Coordinated regulation of gene expression by Brn3a in developing sensory ganglia

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Summary

Mice lacking the POU-domain transcription factor Brn3a exhibit marked defects in sensory axon growth and abnormal sensory apoptosis. We have determined the regulatory targets of Brn3a in the developing trigeminal ganglion using microarray analysis of Brn3a mutant mice. These results show that Brn3 mediates the coordinated expression of neurotransmitter systems, ion channels, structural components of axons and inter- and intracellular signaling systems. Loss of Brn3a also results in the ectopic expression of transcription factors normally detected in earlier developmental stages and in other areas of the nervous system. Target gene expression is normal in heterozygous mice, consistent with prior work showing that autoregulation by Brn3a results in gene dosage compensation. Detailed examination of the expression of several of these downstream genes reveals that the regulatory role of Brn3a in the trigeminal ganglion appears to be conserved in more posterior sensory ganglia but not in the CNS neurons that express this factor.

Supplemental data available online

Key words: Brn3a, POU-domain, Trigeminal ganglion, Microarray, Sensory neuron

Introduction

Studies of the developing vertebrate nervous system have revealed a large number of transcription factors that are expressed in specific populations of neurons or their precursors. In dividing neuroepithelial cells, transcription factors of the bHLH, homeodomain and other classes characterize regions of the neural tube with specific developmental potentials. Later in neurogenesis, other transcription factors are expressed in specific populations of neurons, and may persist in the mature nervous system. Naturally occurring and induced mutations of both the early and late transcription factors have been shown to exert profound effects on neural development.

Transcription factors reside permanently or conditionally in the nucleus, and are presumed to work by interacting with specific cis-acting binding sites in the vicinity of the transcription units they regulate. These ‘target genes’ in turn mediate the effects of the transcription factor on developmental fate decisions, neuronal phenotype and cell survival. However, the downstream targets of these factors cannot necessarily be inferred from their expression patterns, because they are usually not congruent with those of other classes of neural genes, such as neurotransmitters or their receptors. In a few cases, plausible regulatory relationships have been established between neural transcription factors and their targets, but for the vast majority, no clear pathways are known. Using conventional methods applied to individual genes, establishing these transcription factor-target relationships is quite inefficient.

In principle, comparing the transcript pool of neural tissue from a wild type animal to that of an animal under- or over-expressing a given factor should yield a complete set of genes regulated in that cell type. However, because of the tremendous cellular diversity present in most regions of the nervous system, the resulting changes in gene expression in a specific cell type may be obscured by the heterogeneity of the sample. Furthermore, the changes in target gene expression may be regulated indirectly, either as downstream or compensatory effects.

We have been engaged in studies of Brn3a (Pou4f1 – Mouse Genome Informatics), a transcription factor of the POU-domain family which is expressed in terminally differentiating neurons of the sensory peripheral nervous system and caudal CNS. Targeted mutations in mice have shown that Brn3a is necessary for the correct development and/or survival of neurons in the sensory ganglia and some CNS nuclei (McEvilly et al., 1996; Xiang et al., 1996). Sensory neuron death in *Brn3a* knockout mice is preceded by loss of neurotrophin receptor expression (Huang et al., 1999; Ma et al., 2003), and by markedly defective axonal growth (Eng et al., 2001). Despite the success of the knockout approach in demonstrating the importance of Brn3a and related POU factors in neural development, these experiments have yielded little information about what genes these factors regulate, and why they are essential for normal axon growth or neuronal survival.

In the present study, we have used microarrays to compare the patterns of gene expression in the trigeminal ganglia of *Brn3a* knockout and wild-type mice. To maximize the homogeneity of the samples and to minimize secondary effects on gene expression, we have analyzed embryonic ganglia. At the stage chosen for analysis, embryonic day 13.5 (E13.5),

major defects in sensory axon growth are observed in the mutant mice (Eng et al., 2001), but the phase of marked sensory neuron death has not yet commenced (Huang et al., 1999).

Our results demonstrate that Brn3a regulates a coordinated program of gene expression that defines the phenotype of developing trigeminal neurons, including the regulation of neurotransmitters, receptors, ion channels, mediators of axon growth, and other transcription factors. Many of these target genes have known roles in sensory neurons and are strong candidates for mediating the observed effects of Brn3a on axon growth and cell survival. Some of the genes regulated by Brn3a in the trigeminal ganglion are also changed in other sensory ganglia in *Brn3a* knockout mice, but do not appear to be altered in Brn3a-expressing CNS neurons, suggesting that the roles of Brn3a in the sensory system and CNS may be distinct.

