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Direct autoregulation and gene dosage compensation by POU-domain transcription factor Brn3a

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SUMMARY

Brn3a is a POU-domain transcription factor expressed in peripheral sensory neurons and in specific interneurons of the caudal CNS. Sensory expression of Brn3a is regulated by a specific upstream enhancer, the activity of which is greatly increased in Brn3a knockout mice, implying that Brn3a negatively regulates its own expression. Brn3a binds to highly conserved sites within this enhancer, and alteration of these sites abolishes Brn3a regulation of reporter transgenes. Furthermore, endogenous Brn3a expression levels in the sensory ganglia of *Brn3a*$^{+/+}$ and *Brn3a*$^{+/-}$ mice are similar, demonstrating that autoregulation can compensate for the loss of one allele by increasing transcription of the remaining gene copy. Conversely, transgenic overexpression of Brn3a in the trigeminal ganglion suppresses the expression of the endogenous gene. These findings demonstrate that the Brn3a locus functions as a self-regulating unit to maintain a constant expression level of this key regulator of neural development.

Key words: Brn3a, POU-domain, Transcription factor, Autoregulation, Mouse

INTRODUCTION

The generation of cellular diversity in the developing nervous system presents an enormous problem in the regulation of gene expression. We have been engaged in studies of the vertebrate members of the POU4 or Brn3 class of transcription factors, which play a role in determining neuronal identity and survival in diverse organisms. In vertebrates, the most widely expressed member of this class, Brn3a (Pou4f1 – Mouse Genome Informatics), is required for the survival of a majority of peripheral sensory neurons (Huang et al., 1999; McEvilly et al., 1996; Xiang et al., 1996), and is also expressed in specific neurons of the midbrain, hindbrain and spinal cord (Fedtsova and Turner, 1995). Two closely related members of this family, Brn3b (Pou4f2 – Mouse Genome Informatics) and Brn3c (Pou4f3 – Mouse Genome Informatics), are more restricted in their expression and are necessary for the development of retinal ganglion cells and cochlear hair cells, respectively (Erkman et al., 1996; Gan et al., 1996; Xiang et al., 1997).

Recently, we have shown that an enhancer from the mouse *Brn3a* locus targets transgene expression specifically to sensory neurons that express this factor, but not to the CNS. Using the Brn3a sensory enhancer to target a *lacZ* reporter transgene, we showed that mice lacking Brn3a exhibit marked defects in sensory axon growth, and that all of the sensory neurons that express Brn3a require it for survival in late gestation (Eng et al., 2001). Defective growth of sensory axons has also been reported for Brn3a null mutants in the development of the vestibulocochlear system (Huang et al., 2001). Brn3b is required for the normal growth of retinal ganglion cell axons (Erkman et al., 2000; Gan et al., 1999), and in the developing retina, Brn3b and Brn3c appear to have complimentary roles in regulating axon outgrowth (Wang et al., 2002).

The invertebrate Brn3 homologs Unc-86 and Acj6 also regulate sensory neural development. In nematodes, Unc-86 is required for the development of mechanoreceptor neurons, and manipulation of Unc-86 transcriptional activity can cause defects in neuronal migration and axon growth (Sze et al., 1997). In the *Drosophila* antenna, Acj6 is necessary for the expression of a subset of olfactory receptors (Clyne et al., 1999a; Clyne et al., 1999b), and in the CNS Acj6 is required for the correct projection of retinal axons to lamina of the optic lobe, while mis-expression in motoneurons produces abnormal axon outgrowth (Certel et al., 2000).

In vitro studies have shown that all of the POU4 factors have very similar DNA recognition properties, binding with high affinity to the motif ATAATTAAT or very similar sequences (Gruber et al., 1997; Turner, 1996). Presumably, the Brn3 factors regulate specific genes required for sensory neural development via such sites. In a variety of transfected cells in culture, Brn3a enhances the transcription of reporters linked to both synthetic and naturally occurring binding sites. Several downstream targets of the Brn3 genes have been suggested (Budhram-Mahadeo et al., 1995; Erkman et al., 2000; Smith et al., 1997), but a direct role in the regulation of these genes in vivo has not been established. In previous work, we have shown that Brn3a can interact with specific recognition

sequences within its own sensory enhancer region, suggesting that Brn3a may regulate its own expression (Trieu et al., 1999).

We demonstrate that Brn3a negatively regulates its own expression in vivo, and that this regulation is mediated by a direct interaction between Brn3a and its recognition elements within the Brn3a sensory enhancer region. Comparison of the mouse and human *Brn3a* loci reveals that this regulatory region is remarkably conserved across species. Assays of Brn3a mRNA levels in mice with one or two functioning copies of the *Brn3a* gene show that this autoregulatory mechanism leads to a compensatory increase in the transcription of the intact copy of the *Brn3a* gene in heterozygotes, resulting in similar mRNA levels regardless of gene dosage. These findings suggest that Brn3a may function generally as a negative regulator of transcription in the sensory neurons, and that autoregulation of the Brn3a transcription unit can compensate for the loss of one allele, potentially suppressing haploinsufficiency in Brn3a heterozygotes.

MATERIALS AND METHODS

Embryo staging and staining

For all analyses of transgenic mouse embryos, timed matings were performed in the afternoon, and noon on the day of the appearance of a mucous plug was assigned as embryonic day 0.5 (E0.5). Whole embryo staining for β-galactosidase (β-gal) expression was performed as previously described (Eng et al., 2001). For direct comparison of β-gal expression between littermates with different genotypes, embryos were always stained simultaneously under identical conditions. Immunohistochemical analysis of Brn3a expression was performed in paraformaldehyde-fixed sections using a rabbit anti-Brn3a antiserum, which has been previously described (Fedtsova and Turner, 1995).

Gene expression assays

RT-PCR for the relative quantitation of mRNA levels was performed using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturers protocol. Reverse transcriptase reactions included gene-specific primers consisting of 1×10^{-11} mol of each of the 3′ primers required for the intended assays. PCR reactions were conducted with Platinum Taq polymerase (Invitrogen) using ^{32}P-radiolabeled oligonucleotides. For each transcript assayed, preliminary PCR reactions were conducted with varying cycle numbers to determine the appropriate number of cycles for quantitative assays, at which 2-10% of the final product was formed. Assays for quantitation were then performed in triplicate, the products separated in polyacrylamide gels and quantitated on a phosphorimager. Oligonucleotide primers for the RT-PCR reactions were:

GAPDH, 5′ TCATGACCACAGTCCATGCCATCA; 3′, TGAAGGTCGGTGTGAACGGATTTGGC

Brn3a endogenous mRNA, 5′ CGCCAAGATGATGTCCATGAACAG

Brn3a-myc transgene, 5′ GAAGAGGACTTGAGATCTATGAACAG

Brn3a common 3′ oligonucleotide, CTGGCGAAGAGGTTGCTCTGCAG

β-galactosidase, 5′ CTG TAC TGG AGG CTG AAG TTC AGA; 3′ CATCGCAGGCTTCTGCTTCAATCA

neuron specific enolase, 5′ GCAGGATTGCACCAGCCCTCATC; 3′ GCATGGCATCCCGAAAGCTCTCA.

Oligonucleotide primers for the 18S ribosomal RNA were obtained from Ambion.

