brd-3 proteins shows a characteristic heterogeneity among different cells. (OS) Outer segments; (IS) inner segments; (ONL) outer nuclear layer; (OPL) outer plexiform layer; (INL) inner nuclear layer; (IPL) inner plexiform layer; (GCL) ganglion cell layer; (NFL) nerve fiber layer. Bars, 25 μm. Bars in B and O refer to A–D and E–P, respectively. (Reprinted, with permission, from Gan et al. 1996.)
ganglion cell precursors become postmitotic and migrate from the proliferative zone in the outer retina to the future ganglion cell layer at the inner surface of the retina (Sidman 1961). As seen in Figure 3, G–J, Brn-3b expression commences in presumptive ganglion cells during their migration from the proliferative zone; Brn-3a and Brn-3c expression begins 1-2 days later (M. Xiang, unpubl.). In the Brn-3b (-/-) retina, a significant decrease in cell number or an increase in cell death in the ganglion cell layer is not seen during the prenatal period. However, a five-fold decrease in the number of cells expressing Brn-3a and Brn-3c is observed beginning at e13.5, the earliest times at which Brn-3a and Brn-3c immunoreactivity can be detected. Recently, experiments in which a β-galactosidase reporter has been targeted to the deleted Brn-3b-coding region indicate that in the Brn-3b (-/-) retina, those cells that were destined to express Brn-3b persist into early postnatal life (L. Gan and W. Klein, unpubl.). Thus, the reduction in the number of ganglion cells expressing Brn-3a and Brn-3c in the embryonic retina reflects a block in the expression of the Brn-3a and Brn-3c genes, indicative of an early developmental defect in these cells. The ultimate fate of these aberrant cells remains to be determined.

**Brn-3c Is Required for Auditory and Vestibular Hair Cell Development**

Brn-3c (-/-) mice have normal viability, but are 10–20% smaller than wild type, have low fertility, and spend much of their time running in circles, a behavior that has been described for a number of mouse lines with inner ear defects (Fuller and Wimer 1966). Brn-3c (-/-) mice also lack a startle response to sound and show no auditory brainstem response, even at stimulus levels 60 dB higher than the threshold for wild-type mice (Fig. 7A). To test vestibular function, mice were placed individually on a horizontal drum, and the time elapsed until they fell was recorded (Fig. 7B). Brn-3c (+/-) and (+/-) animals rarely fell from the drum, even when it was slowly rotated. In contrast, Brn-3c (-/-) mice exhibit extremely poor balance, typically falling from a stationary drum within 10 seconds. When the drum was slowly rotated, forcing the Brn-3c (-/-) animals to walk, none remained on the drum after 5 seconds. As a second test of vestibular function, we monitored the ability of mice to remain upright and swim effectively. In this test, Brn-3c (+/-) and (+/-) mice remained upright and swim, whereas Brn-3c (-/-) mice tumbled about ineffectively.