Materials and methods

Matings, embryos and immunohistochemistry

To generate tissue for microarray analysis, timed matings of Brn3a heterozygote animals were performed, and the embryos harvested at embryonic day (E)13.5. Only embryos at E13.5±0.5 days, based on the staging system of Theiler (Theiler, 1972), were pooled for microarray analysis. Trigeminal ganglia were removed by blunt dissection and carefully freed of adherent non-neural tissue with fine forceps. Only complete ganglia were used for analysis. Dissected ganglia were placed in RNAse inhibitor solution (RNAlater, Ambion) and frozen until enough tissue was harvested to be pooled. Embryos were genotyped from a sample of tail or hindlimb tissue harvested at the time of ganglion dissection. Genotypes were determined for the native *Brn3a* allele and the neomycin resistance cassette included in the null allele as previously described (Eng et al., 2001). Approximately 10-12 genotyped ganglia were sufficient to provide 5 μg of total RNA for a single analysis, which was extracted using the RNeasy system (Qiagen). The generation of cDNA, production of labeled cRNA, and hybridization to GeneChip arrays were all performed according to standard protocols provided by the manufacturer (Affymetrix).

Non-isotopic in situ hybridization was performed as previously described (Birren et al., 1993). A table of probes used and their sources appears in the Supplemental Data (Table S1, http://dev.biologists.org/supplemental/). Immunofluorescence for Brn3a was performed with rabbit polyclonal antisera as previously described (Fedtsova and Turner, 1995). Immunofluorescence for other antigens was performed with commercially available antibodies, including rabbit anti-calretinin (Swant), rabbit anti-galanin (Peninsula Laboratories), rabbit anti-somatostatin-14 (Peninsula Laboratories), and rabbit anti-tyrosine hydroxylase (Chemicon).

Analysis of expression array data

The primary analysis of microarray data, including determination of the absence/presence of the assayed transcripts, transcript expression levels, and the probability of change in transcript expression between genotypes ('change-p') was performed with Microarray Suite 5.0 (Affymetrix). Two proprietary databases were used to relate microarray results for ESTs to the identity of the expressed transcripts, NetAffx (Affymetrix) and GeneSpring (Silicon Genetics). The results for those transcripts identified in both databases were discordant in less than 1% of cases.

Results

Microarray analysis of gene expression in the developing trigeminal ganglion

To begin to address the complement of genes regulated by Brn3a, we chose to analyze gene expression in the trigeminal ganglia of *Brn3a* wild-type, heterozygous and knockout mice at E13.5. These ganglia do not represent a homogeneous population with respect to their eventual sensory subtype, but at this stage most of the cells in the trigeminal ganglion exhibit Brn3a immunoreactivity (Trieu et al., 2003) and thus it is likely

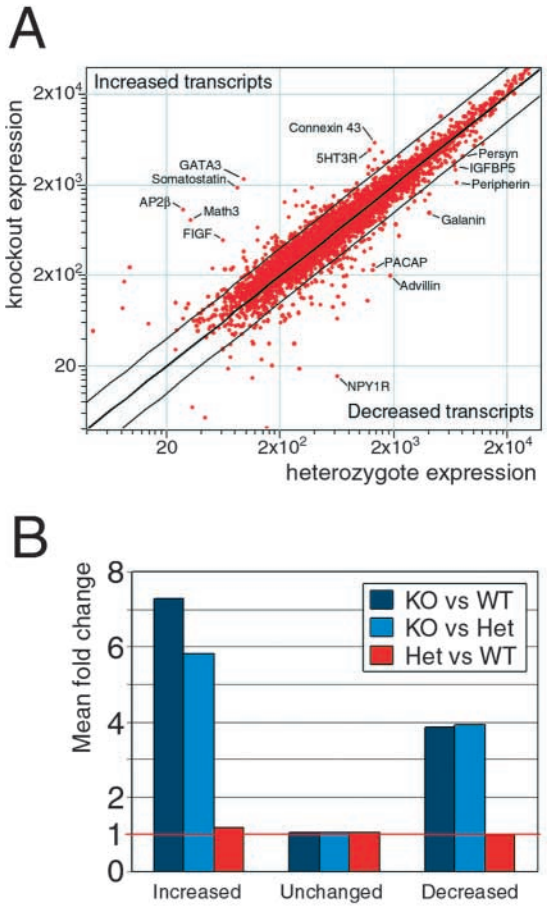

Fig. 1. Expression array analysis of E13.5 trigeminal ganglia. (A) A two-dimensional plot of the hybridization signal for ~4,000 present transcripts, including ESTs, in one of the two experiments comparing *Brn3a* heterozygous and knockout trigeminal ganglia. Values for the expression of the majority of transcripts fall along the central diagonal line representing equal expression in the two genotypes. A few transcripts fall outside the parallel lines indicating a greater than twofold change in expression, and examples of some highly changed transcripts are indicated. (B) To determine whether *Brn3a* heterozygosity results in intermediate levels of target gene expression, two-way comparisons were made between transcript levels in knockout versus wild-type, knockout versus heterozygous and wild-type versus heterozygous ganglia for the 41 increased and 62 decreased transcripts from the U74Av2 array, Experiment 1. Expression levels in wild type and heterozygotes showed a similar mean fold increase or decrease when compared to those in the knockout. However, comparison of expression levels in heterozygotes with those in wild type revealed no significant difference (fold change ~1, red horizontal line) for either the increased or decreased transcripts. As expected, the fold change was also approximately 1 for two-way comparisons between the genotypes for a group of 160 unchanged neural transcripts (0.003<change-p<0.997; see Supplemental Data, http://dev.biologists.org/supplemental/).