Genomic cloning and sequencing

Human BAC genomic clones encompassing the *Brn3a* locus were obtained by PCR-based screening of a human BAC library as a commercial service of Incyte Genomics. BAC clones were mapped by Southern hybridization using probes derived from mouse Brn3a cDNA, and subcloned into pBKS. Approximately 10 kb of the human *BRN3A* gene (*POU4F1* – Human Gene Nomenclature Database, extending upstream 8 kb from the open reading frame, were sequenced by the shotgun method. Plasmid insert DNA encompassing this region was sheared by sonication to an average fragment size of 200-400 bases, repaired with Mung Bean Nuclease and blunt end ligated into the vector pBKSII. About 50 overlapping cloned fragments were sequenced and assembled to produce a contiguous map. Pairwise alignment of the homologous mouse and human genomic sequences was performed using MegAlign software (Lasergene).

DNA binding and transcription assays

Complex-stability electrophoretic mobility shift assays (EMSAs) were performed with DNA fragments excised from plasmid vectors, which were then dephosphorylated, and end-labeled with ^{32}P. Each assay contained 5×10^{-14} moles of radiolabeled DNA and 1×10^{-14} moles of Brn3a-GST fusion protein. At the zero time point of each dissociation assay, 4×10^{-12} moles of specific competitor oligonucleotide containing a consensus Brn3a recognition sequence were added. The sequence of the oligonucleotide competitor and the assay and electrophoresis conditions have been previously described (Trieu et al., 1999). Under the conditions of this assay, complexes of Brn3a with DNA sites that have high enough affinity to mediate effectively the transcriptional effects of this factor exhibit dissociation half lives of 5-100 minutes.

RESULTS

Brn3a negatively regulates its own expression in vivo

Brn3a is expressed in terminally differentiating neurons in the sensory peripheral nervous system (PNS) and in specific neurons of the midbrain, hindbrain and spinal cord. In prior work, we have shown in transgenic mice that 11 kb of Brn3a upstream flanking sequence (Fig. 1A) is sufficient to direct reporter gene expression to the sensory neurons expressing Brn3a, but not to Brn3a neurons in the CNS. In an E13.5 embryo expressing this transgene, βgal activity is evident in the trigeminal ganglion, vestibulocochlear ganglion, the IX/X ganglion complex, the dorsal root ganglia, and the central and peripheral axons of these groups of neurons (Fig. 1B).

In prior experiments, we interbred this Brn3a/LacZ reporter strain with mice carrying a Brn3a null allele to demonstrate defects in axonal growth in Brn3a knockout mice (Eng et al., 2001). In the subsequent examination of a large number of embryos, we noted that when littermates were stained for β-gal expression under identical conditions, the signal intensity was inversely proportional to Brn3a gene dosage, with a marked upregulation of β-gal expression in Brn3a knockout embryos. Fig. 1C shows this differential β-gal activity in the cervical dorsal root ganglia of Brn3a wild-type, heterozygote and knockout littermate embryos carrying the Brn3a/*lacZ* reporter. A similar inverse relationship between transgene expression and Brn3a gene dosage was also observed for the trigeminal ganglion and the other cranial sensory ganglia expressing Brn3a.

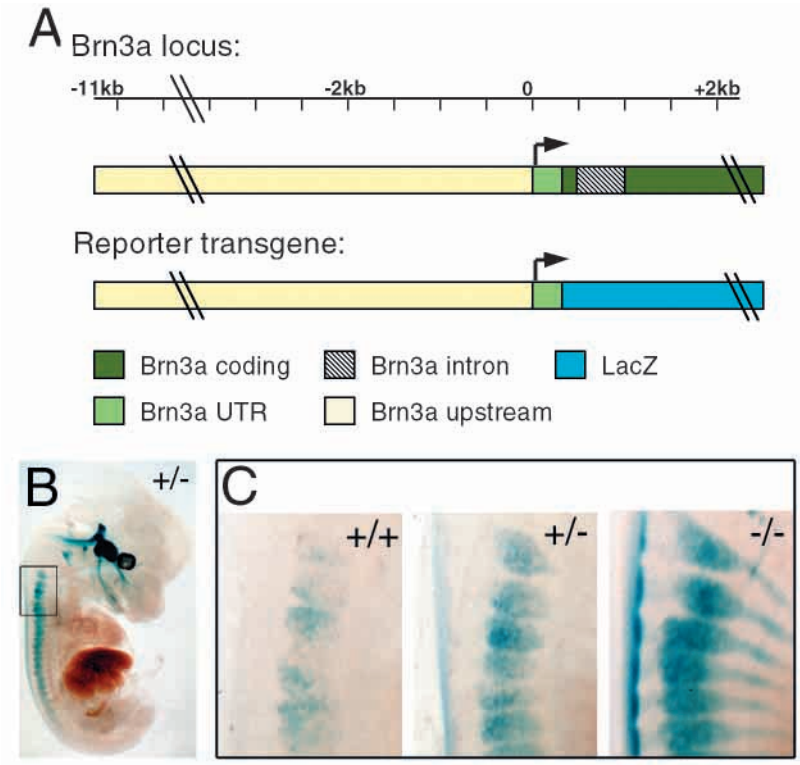


Fig. 1. Transgene expression controlled by a Brn3a sensory enhancer is strongly autoregulated. (A) Diagram of the mouse *Brn3a* locus and the structure of a *Brn3a*/*lacZ* transgene. (B) The *Brn3a* upstream flanking sequences target β-galactosidase expression to the cranial sensory and dorsal root ganglia and their axons in an E13.5 *Brn3a* heterozygote embryo. (C) Enlargement of the cervical dorsal root ganglia of three littermate embryos with different *Brn3a* genotypes, stained under identical conditions, in which β-gal activity is inversely proportional to *Brn3a* gene dosage.

RT-PCR assays were then used to examine the developmental relationship between Brn3a genotype and Brn3a/*lacZ* transgene expression, and to verify that the observed changes in β-gal activity represented altered mRNA levels, and not a difference in the stability or distribution of β-gal protein. Because β-gal expression from the Brn3a/*lacZ* transgene was restricted to the sensory ganglia, RNA isolated from whole embryonic head or trunk tissue could be used for these assays. At E10.5, β-gal activity was not yet detectable in sensory neurons by enzymatic staining, and no consistent difference in transgene expression between genotypes could be demonstrated by RT-PCR. At E12.5, cranial expression of β-gal mRNA was about 2.5-fold elevated in knockouts relative to wild type (Fig. 2A).

At E13.5, β-gal mRNA levels were consistently increased three- to fourfold in both the cranial sensory ganglia (head) and the dorsal root ganglia (trunk) of Brn3a knockout embryos relative to wild type (Fig. 2B,C), exhibiting a greater effect of gene dosage than that observed at E12.5. The onset of cell death in *Brn3a*$^{-/-}$ embryos prevented the examination of relative β-gal expression at stages later than E13.5. A likely explanation for the increasing effect observed with advancing developmental age is that the cellular level of Brn3a protein increases in early development, as sensory neurons exit the cell cycle and differentiate (Fedtsova and Turner, 1995), and by E13.5 reaches a threshold level that effectively suppresses transgene expression in heterozygote and wild-type embryos. To demonstrate that the gene dosage