Figure 7. Physiologic defects in Brn-3c (-/-) mice. (A) Auditory brainstem responses in Brn-3c (+/-) and Brn-3c (-/-) mice. Representative auditory brainstem responses to a click stimulus are shown at different stimulus intensities. Brn-3c (+/-) mice show a threshold between 49 and 59 dB SPL with robust responses at and above 59 dB SPL. Brn-3c (-/-) mice show no response at any stimulus level, including 109 dB SPL, the highest level shown here. The traces at 109 dB SPL show a stimulus artifact in the first 1 msec. Traces were produced by averaging more than 1000 responses. (Bottom) Percent of animals remaining on the drum during the 60 sec following their placement on it. Each of seven animals of the indicated genotypes was tested in ten trials in which the drum was stationary and in ten trials in which the drum was rotating at 7 rpm, a total of 70 trials for each genotype and test condition. (Reprinted, with permission, from Xiang et al. 1997.)
Figure 8. Anatomic defects in Brn-3c (-/-) mice. (A–D) Absence of hair cells in the organ of Corti and defects in the spiral ganglion in Brn3c (-/-) mice. (A) The organ of Corti from a Brn-3c (+/-) mouse. The single inner hair cell, the three outer hair cells, and the three underlying Deiter’s cells are clearly visible. (B) Part of a spiral ganglion from a Brn-3c (+/-) mouse showing a dense packing of myelinated axons and neuronal cell bodies. (C) The organ of Corti from a Brn-3c (-/-) mouse. Inner and outer hair cells are missing and the epithelium beneath the tectorial membrane contains only supporting cells. (D) The spiral ganglion from a Brn-3c (-/-) mouse contains fewer than 10% as many myelinated axons and neuronal cell bodies as the spiral ganglia of Brn-3c (+/+) or (+/-) mice. (E–H) Absence of hair cells in the otolith organs and cristae of Brn-3c (-/-) mice. (E) An otolith organ from a Brn-3c (+/-) mouse. Type I hair cells, the most abundant hair cell class, have a lightly stained nerve chalice surrounding a darkly stained cell body. Ciliary bundles can be seen protruding into the densely stained otoliths at the top. A single layer of supporting cells lies beneath the layer of hair cells. (F) The crista from a Brn-3c (+/+) mouse. Type I hair cells are abundant and their ciliary bundles are seen at the left side of the ampullary crest where the section is nearly perpendicular to the apical surface. (G, H) An otolith organ (G) and a crista (H) from a Brn-3c (-/-) mouse are devoid of hair cells. Presumptive supporting cells are seen within the epithelium. The density of axon bundles beneath the otolith organ is greatly reduced in the Brn-3c (-/-) animal. Tissues were embedded in Spurr’s resin and 1-μm sections were stained with methylene blue. (I–L) Whole-mount preparations of the organ of Corti in Brn-3c (+/+, (+/-), and (-/-) mice. (I) The organ of Corti from a Brn-3c (+/-) mouse stained with cresyl violet shows the wild-type arrangement of a single row of inner hair cells (out of focal plan) and three rows of outer hair cells (in the focal plane). (K) The organ of Corti from a Brn-3c (+/-) mouse lacks identifiable hair cells (J) and cholinergic innervation (L). Bars: (A–H) 25 μm; (I–L) 50 μm. (Reprinted, with permission, from Xiang et al. 1977.)

Anatomically and histologically, Brn-3c (-/-) mice show a rapid and progressive loss of auditory and vestibular hair cells during late gestation and early postnatal life. Spiral and vestibular ganglion neurons degenerate over the ensuing weeks, but the middle ear and the overall architecture of the inner ear are unaffected. By adulthood,
auditory and vestibular ganglia contain few neuronal cell bodies or fibers (Fig. 8). Since Brn-3c is not expressed in supporting cells or in the spiral or vestibular ganglia during development, loss of spiral and vestibular ganglion neurons is likely to be secondary to the loss of hair cells. Brn-3c (−/−) mice show no differences in the size, number, or arrangement of neurons in the retina, dorsal root and trigeminal ganglia, and midbrain.

DISCUSSION

The high degree of sequence similarity between the POU-domains of unc-86 and the Brn-3 family, together with the conserved Brn-3 gene structures, suggests that the Brn-3 genes arose by duplication and divergence from 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, and the unc-86 phenotype includes defects in mechanosensation, chemosensation, and egg laying (Hodgkin et al. 1979; Chalfie et al. 1981; Finney and Ruvkin 1990). 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.

In mammals, each member of the Brn-3 family is expressed in a small subset of neurons in the brainstem and in the auditory/vestibular, somatosensory, and visual systems. Expression in each sensory system is confined to cells close to or at the site of sensory transduction: cochlear and vestibular hair cells and their associated ganglia, 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; and the only defects apparent in Brn-3c (−/−) mice are a loss of vestibular and cochlear hair cells and a secondary degeneration of their associated sensory ganglia. Brn-3a (−/−) mice show a decrease in the number of neurons in the trigeminal ganglia and in select brainstem nuclei but do not show any abnormalities in the retina. However, the early lethality of Brn-3a (−/−) animals means that a requirement for Brn-3a in the postnatal survival of other neuronal populations would have been missed in the analyses performed thus far.

The functional diversification of Brn-3 family members indicates that the expansion and evolution of specialized sensory systems in more complex organisms have been accompanied by a parallel expansion and evolution of genetic regulatory proteins. These observations suggest that despite the great differences in transduction mechanism, sensory organ structure, and central information processing between the auditory/vestibular, somatosensory, and visual systems, there may be fundamental homologies in the genetic regulatory events that control their development.

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REFERENCES


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