that Brn3a regulates genes common to a majority of the neurons present in these ganglia. E13.5 was chosen as the time point for analysis because in mice lacking Brn3a, a clear defect in the growth of axons from the trigeminal ganglion is evident by this stage, with aberrant innervation of peripheral and central targets. Furthermore, the extensive sensory cell death observed in *Brn3a* knockout mice does not take place until E14.5-E16.5 (Huang et al., 1999), so any effects from cell loss should be minimal at this stage.

Although no differences in the phenotype of *Brn3a* wild-type and heterozygous embryos have been identified (Eng et al., 2001), tissue samples from these genotypes were analyzed separately. The comparison of gene expression across all three genotypes was performed to look for subtle differences in heterozygotes, and to provide a partial replication of the results within each experiment. All three genotypes were analyzed in two completely independent experiments.

Trigeminal RNA were analyzed using the commercial oligonucleotide-based U74Av2 and U74Bv2 microarrays (Affymetrix). The U74Av2 array represents 12,422 transcripts, including 5993 known genes and 6429 ESTs, and the U74Bv2 array includes an additional 12,411 EST sequences. A significant number of the EST sequences present on both arrays have subsequently been related to identified genes in public and proprietary databases. Of all the transcripts represented on the U74Av2 array, 4885 were detected as 'present' in both experiments in at least one of the three *Brn3a* genotypes, using the manufacturer's standard criteria for array analysis. The transcripts that were reproducibly present on the U74Av2 and U74Bv2 arrays were then further analyzed with respect to their relative expression in the three genotypes.

Two measures were used to compare transcript levels between samples from different genotypes, the change-p value and the fold change in the intensity of the hybridization signal. The change-p value is calculated by proprietary data analysis software (Affymetrix) using the Wilcoxon's signed rank test applied to the hybridization signals for the 16 matched and mismatched oligonucleotide probe pairs representing each transcript in the array. Change-p values <0.003 (increased expression in the arbitrarily designated 'experimental' sample) or >0.997 (decreased in the experimental sample) are considered highly significant. For abundant transcripts, change-p values may be significant even when the fold change in expression is small, because for strong signals even minor relative differences may achieve statistical significance by this method. Because small relative changes in expression are not easily verified, and have uncertain biological significance, a minimum twofold increase or decrease in expression was used as an additional criterion for determining the changed transcripts of interest. More detailed information on the transcripts included and excluded by these criteria are given in Fig. S1, http://dev.biologists.org/supplemental/.

Figure 1A compares the relative expression of all present transcripts in heterozygote and knockout ganglia in one analysis using the U74Av2 array. The vast majority of the expressed transcripts fall between parallel lines designating less than a twofold change in expression. The expression values for significantly changed transcripts are located off the central axis,

Table 1. Increased transcripts in the trigeminal ganglion of *Brn3a* mutant mouse embryos

Description	GenBank	Experiment 1			Experiment 2			Fold
Increased transcripts	acc. no.	Wild type	Heterozygote	Knockout	Wild type	Heterozygote	Knockout	Knockout/ control
Cardiac responsive adriamycin protein	AF041847	4	4	170	11	10	244	34.3
Gata3	X55123	61	61	2524	85	96	2236	33.0
AP-2βa	X78197	23	99	1115	27	28	1037	28.1
Somatostatin	X51468	56	69	1662	63	85	1843	25.7
Calretinin (calbindin 2)	X73985	165	147	1838	57	219	1265	10.5
C-fos induced growth factor FIGF	X99572	17	103	508	38	63	478	9.0
HLH transcription factor Math3	AF036257	98	111	522	120	33	800	7.8
HLH transcription factor Musculin/MyoR	AF087035	195	277	1578	191	274	1685	7.0
Serotonin receptor 3A	M74425	1049	1129	5244	854	1242	4788	4.7
Connexin 43 (2 probe sets)	M63801	994	874	3211	1327	1381	5790	3.9
ART3	Y08027	42	32	154	79	108	214	3.2
Neuroserpin	AJ001700	1088	1316	4134	1564	1590	4559	3.2
Thrombospondin	M62470	55	87	198	79	96	307	3.2
HLH transcription factor NeuroD1	U28068	500	416	1005	378	351	1256	2.8
Brain natriuretic peptide	D16497	163	186	377	154	171	515	2.7
Eyes absent 2	U81603	716	693	1460	405	314	1172	2.7
Galectin 7	AF038562	556	317	877	413	391	1107	2.4
Short stature homeobox protein 2	U66918	1295	1925	3361	1434	1364	3742	2.4
Cytochrome P450, Cyp1b1	X78445	291	261	549	327	397	975	2.3
PG-M core protein	D45889	370	541	1159	478	525	1044	2.3
Tachykinin 1	D17584	95	38	160	152	124	286	2.2
Regulator of G-protein signaling 4	AB004315	78	162	329	162	173	282	2.2
LIM only 4	AF074600	754	789	1397	1224	1205	3132	2.2