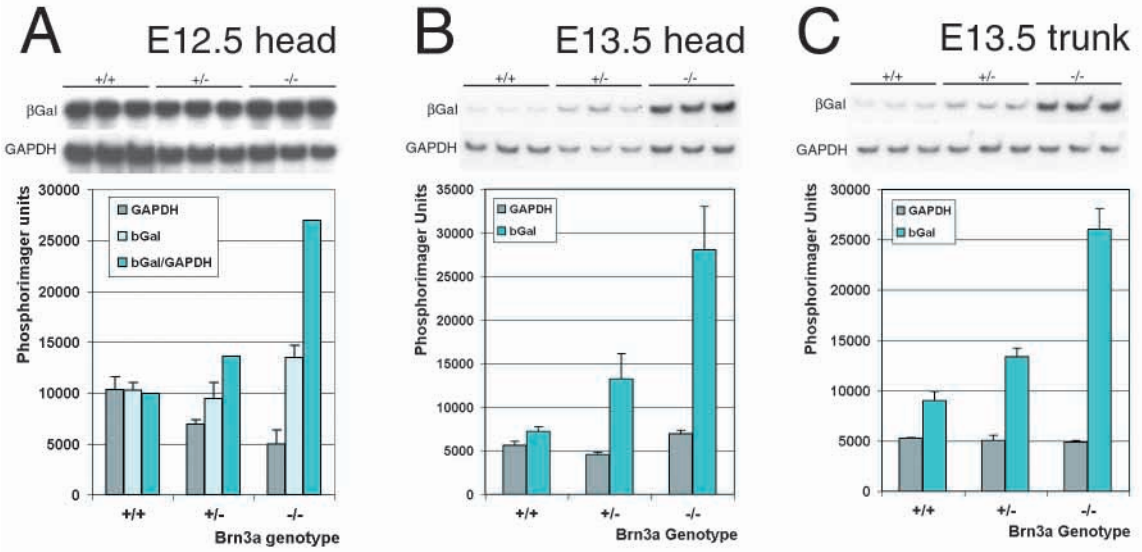


Fig. 2. *Brn3a* autoregulation increases with developmental age. The expression of β-gal mRNA from the *Brn3a*/*lacZ* transgene was measured in embryonic tissues by RT-PCR. Autoradiograms and the corresponding phosphorimager integration of the triplicate assays are shown. (A) E12.5 head, representing β-gal expression in the cranial sensory ganglia. (B) E13.5 head and (C) trunk, representing expression in the dorsal root ganglia. To confirm that Brn3a autoregulation is not dependent on the transgene insertion site, the relationship between β-gal expression and gene dosage was examined for two independent reporter lines, which gave indistinguishable results. The β-gal staining in Fig. 1B,C, and the quantitative assays in B represent the first of these lines, and the results shown in A,C are from the second line. Assays represent the mean±s.d. of triplicate PCR assays, and all determinations were repeated in at least three experiments. Phosphorimager units are arbitrarily scaled and cannot be compared between different sets of assays.

dependence of β-gal expression was intrinsic to the 11 kb Brn3a sensory enhancer, and not an artifact of the transgene insertion site, we replicated these results using β-gal staining and quantitative mRNA assays in two independent strains of reporter mice and obtained very similar results.

Brn3a interacts directly with a highly conserved autoregulatory domain

The expression pattern of Brn3a is very similar in the mouse (Fedtsova and Turner, 1995), rat (Turner et al., 1994) and chick (Fedtsova and Turner, 2001), and is likely to be conserved throughout the higher vertebrates. Thus, it would be reasonable to expect that the sequences that regulate Brn3a expression would also be somewhat conserved between species. To search for such regulatory regions, we cloned and sequenced the human Brn3a gene, including ~8 kb of upstream flanking DNA. Alignment of the mouse *Brn3a* gene and the corresponding human sequence revealed regions of partial sequence conservation, such as the G/C-rich region surrounding the transcription initiation site (Fig. 3), alternating with regions of divergence. In addition to these expected regions of partial sequence conservation, this comparison revealed an 'island' of highly conserved sequence residing between 5400 and 5900 bases upstream from the mouse Brn3a transcription start site, including a region of 244 bp that displayed 100% conservation between the mouse and human sequences. Although we considered the possibility that this conserved sequence represented the coding region of a closely adjacent gene, analysis of stop codons and codon usage did not reveal an open reading frame on either DNA strand. In previous studies, we have identified a cluster of Brn3a-binding sites that reside ~5.5 kb upstream from the mouse Brn3a transcription start site (Trieu et al., 1999). These sites are all included within the region of highest conservation between the mouse and human Brn3a sequences, and each of the Brn3a recognition elements is entirely conserved.

Although such an extensive region of exact sequence identity between mouse and human regulatory sequences is unusual, enhancer sequences of similar extent that are 80-90% conserved between these species have been reported for several genes, particularly for regulators of neural development. For example, the neural bHLH gene Math1 (Atoh1 – Mouse Genome Informatics), which is required for correct development of the spinal neural tube and cerebellum, is regulated by two 3′ enhancer elements of ~500 bp that show 87% and 92% homology between mouse and human (Helms et al., 2000). The mouse gene for another neurogenic bHLH factor, Ngn2, contains multiple 5′ and 3′ enhancers, with 80-

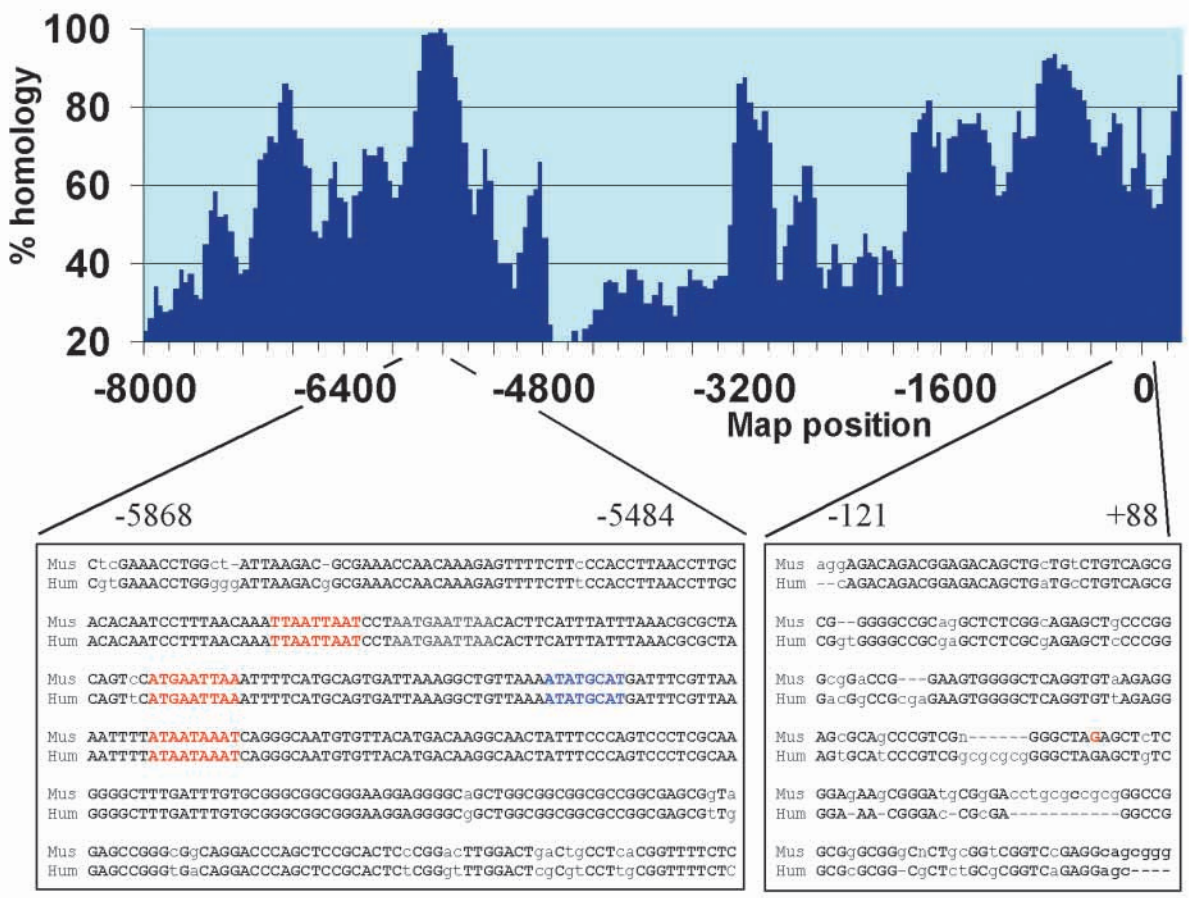


Fig. 3. Brn3a-binding sites are highly conserved in the mouse and human sensory enhancer regions. Pairwise alignment of the mouse and human Brn3a sequences reveals several regions of upstream homology, the most extensive of which encompasses a cluster of Brn3a binding sites (red), and an octamer site (blue). The *y*-axis represents the percent of base pair identity within a moving window of 120 bases, at intervals of 40 bases. The designated map positions are for the mouse sequence and are specified relative to the most frequently used transcription start site (red G residue in the right-hand box).

90% homology to the human sequence extending up to 800 bp (Scardigli et al., 2001; Simmons et al., 2001). Shorter functional regulatory sequences with a similar extent of conservation have been identified in the mouse and human *PAX6* (Kammandel et al., 1999), *PAX4* (Brink et al., 2001) and *PDX1* (*IPF1* – Human Genome Nomenclature Database) (Marshak et al., 2000) genes, among others. It is unclear whether the difference between the 80-90% conservation of the mouse and human sequences of the previously described enhancers and the 100% conservation of the Brn3a-binding region of Brn3a sensory enhancer has any regulatory significance, and it may be fortuitous. It is likely that all of these domains mediate conserved regulatory functions, although their roles in human development cannot be directly demonstrated.

Previously, we have shown that the Brn3a-binding sites in the sensory enhancer mediate transcriptional activation in co-transfection assays performed in an epithelial cell line, suggesting that Brn3a is a positive regulator of its own expression. In the transgenic experiments shown here, however, Brn3a clearly inhibits the activity of its sensory enhancer in developing sensory neurons. There are two possible explanations for this discrepancy between the cell transfection and the transgenic results. First, Brn3a might act to inhibit the activity of this enhancer through an indirect mechanism in vivo, perhaps through the induction of a downstream regulatory factor that negatively regulates Brn3a through distinct binding sites. Second, Brn3a may act directly in both cases, but with opposite effects on transcription, perhaps due to differing populations of co-activators or co-repressors in the different cell types.

To determine whether Brn3a regulates its own expression by direct interaction with its own upstream sequences in vivo, we introduced a number of single base-pair mutations in the Brn3a autoregulatory region. Several mutagenesis steps were required to eliminate Brn3a binding to this region completely, the last two of which are shown in Fig. 4A. In the final altered enhancer sequence, 19 bp changes were introduced in four Brn3a consensus binding sites, a variant octamer site and two AT-rich sites that weakly matched the Brn3a consensus. As shown in Fig. 4B, the elimination of significant Brn3a-binding activity to the mutant enhancer sequences was then demonstrated using by complex stability electrophoretic mobility shift assays (Trieu et al., 1999). In these assays, recombinant Brn3a protein is allowed to form a complex with radiolabeled DNA. Then, at various times prior to electrophoresis, a large excess of unlabeled competitor oligonucleotide is added. In the presence of the competitor, the dissociation of the complex between Brn3a and the labeled site exhibits first-order kinetics and a constant half-time ($T_{1/2}$) of dissociation (Gruber et al., 1997). In prior work, we have shown that the stability of Brn3a binding to its recognition sites is highly correlated with its ability to mediate transcriptional effects, and under the

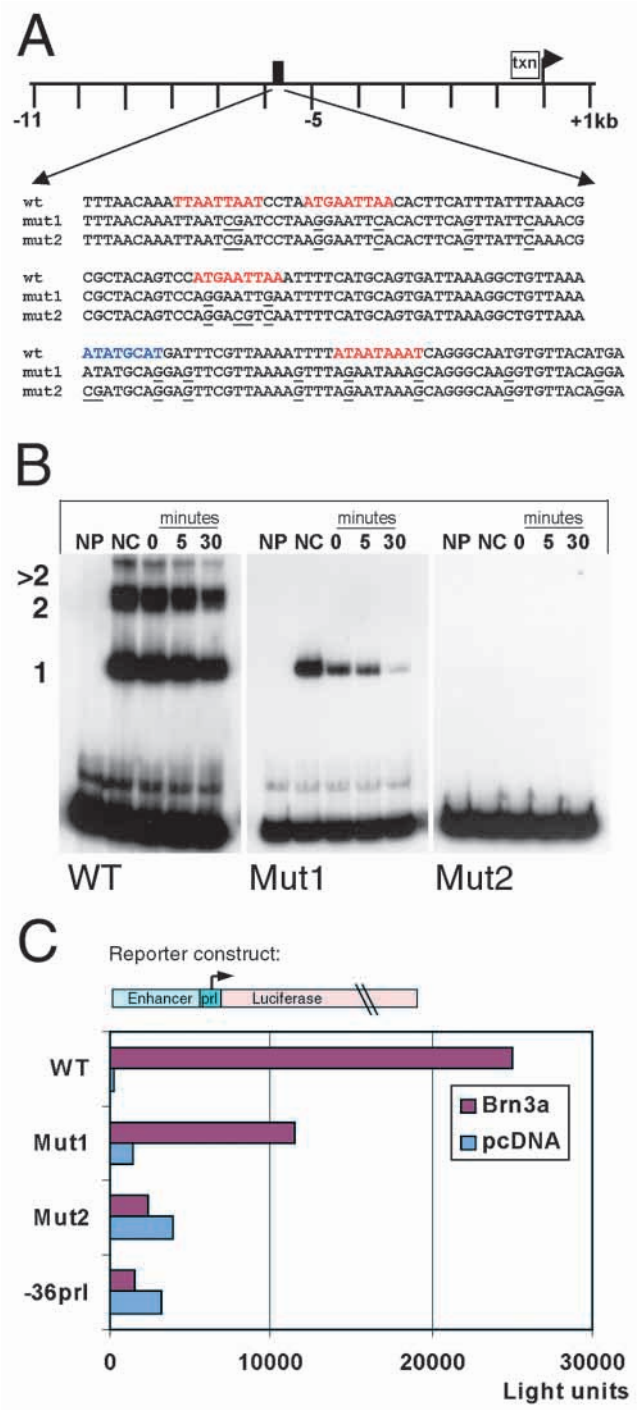


Fig. 4. Mutagenesis of the autoregulatory region of the Brn3a enhancer. (A) Shows the location of the Brn3a autoregulatory region, including four consensus Brn3a-binding domains (red) and one octamer binding site (blue). Two rounds of mutagenesis were required to eliminate Brn3a binding completely, finally incorporating 19 nucleotide changes (mut2). (B) The stoichiometry and stability of Brn3a/DNA complexes were assessed in complex-stability EMSA assays. In these assays, a competitor oligonucleotide was added at the stated time prior to the start of electrophoresis. The wild-type enhancer forms a stable complex with multiple Brn3a molecules, while the Mut1 enhancer retains a single stable binding site and binding is eliminated in Mut2. NP, no protein; NC, no competitor. (C) Luciferase reporter constructs containing wild-type or mutant enhancer domains, or a minimal promoter (–36prl) were co-transfected into CV1 epithelial cells with a Brn3a expression plasmid or vector alone (pcDNA). Elimination of the identified Brn3a-binding sites in the mutant enhancer construct eliminated transactivation by Brn3a. Bars represent the mean of triplicate assays, and similar results were observed in three experiments.

conditions of these assays, sites with a dissociation $T_{1/2}$ of less than 5 minutes are unlikely to be transcriptionally active.