This table and Table 2 show expression data for all transcripts from among the 5993 known genes represented on the U74Av2 chip that met the following criteria: (1) Present in at least one genotype in both experiments; (2) change-p value for the comparison of heterozygote to knockout transcript levels showed significantly changed expression (pΔ <0.003 or >0.997) in both experiments; (3) a minimum of a twofold increase or decrease in signal intensity in both experiments. Transcript levels in each experiment were normalized to a mean value of 500. The fold change is calculated as the ratio of knockout/control expression and represents the mean of the individual ratios for the two experiments. A list of genes that exhibited changed expression but did not meet these criteria appears in the Supplemental data, http://dev.biologists.com. Numbers in parentheses following gene names represent the number of probe sets for a given gene when represented by multiple probe sets on the array. These were in every case concordant and results for only one probe set are given.

Table 2. Decreased transcripts in the trigeminal ganglion of Brn3a mutant mouse embryos

Description	GenBank	Experiment 1			Experiment 2			Fold
Decreased transcripts	acc. no.	Wild type	Heterozygote	Knockout	Wild type	Heterozygote	Knockout	Knockout/ control
NPY1 receptor	Z18280	474	242	59	489	635	15	21.4
HoxD1 (Hox-4.9) (2 probe sets)	M87802	723	713	38	837	765	193	11.6
Advillin	AF041448	1455	1407	242	2022	1862	195	7.9
Na^+ channel Scn6a	L36179	211	316	25	220	240	72	6.8
Basonuclin	U88064	450	426	80	796	589	89	6.6
Homeobox transcription factor Hmx1	AF009614	612	542	102	682	728	94	6.6
Phospholipase A2 group VII	U34277	300	321	49	604	457	93	6.0
Runt related transcription factor Runx1	D26532	742	721	126	720	740	149	5.3
PACAP	AB010149	880	697	175	1261	1318	226	5.1
K^+ channel Kcnab2	U65592	1019	827	289	1750	1083	221	4.8
Galanin	L38580	4088	3753	938	3816	4093	952	4.2
Olfactomedin 1 (noelin, pancortin)	D78265	1216	1250	390	1494	1248	386	3.4
Bicarbonate transporter SLC4A4	AF020195	369	375	163	382	288	76	3.4
Insulin-like growth factor binding protein 5 Igfbp5	L12447	9683	10278	2283	7560	6864	3210	3.3
Myotubularin related protein 7	AF073882	370	526	97	686	528	325	3.2
Apolipoprotein E	D00466	1857	1404	405	2029	2694	1086	3.1
FK506 binding protein 1b	AF060872	9309	8866	3041	5809	7165	2071	3.1
Hivep2	Y15907	413	373	128	463	477	167	2.9
Latexin	D88769	1005	1181	402	1816	1596	628	2.7
HLH transcription factor Id1	M31885	1160	1003	366	1259	1210	502	2.7
Synaptotagmin 1	D37792	412	244	111	563	569	242	2.6
Na^+ channel Scn9a	L42338	260	426	140	566	412	178	2.6
C-kit	Y00864	702	484	263	847	795	304	2.5
Elongation factor 1 alpha 2	L26479	3197	3258	1326	3248	3081	1305	2.4
Tyrosine hydroxylase	M69200	3078	3304	1391	3375	4126	1563	2.3
RNA polymerase II 3	D83999	1455	1159	526	997	1218	502	2.3
Synuclein, gamma	AF017255	7459	9458	3209	8466	8145	4084	2.3
Activated leukocyte cell adhesion molecule	L25274	1320	1574	555	1998	1727	981	2.3
Peripherin 1	X15475	3746	4563	2009	6947	6982	2858	2.3
Annexin A2	M14044	9444	11497	4164	9051	12268	5639	2.2
Fgf13	AF020737	926	1137	461	932	1055	463	2.2

Transcripts were selected from among 5993 known genes on the U74aV2 chip according to the criteria described in Table 1. The threshold change-p value for decreased transcripts is pΔ >0.997. The fold change is calculated as the ratio of control/knockout expression.

and the positions of selected mRNAs encoding proteins with known roles in sensory development or function are indicated.