The altered enhancer sequences were then tested in transfection assays in CV1 epithelial cells (Fig. 4C). As expected, the Mut1 enhancer shows significantly reduced transcriptional activation compared with wild type (eightfold compared with 90-fold), and the Mut2 enhancer showed no transcriptional effect of Brn3a. The Mut2 enhancer sequence was then used to construct a new *lacZ* transgene (Brn3a-mut/*lacZ*), which differed from the original Brn3a/*lacZ* transgene only by the introduced point mutations in the Brn3a recognition sites. The Brn3a-mut/*lacZ* transgene construct was then used to generate new lines of transgenic mice. Elimination of Brn3a binding to the sensory enhancer had no effect on the qualitative expression pattern of the transgene, with β-gal activity detectable in the cranial sensory ganglia, the dorsal root ganglia, and their central and peripheral axons (Fig. 5A,C).

Mice carrying the Brn3a-mut/*lacZ* transgene were then interbred with mice heterozygous for a Brn3a null allele, and the resulting *Brn3a*$^{+/-}$, Brn3a-mut/*lacZ* reporter mice were again crossed with *Brn3a*$^{+/-}$ mice to generate littermate embryos containing the mutant reporter and all three Brn3a genotypes (Fig. 5A-D). These embryos were examined by whole-mount β-gal staining at E13.5. Those embryos lacking Brn3a exhibited several defects consistent with previous findings (Eng et al., 2001), including defasciculation of axon bundles and aberrant axons, failure to correctly innervate peripheral targets, and an ectopic mass of cell bodies and fibers adjacent to the caudal hindbrain. Trigeminal innervation of the CNS was also abnormal, as shown in Fig. 5B, where the normal patterns of trigeminal innervation of the principal (pr5) and spinal (sp5) trigeminal nuclei are outlined.

Although β-gal expression in Brn3a-mut/*lacZ* embryos was qualitatively indistinguishable from that regulated by the wild-type enhancer, the relationship between β-gal expression and Brn3a gene dosage was nearly eliminated. Staining for β-gal activity appeared equal in Brn3a wild-type, heterozygote and knockout embryos, and this effect was consistent in two

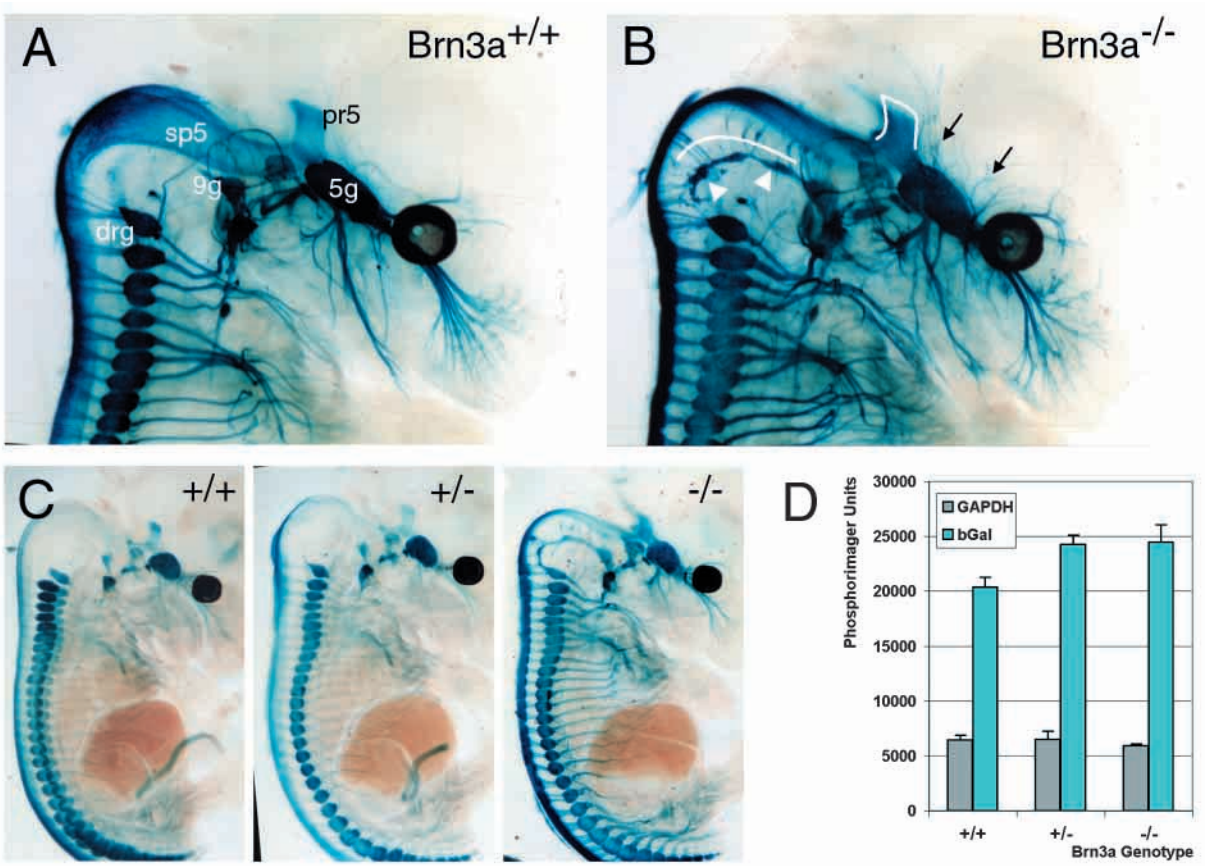


Fig. 5. Elimination of Brn3a binding to its own enhancer abolishes autoregulation. Embryos expressing a Brn3a-mut/*lacZ* transgene and varying Brn3a gene dosage were analyzed for β-gal expression. To ensure that the loss of autoregulation by the mutant enhancer was not an artifact of transgene insertion, two independent Brn3a-mut/*lacZ* lines were analyzed. (A,B) β-gal staining is similar in wild-type and knockout embryos in the first of two lines analyzed. β-gal expression in *Brn3a*$^{+/+}$ embryos (A) is qualitatively indistinguishable from embryos expressing the wild-type Brn3a/*lacZ* transgene. Abnormal features of the *Brn3a*$^{-/-}$ embryos are indicated in B, including defasciculation of axon bundles and aberrant axons (small arrows), an ectopic mass of cell bodies and fibers adjacent to the caudal hindbrain (arrowheads), and abnormal trigeminal innervation of the CNS [the normal extent of trigeminal innervation of the principal (pr5) and spinal (sp5) trigeminal nuclei is outlined]. 5g, trigeminal ganglion; 9g, 9/10 cranial ganglion complex; drg, dorsal root ganglion. (C) A second transgenic line in which β-gal expression is independent of Brn3a gene dosage for all three Brn3a genotypes. (D) RT-PCR assays of β-gal mRNA in E13.5 embryonic head demonstrate that transgene expression is only slightly increased in Brn3a knockout embryos.

independent Brn3a-mut/*lacZ* reporter lines. RT-PCR assays of RNA samples from E13.5 embryonic heads revealed that β-gal mRNA levels in knockout embryos were 120% of wild-type (Fig. 5D), compared with the 300-400% increase in expression observed for the wild-type sensory enhancer in Brn3a knockout embryos (Fig. 1). These findings demonstrate that negative autoregulation of the Brn3a sensory enhancer is mediated by a direct interaction between Brn3a and its specific upstream recognition sequences.

Autoregulation provides a mechanism for Brn3a gene dosage compensation

Brn3a heterozygote mice are viable, fertile and exhibit no obvious behavioral abnormalities. Detailed studies of sensory neuron survival (Huang et al., 1999) and axon growth (Eng et al., 2001) in Brn3a knockout mice also have not detected significant defects in heterozygotes. There are several possible explanations for the lack of haploinsufficiency in heterozygous Brn3a animals. However, Brn3a regulation of its own sensory enhancer suggests a direct transcriptional mechanism for gene dosage compensation in mice with only one functional Brn3a allele.

To test whether Brn3a mRNA levels were normalized toward wild-type expression levels in heterozygotes, we measured Brn3a mRNA in neural tissues from wild-type and heterozygous embryos (Fig. 6). In the E13.5 embryonic head, heterozygote Brn3a levels were 83±8% of wild-type. Brn3a-expressing neurons in the embryonic head include the cranial sensory ganglia and also developing Brn3a interneurons in the midbrain and hindbrain. Therefore, to determine whether autoregulation pertains to Brn3a-expressing neurons in both the sensory PNS and the CNS, we also assayed Brn3a mRNA in dissected E13.5 trigeminal ganglia and midbrain tecta from wild-type and heterozygous embryos. In the trigeminal ganglion three sets of triplicate assays yielded heterozygote Brn3a mRNA levels corresponding to 89%, 89% and 97% of wild type. For assays of Brn3a expression in the CNS, we harvested tissue samples from the developing tectum of E13.5 embryos as shown in Fig. 6C. The analysis of three matched pairs of midbrain samples from heterozygote and wild-type littermates showed heterozygote Brn3a expression levels that were 77%, 78% and 85% of wild type, suggesting that Brn3a is also autoregulated in CNS neurons, but possibly to a lesser extent than in sensory ganglia. Because the enhancer sequences regulating Brn3a expression in the CNS have not been identified, it is not possible to determine whether the autoregulatory sites in the Brn3a sensory enhancer also confer autoregulation in CNS neurons, or whether the regulation of Brn3a in the CNS and PNS is entirely independent.

To further test the effect of Brn3a on the regulation of its own transcription, we used the 11 kb Brn3a sensory enhancer to overexpress Brn3a in sensory ganglia. Fig. 7 shows the structure of the transgene construct used (Brn3a/Myc), which includes the Brn3a 11 kb sensory enhancer and a Brn3a cDNA insert that has been modified by the inclusion of a Myc epitope sequence immediately after the Brn3a initiator methionine codon. Because the native Brn3a sensory enhancer used to make this transgene is negatively autoregulated by Brn3a, it was anticipated that the transgene would be weakly expressed in *Brn3a*$^{+/+}$ animals, but show increased expression in Brn3a heterozygote and knockout mice. Negative autoregulation of the transgene in wild-type mice has the advantage of minimizing any deleterious effects of overexpressing Brn3a, and three transgenic lines carrying the Brn3a/Myc transgene were viable, fertile and showed no obvious behavioral deficits.

Brn3a/Myc transgenic mice were then interbred with *Brn3a*$^{+/-}$ mice, and Brn3a heterozygotes from this cross that expressed the Brn3a/Myc transgene were again bred to *Brn3a*$^{+/-}$ mice to generate *Brn3a*$^{-/-}$, *Brn3a/Myc*$^{+}$ embryos. The expression of Brn3a protein at E13.5 in these embryos was compared with wild-type littermates by immunohistochemistry. As expected, the normal pattern of Brn3a expression in spinal cord and hindbrain interneurons (Fig. 7B,D) was entirely absent in *Brn3a*$^{-/-}$, *Brn3a/Myc*$^{+}$ embryos (Fig. 7C,E). However, transgenic Brn3a expression in the dorsal root ganglia and trigeminal ganglion appeared qualitatively and quantitatively similar to wild type.

The loss of the majority of sensory neurons in late gestation

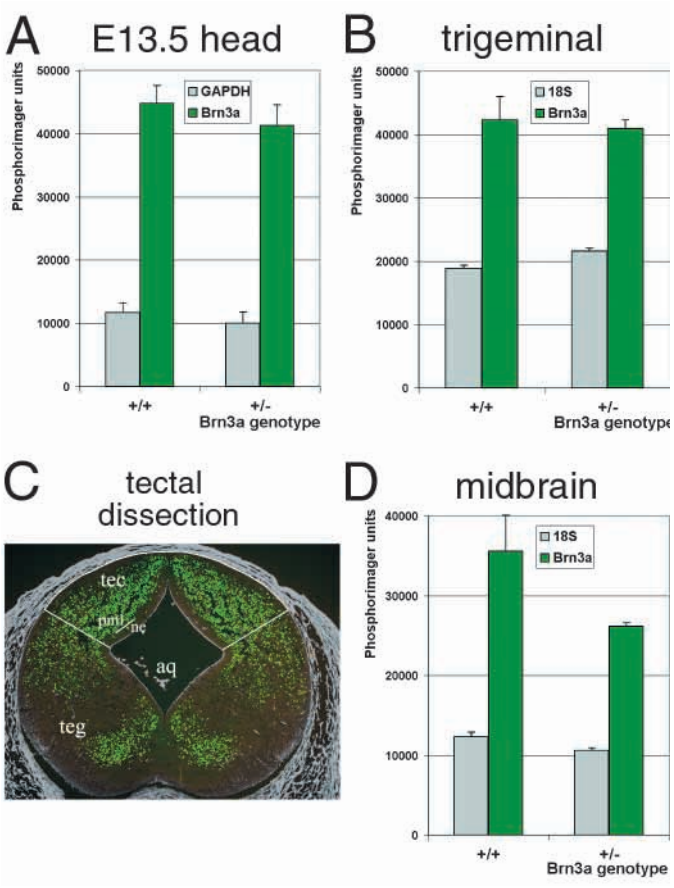
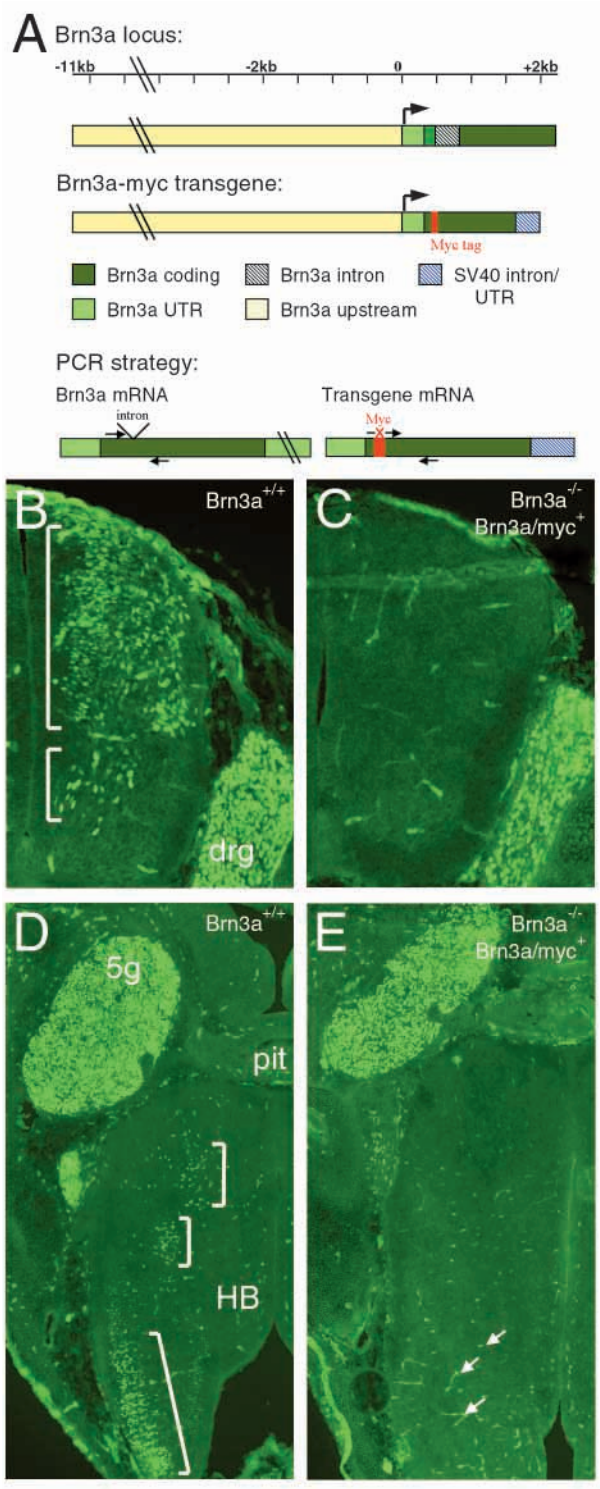