A complete list of the known transcripts that met the criteria for significantly increased expression appears in Table 1, and the significantly decreased genes are shown in Table 2. Most of the known transcripts that were significantly changed in *Brn3a* knockout ganglia encode proteins with established roles in neural function or neural development, including neurotransmitters and their receptors, enzymes of neurotransmitter synthesis, ion channels, specialized components of axons and synapses, mediators of intracellular signal transduction, and transcription factors. The specificity of these changes is underscored by the fact that we observed very few significant changes in transcripts associated with processes other than neurogenesis and neural function, such as factors regulating metabolic pathways, the cell cycle, or apoptosis. A more extensive list of 271 known neural genes, which were expressed in E13.5 trigeminal ganglia but were either unchanged in the *Brn3a* knockout or changed but did not meet the inclusion criteria, appears in Table S2, http://dev.biologists.org/supplemental/.

In addition to the previously known transcripts represented on the U74Av2 array, the U74Av2 and U74Bv2 arrays include oligonucleotide probes for nearly 19,000 ESTs, some of which have subsequently been linked to known genes. Although many significantly changed ESTs on these arrays could not be identified, searches of public and proprietary databases allowed a significant number to be assigned with confidence, and these are summarized in Table 3. Several of the ESTs confirmed results obtained for the known transcripts, including GATA3, AP2β, NeuroD1, Scn9a and Runx1. In addition, analysis of the ESTs contributed a number of novel changed genes, particularly transcription factors and mediators of intracellular transduction pathways.

Target gene expression is unaltered in the trigeminal ganglia of *Brn3a* heterozygotes

Brn3a heterozygous mice are viable and do not exhibit the defects in sensory axon growth or neuronal survival observed in knockouts. In previous studies we have shown that Brn3a attenuates its own expression via an autoregulatory enhancer (Trieu et al., 2003; Trieu et al., 1999). In heterozygotes, increased *Brn3a* expression from the intact allele restores Brn3a transcript levels to approximately 90% of the wild-type level, effectively compensating for reduced gene dosage. If this mechanism in fact normalizes Brn3a regulatory activity in heterozygotes, one would predict that all downstream targets of Brn3a, whether directly or indirectly regulated, would have nearly normal expression levels in heterozygous mice.

To test this hypothesis, we compared the expression levels of 41 increased, 62 decreased and 160 unchanged transcripts in ganglia from the three *Brn3a* genotypes. For both the increased

Table 3. Significantly changed ESTs in Brn3a mutant mice

Description	GenBank acc. no.	Mean ratio§
Increased transcripts		
Transcription factor Gata3‡	AA717155†	52.1
Transcription factor AP2β‡	AI021690†	18.6
Homeobox protein Iriquois 2	AI851291†	18.4
Homeobox protein Iriquois 1 (2 probe sets)	AA028275†	6.5
LIM only protein Testin 2	AI845609†	6.1
HLH transcription factor NeuroD1‡	AI848674†	3.5
Solute carrier family 1, member 3	AW121315†	3.4
Neuropilin 2	AW046112†	3.1
Protein kinase C-binding protein NEL	AI838010†	2.9
Thymocyte selection-associated HMG box gene	AA688946†	2.9
Bruno-like 4 (2)	AI839880*	2.5
Dual specificity phosphatase 6 Dusp6 (=MKP3)	AI845584*	2.2
Catenin beta interacting protein 1	AI842284†	2.1
Regulator of G-protein signaling 4	AI854153†	2.1
Decreased transcripts		
Regulator of G-protein signaling 10 (2)	AI847399*	13.6
Na^+ channel Scn9a‡	AI593229†	8.6
Ras family GTPase RAP	AA920095†	6.9
Runt-related transcription factor Runx1‡	AI643935†	5.4
Downstream of tyrosine kinase 4 Dok4 (3)	AA982457†	4.8
Protein tyrosine phosphatase H	AI851090†	4.7
Insulin-like growth factor 1 (2)	AA544955†	4.6
Synaptotagmin-like 2	AW124071†	4.1
Doublecortin-like kinase	AI853072†	3.8
G-protein coupled receptor 64	AI132005†	3.6
Src homology 2 containing protein C1 Shc1	AI050321*	3.4
Ataxin-2 binding protein A2bp1 (2)	AW047913†	3.4
Wnt1 responsive Cdc42 homolog Wrch1	AV246963†	3.3
CRB1 isoform II precursor	AW046426†	3.1
Microsomal glutathione S-transferase 3	AI843448*	2.9
Oxysterol binding protein-like 3	AI591488†	2.9
Bicarbonate transporter SLC4A4‡ (2)	AW124701†	2.8
Nerve growth factor receptor p75	AA050723†	2.8
FXYD domain ion transport regulator 7	AI844246*	2.7
Intermediate filament protein Desmuslin (3)	AI852401†	2.7
ELKL motif serine-threonine protein kinase 3	AI430097†	2.6
Actin-binding double zinc finger protein	AI840403†	2.6
Growth factor receptor bound protein 14 Grb14	AW124221†	2.6
N-chimaerin (2)	AI848376†	2.5
Calcium channel, Cacna2d2	AA008996†	2.4
Synaptotagmyn 1‡ (2)	AW125093†	2.4
Axonin, (TAG1, contactin 2)	AW049675†	2.3
Calsyntenin 2	AI842881†	2.3
Neuritin	AI839544†	2.2

Data are given for the relative expression of selected transcripts from amongst approximately 18,000 ESTs represented on the U74Av2 and U74Bv2 arrays.