Fig. 6. Brn3a expression is normalized towards wild-type levels in Brn3a heterozygotes. Brn3a mRNA levels were assayed in E13.5 embryonic tissues by RT-PCR; the mean ±s.d. of triplicate assays are shown. (A) Brn3a expression in the intact E13.5 head, including Brn3a neurons in the midbrain, hindbrain and cranial sensory ganglia. (B) Brn3a expression in isolated trigeminal ganglia. (C) The location of tissue samples dissected from the E13.5 midbrain tectum, in which Brn3a expressing neurons are labeled by immunohistochemistry (green). aq, aqueduct; pml, post-mitotic layer; ne, neuroepithelium; tec, tectum; teg, tegmentum. (D) Brn3a expression in the isolated E13.5 tectum.



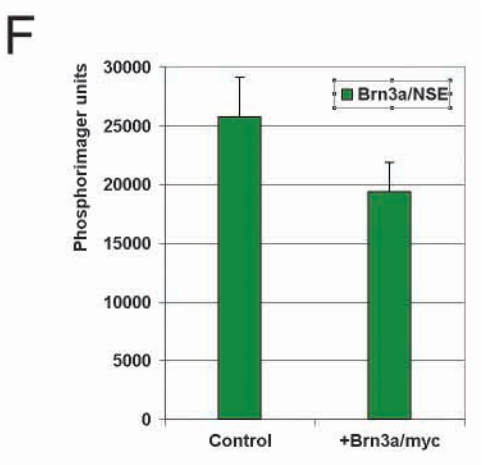


Fig. 7. Transgenic Brn3a overexpression suppresses endogenous Brn3a transcription. (A) Structure of a transgene in which the 11 kb Brn3a sensory enhancer is used to drive the expression of a Brn3a cDNA transgene. The transgene product can be distinguished from the endogenous *Brn3a* gene by the presence of a Myc epitope sequence immediately after the transcriptional initiation site. (B-E) Expression of the *Brn3a*/*Myc* transgene in E13.5 embryos. In the wild-type embryos shown in B,D, *Brn3a* is detected in the dorsal root and trigeminal ganglia, and in specific groups of spinal cord and hindbrain interneurons (brackets). In the *Brn3a* knockout mice shown in C,E, only *Brn3a* expression from the sensory transgene is detected, and Brn3a is not expressed in the CNS. The arrows in E indicate signal originating from vascular artifacts. 5g, trigeminal ganglion; drg, dorsal root ganglion; HB, hindbrain; pit, pituitary. (F) Quantitative assays of the expression of the endogenous *Brn3a* message in isolated trigeminal ganglia from Brn3a wild-type and heterozygous E13.5 embryos in the presence and absence of the *Brn3a*/*Myc* transgene. In these assays, *Brn3a* mRNA encoded by the native locus was assayed selectively by the use of a 5′-PCR oligonucleotide that is interrupted by the Myc epitope sequence inserted into the transgene. *Brn3a* data are normalized to the expression of neuron-specific enolase (NSE). It is unlikely that the reduction in *Brn3a* expression observed in the presence of the *Brn3a*/*Myc* transgene is due to the presence of regulatory sequences in the transgene itself because in the transgenic line used, the transgene was present at only one or two copies, based on quantitative RT-PCR comparison with a single copy gene.

is probably a sufficient cause of neonatal death in *Brn3a* knockout mice, as other mutant mice with extensive sensory loss, such as *Bdnf/Nt3* double knockouts, also die at birth (Liebl et al., 1997). We examined newborn mice (n=20) from $Brn3a^{+/-}$, $Brn3a/Myc^{+}$ crosses with $Brn3a^{+/-}$ mice to see if the transgenic expression of Brn3a in the sensory ganglia would rescue neonates from the expected lethal effects of the Brn3a null mutation. However, $Brn3a^{-/-}$, $Brn3a/Myc^{+}$ pups died within 24 hours of birth, as did the knockout neonates without the transgene. The failure of the transgenic expression of Brn3a in the sensory system to rescue pups from neonatal lethality suggests that the function of Brn3a in the CNS is also essential for survival.

Next, we determined the effect of the Brn3a/Myc transgene on Brn3a expression from the endogenous Brn3a locus in trigeminal ganglia isolated from E13.5 $Brn3a^{-/-}$, $Brn3a/Myc^{+}$ embryos. Transcripts originating from the native Brn3a locus were distinguished from the transgenic mRNA in RT-PCR assays by the use of a 5′ PCR oligonucleotide that was interrupted by the Myc epitope and thus did not amplify the transgene product (Fig. 7A). Expression of the Brn3a/Myc transgene led to a significant but modest reduction in levels of the endogenous *Brn3a* mRNA (Fig. 7F). The limited effect of the Brn3a transgene on the expression of the endogenous Brn3a mRNA is probably due to the fact that the transgene itself is negatively autoregulated in $Brn3a^{+/+}$ embryos, lessening the extent to which Brn3a can be overexpressed, and thus limiting the repression of the native Brn3a locus.

DISCUSSION

In the developing neural tube and neural crest, classes of progenitor cells are defined by the expression of specific transcription factors, often of the HLH variant homeodomain, and Pax family members. As these developing neurons become postmitotic, a second set of factors appear, many of which have been shown to have profound effects on neural phenotype and survival. In the spinal cord, these factors include Brn3a and several members of the Lim and variant homeodomain families (Briscoe and Ericson, 2001; Gross et al., 2002; Lee and Pfaff, 2001). In the sensory ganglia, terminally differentiating neurons are characterized by the co-expression of Brn3a and the Lim domain factor Isl1 (Fedtsova and Turner, 1997; Fedtsova and Turner, 2001).