*Affymetrix U74A chip.

†Affymetrix U74B chip.

‡Also appears as a known transcript on U74Av2 array.

§Calculated for increased and decreased transcripts as described in Table 1 and 2, respectively.

and decreased transcripts, the target gene expression levels in wild-type and heterozygous ganglia showed similar differences from those of the knockout (Fig. 1B). In contrast, the wild-type and heterozygote ganglia did not significantly differ from each other for either class of target genes. These results confirm the complete suppression of a heterozygous phenotype at this stage in the trigeminal ganglia of *Brn3a* knockout mice.

Expression of genes previously reported to be regulated by Brn3a

Numerous genes have been previously reported to be transcriptionally activated by transfected Brn3a in cell culture models of sensory ganglia. These putative Brn3a targets include structural components of axons and synapses, neurotransmitter receptors and oncogenes (Table 4). Almost all of these proposed downstream genes are represented on the U74A and B arrays, and were detected (present call) in E13.5 trigeminal ganglia. However, aside from a modest but statistically significant decrease in the neurofilament NF-H, none of these genes were markedly affected by the loss of Brn3a expression in vivo.

Work in cell line models has also led to the hypothesis that decreased expression of the anti-apoptotic gene *Bcl2* may contribute to the extensive death of sensory neurons in *Brn3a* knockout mice (Smith et al., 1998). In recent work, we have shown that Bcl2 mRNA levels are unaltered in *Brn3a* knockout mice just prior to the onset of cell death (Eng et al., 2003), suggesting that loss of *Bcl2* expression is not a primary defect in *Brn3a* knockout mice. Consistent with this result, two probe sets on the array gave unchanged signals for *Bcl2*. Overall, the results reported here do not support the in vivo regulation of the putative Brn3a targets from previous over-expression studies in transfected cell lines, and illustrate the difficulty of identifying physiological regulatory pathways in these model systems.

Previous studies of *Brn3a* knockout mice have also revealed changes in the expression of several genes in the sensory ganglia. A major focus of these studies has been the neurotrophins and their receptors. We have previously reported microarray assays showing a reduction in TrkA transcripts in *Brn3a* knockout trigeminal ganglia at E13.5 (Ma et al., 2003), and our array analysis is consistent with previous reports that the TrkA neurotrophin receptor is decreased in these mice (Huang et al., 1999; McEvilly et al., 1996). Transcripts for the p75 NGF receptor have been reported to be significantly decreased in mice lacking Brn3a (McEvilly et al., 1996), whilst immunohistochemistry for p75 protein has been reported as unchanged in mid-gestation knockout ganglia (Huang et al., 1999). In the present study, knockout levels of p75 mRNA were approximately 40% of wild type. Transcript levels for BDNF, previously reported to be reduced to undetectable levels at E12.5 in the trigeminal ganglia of *Brn3a* null mice (McEvilly et al., 1996), were found to be unchanged from controls in the present analysis. Loss of expression of the TrkB and TrkC neurotrophin receptors has also been reported in Brn3a null mice, but transcripts for the TrkB and TrkC receptors were not detected (absent call) in any genotype by the probe sets designed for these genes on the U74A array. This is an inconclusive result, which may reflect a problem in array design.

Brn3a regulates the expression of neurotransmitter systems and other transcription factors in multiple sensory ganglia

In addition to the trigeminal ganglion, Brn3a is expressed in neurons of the vestibulocochlear (VIII) ganglion complex, IX/X ganglion complex, and in the dorsal root ganglia (Fig. 2B). In order to verify the gene expression changes noted in the trigeminal array analysis, and to determine whether the trigeminal target genes are regulated elsewhere in the nervous system, we examined the expression of several Brn3a regulatory targets by in situ hybridization and immunohistochemistry in E13.5 embryos.

Table 4. Relative expression of previously reported Brn3a target genes in the trigeminal ganglia of Brn3a mutant embryos

Description	Probe set acc. no.	Reference	Wild type	Heterozygote	Knockout	Knockout × heterozygote change call*	Knockout/ heterozygote ratio
Cell transfection studies							
α-internexin	L27220	Budhram-Mahadeo et al., 1995	9514	10729	13470	I	1.26
Neurofilament, heavy	M35131	Smith et al., 1997	794	807	381	D	0.47
Neurofilament, medium	X05640	Smith et al., 1997	6410	7503	7956	NC	1.06
Neurofilament, light	M55424	Smith et al., 1997	3514	3056	1876	NC	0.61
Synapsin I	AF085809	Morris et al., 1996	10158	11068	13351	I	1.21
SNAP-25	M22012	Lakin et al., 1995	1976	2338	2359	NC	1.01
Bcl2	L31532	Smith et al., 1998	578†	612†	466†	NC	0.76
p53	AB021961	Budhram-Mahadeo et al., 2002	533	578	641	NC	1.11
Nicotinic AchRα3	(§)	Milton et al., 1996	–	–	–	–	–
Knockout studies							
trkA‡	AW124632	Huang et al., 1999	11675	13334	7083	D	0.53
BDNF	X55573	McEvilly et al., 1996	408	327	433	NC	1.33
NGF Receptor p75‡	AA050723	McEvilly et al., 1996	2745	3655	1107	D	0.30
Parvalbumin¶	X59382	Ichikawa et al., 2002b	1412†	1808†	1330†	NC	0.74
Calbindin¶	D26352	Ichikawa et al., 2002b	177	196	302	NC	1.54
Vanilloid-like receptor 1¶	AB021665	Ichikawa et al., 2002a	582	645	490	NC	0.76

All of the listed targets have been proposed as positively regulated target genes of Brn3a, and would thus be predicted to have decreased transcript levels in Brn3a knockout mice. The expression values are derived from the same array experiments as Tables 1-3. Only one experiment is shown, similar results were obtained in both experiments, and complete data appear in Table S1, http://dev.biologists.org/supplemental/.

*Change call values: I, increased, change-p <0.003; NC, no change, 0.003<change-p<0.997; D, decreased, change-p >0.997.

†Absent call. Absent calls in the presence of a strong hybridization signal can result from high background hybridization for a particular set of oligonucleotides on the array. For this reason these results are suggestive of unchaged expression, but are not conclusive.

‡U74B chip EST. All others are known transcripts from the U74Av2 array.

§Not represented on array.

¶Measured in late gestation, and may represent selective cell death.

Among the Brn3a-regulated gene products related to neurotransmitter systems, examination of the 5HT3 receptor mRNA by in situ hybridization confirmed markedly increased expression in the trigeminal ganglion, the IX/X ganglion (Fig. 2C), and the cervical dorsal root ganglion (not shown). Conversely, the regulator of G-protein signaling RGS10 exhibited strong expression in the trigeminal ganglion, IX/X ganglion (Fig. 2D), and dorsal root ganglion (not shown) of control mice, which fell to background levels in *Brn3a* knockout embryos. However, the VIII ganglion did not show increased expression of 5HT3R in *Brn3a* knockout embryos, or endogenous expression of RGS10 in controls.

The expression patterns of the mediator of Ca^{2+} signaling, calretinin, the neuropeptides somatostatin and galanin, and the enzyme of catecholamine synthesis, tyrosine hydroxylase were examined in the trigeminal ganglion by immunohistochemistry. Consistent with cell-autonomous regulation by Brn3a, galanin and tyrosine hydroxylase co-localized with Brn3a protein in the trigeminal neurons of control ganglia (Fig. 2E,F), and the direction and approximate extent of the expression changes in each of these proteins was entirely consistent with the array results (Fig. 2G). We also examined the DRG and spinal cord for changes in the expression of these four proteins (data not shown). Galanin immunoreactivity was markedly decreased in the DRG, but no significant changes in calretinin or tyrosine hydroxylase were evident. Somatostatin immunoreactivity accumulated abnormally in the dorsal root entry zone of *Brn3a* knockout mice, a finding which may reflect either increased expression, or the failure of sensory axons to appropriately enter the CNS in these mutants (Eng et al., 2001), or both. No changes were noted in any of these markers in the Brn3a-expressing neurons of the dorsal spinal cord.

Several transcription factors were also prominent among the most changed transcripts in the array analysis. To verify the array results for the trigeminal ganglion, and examine the expression of these factors in other cranial sensory ganglia and the caudal CNS, we performed in situ hybridization for the increased transcripts GATA3, Irx1, Irx2, AP2b, MyoR, Math3 (Fig. 3A), and NeuroD1 (not shown), and for the decreased transcripts HoxD1 and Runx1 (Fig. 3B), in E13.5 wild-type and *Brn3a* knockout embryos. In each case the direction and magnitude of change in the in situ hybridization signal in the trigeminal ganglion correlated well with the array results.

Further examination of these transcripts in the cranial sensory ganglia clearly indicate a role for Brn3a in the coordinated regulation of gene expression in the sensory system. GATA3, Irx1, Irx2, MyoR and NeuroD1 were all expressed in the vestibulocochlear ganglion (VIII) complex in control mice, but were weakly expressed to undetectable in the trigeminal, IX and dorsal root ganglia. In mice lacking Brn3a, the expression of these factors was markedly increased in the trigeminal ganglion and IX/X complex (Fig. 3A). GATA3, MyoR and NeuroD1, but not the Irx transcripts, were also increased in the dorsal root ganglion (not shown). AP2β and Math3 were not detectable in the VIII ganglion of control mice, but showed a similar coordinated increase in expression in the trigeminal and IX ganglia in embryos lacking Brn3a.