In sensory neurons, Brn3a is essential for normal axon growth and for cell survival in late gestation (Eng et al., 2001; Huang et al., 1999; Huang et al., 2001). The role of Brn3a is less well understood in the CNS, but it appears to affect the migration and survival of at least some of the CNS neurons in which it is expressed (McEvilly et al., 1996; Xiang et al., 1996). Brn3a, like all transcriptional regulators of development, is assumed to affect gene expression by interacting with *cis*-acting elements in the regulatory regions of its target genes, and the DNA-binding properties of Brn3a and other neural POU-domain factors have been extensively characterized in vitro and in cell transfection models (Gruber et al., 1997; Rhee et al., 1998; Turner, 1996). However, very few of the direct regulatory targets of these late neural transcription factors have been identified, and in most cases it is not known whether they act primarily as enhancers or repressors of transcription.

In previous work, we have demonstrated that Brn3a expression in the sensory system, but not in the CNS, is regulated by an upstream sensory enhancer region containing multiple high-affinity Brn3a-binding sites (Eng et al., 2001). Here, we have shown, by manipulating the Brn3a-binding sites within this enhancer, that Brn3a directly attenuates its own expression in vivo. In sensory ganglia containing two intact copies of the *Brn3a* gene, transcriptional activity of each copy is partly suppressed. Conversely, when one allele is disrupted by a targeted mutation, the transcriptional activity of the intact allele is increased ~80%, nearly compensating for the inactive gene copy. When both alleles are disrupted, a functional Brn3a mRNA cannot be transcribed, but the activity of a reporter transgene driven by the Brn3a sensory enhancer is about fourfold increased. Partial compensation for Brn3a gene dosage also appears to occur in the midbrain tectum, but to a lesser extent than in the trigeminal ganglion. Whether Brn3a autoregulation in the tectum is controlled by the same upstream Brn3a recognition sites, or by distinct elements, cannot presently be determined, because a specific enhancer that regulates Brn3a expression in the CNS has not yet been identified.

Negative autoregulation by Brn3a raises the possibility that the general function of this factor will be to repress the transcription of all of its direct targets, at least in the developing sensory system. In the developing spinal cord, where the molecular mechanisms of neural specification are best understood, several homeodomain proteins that characterize progenitor cell domains in the spinal neuroepithelium act as transcriptional repressors (Lee and Pfaff, 2001). These repressive activities prevent the inappropriate expression of transcription factors that characterize adjacent domains, and refine the boundaries between their domains. By contrast, examples of direct positive regulation of downstream target genes by neural transcription factors are relatively few, and it appears increasingly likely that neurons are transcriptionally defined to a large extent by the repression of inappropriate gene expression.

The role of Brn3a as a negative regulator of its own expression in vivo is somewhat surprising given that it strongly activates transcription when co-transfected with reporter plasmids in cultured epithelial cells (Gruber et al., 1997; Trieu et al., 1999). This difference cannot be accounted for by the Brn3a recognition elements used or their immediate context, because in transfection assays Brn3a strongly activates transcription from reporters containing the same ~200 base pair autoregulatory region that confers negative regulation in vivo. Although it is possible that this reversal of transcriptional effect depends on the broader context of the *cis*-acting sequences, it seems more likely that sensory neurons express a co-repressor that converts Brn3a from a positive to a negative regulator of transcription, or that dividing epithelial cells express an essential Brn3a co-activator.

One set of candidates for a Brn3a co-repressor that could act in the sensory system are the murine Gro/TLE proteins, homologs of *Drosophila* groucho (Smith and Jaynes, 1996), which have recently been shown to mediate transcriptional repression by many of the progenitor zone homeodomain proteins in the developing spinal cord (Muhr et al., 2001). The Gro/TLE repressors interact with a variety of homeodomain proteins through a conserved Engrailed homology (eh1) domain. The locus for one factor containing an eh1 domain, the motoneuron transcription factor HB9, exhibits autoregulatory behavior very similar to Brn3a, in that reporter genes linked to this locus are markedly upregulated in HB9 knockout mice (Arber et al., 1999). However, it is not known whether HB9 autoregulation occurs by a direct or indirect mechanism. The Gro/TLE protein Grg4 is expressed in the developing cranial and dorsal root ganglia where it might be available to interact with Brn3a (Koop et al., 1996), but Brn3a does not have a readily apparent eh1 domain to mediate this interaction.

Autoregulation and haploinsufficiency

In humans, a majority of the known transcription factor diseases exhibit dominant inheritance (Veitia, 2002). This may reflect in part an ascertainment bias, because homozygous mutations in many transcription factors result in embryonic or neonatal lethality and thus would not be represented in human populations. In some cases, dominant inheritance has been attributed to the dominant negative effect of toxic proteins, or to allele-specific regulation. However, the default explanation for dominance is haploinsufficiency caused by reduced protein levels (Nutt et al., 1999).

In mice, null mutants have been generated for a large number of transcriptional regulators of neurodevelopment. For a majority of the members of the LIM, POU, variant homeodomain and bHLH families, these mutations have no known phenotype in heterozygous animals. By contrast, several members of the Pax gene family exhibit

haploinsufficiency in both mice and humans (Chi and Epstein, 2002).

Among the members of the POU family expressed in the nervous and endocrine systems, loss-of-function mutations appear to be consistently recessive. Mutations in Pit1, a regulator of pituitary development, cause recessive dwarfism in mice and in humans. Autosomal dominant Pit1 mutations are also known, but these result from mutations outside the DNA-binding domain of the molecule, and most probably cause dominant-negative transcriptional effects (Parks et al., 1999). Pit1 exhibits positive autoregulation that maps to a conserved distal enhancer, and is necessary for the sustained but not the embryonic expression of the gene (DiMattia et al., 1997; Rhodes et al., 1993).

Within the POU4 transcription factor class, the effects of Brn3a loss-of-function on sensory neuron survival and axon growth and fasciculation observed in Brn3a knockout mice do not appear in heterozygotes (Eng et al., 2001; Huang et al., 1999). Similarly, the excessive retinal ganglion cell death and aberrant axon growth observed in Brn3b-null mutants does not occur in heterozygotes (Gan et al., 1999), and the auditory defects observed in Brn3c-null mice are also recessive (Keithley et al., 1999; Xiang et al., 1997). Mutations in Brn3c have also been identified as a causes of progressive heritable hearing loss in humans (Vahava et al., 1998). This syndrome exhibits autosomal dominant inheritance, which has been attributed to the dominant-negative effects of the mutant protein. No enhancers have been characterized that accurately reproduce the in vivo expression patterns of Brn3b and Brn3c, so it is not known if autoregulatory elements are present in these genes.

Haploinsufficiency can be suppressed by several mechanisms, including the expression of functionally redundant genes, expression of sufficient protein from a single functional allele to saturate binding sites, post-translational modulation of transcription factor activity or by regulatory feedback from downstream targets. In the case of Brn3a, the loss of one allele partly relieves direct transcriptional suppression, leading to an increase in expression of the intact gene copy. This is a particularly economical, self-contained homeostatic mechanism for maintaining the expression level of a crucial developmental regulator. Given that a large number of transcription factors regulating neurodevelopment appear to act as transcriptional repressors, and that the null mutations for a majority of these do not exhibit heterozygote phenotypes, it will be interesting to see if such a mechanism is widely employed.

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