The transcription factors HoxD1 and Runx1 showed decreased expression in the array analysis of *Brn3a* knockout mice. In situ hybridization for these transcripts confirmed markedly decreased expression in the trigeminal and IX

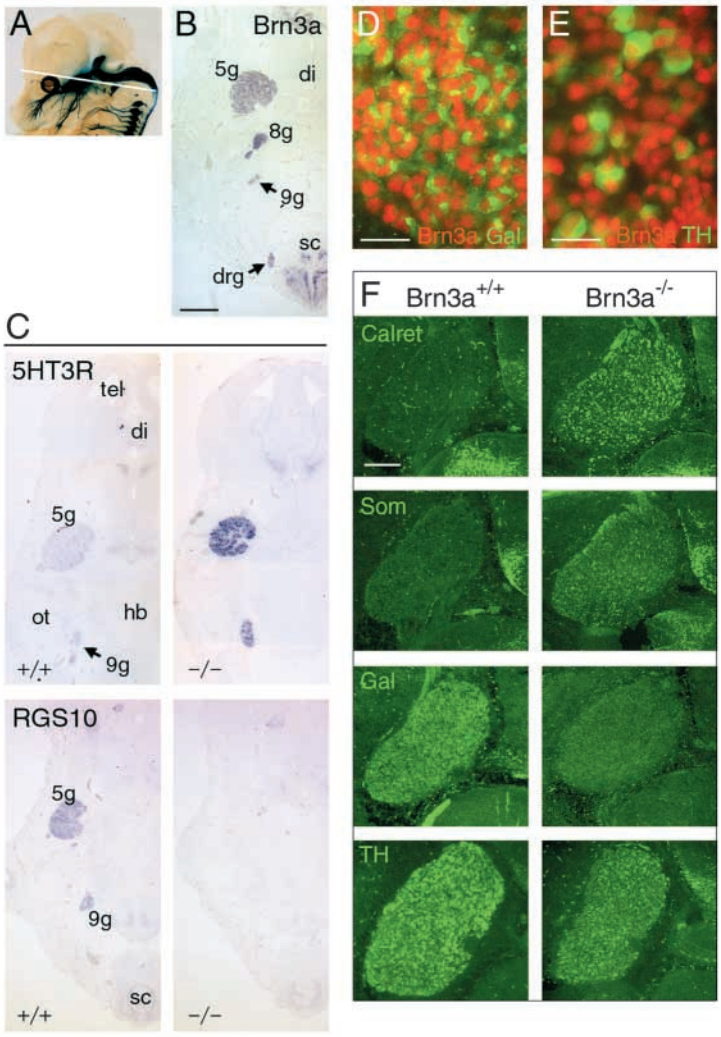

Fig. 2. Brn3a regulates sensory neurotransmitter systems. The cranial sensory ganglia of control (*Brn3a*$^{+/+}$) and knockout (*Brn3a*$^{-/-}$) E13.5 embryos were examined for the expression of components of neurotransmitter systems. (A) The plane of section used in subsequent views is illustrated using an E13.5 embryo stained for the expression of β-galactosidase regulated by a Brn3a sensory enhancer (Eng et al., 2001). (B) In situ hybridization showing the expression of the Brn3a mRNA in the cranial sensory ganglia. (C) In situ hybridization for the 5HT3 receptor, increased in the microarray analysis of *Brn3a* knockout mice, and the mediator of G-protein signaling RGS10, decreased in the microarray. (D-F) Immunohistochemistry for the products of Brn3a target genes in the trigeminal ganglia of E13.5 embryos. (D) Galanin immunoreactivity in the trigeminal ganglion co-localized with Brn3a in a majority of trigeminal neurons. (E) Tyrosine hydroxylase was expressed in a more limited subset of trigeminal neurons, most of which also expresses Brn3a. (F) A comparison of trigeminal ganglia from control mice and *Brn3a* knockouts, showing that, as predicted from the microarray studies, calretinin (Calret) and somatostatin (Som) immunoreactivity is markedly increased in the absence of Brn3a, whilst galanin is reduced to below the threshold of detection, and tyrosine hydroxylase (TH) is also significantly decreased. 5g, trigeminal ganglion; 8g, vestibulocochlear ganglion; 9g, IX/X ganglion complex; di, diencephalon; drg, dorsal root ganglion; hb, hindbrain; ot, otic region; tel, telencephalon; sc, spinal cord. Scale bars: B, 400 μm, D,E, 25 μm; F, 200 μm.

ganglion. Endogenous expression of Runx1 in the VIII ganglion appeared to be less affected.

The regulatory role of Brn3a may be distinct in the CNS

In addition to the sensory ganglia, Brn3a is expressed in specific neurons of the CNS, residing in the habenula, midbrain tectum and tegmentum, hindbrain, dorsal spinal cord and retina. The examination of the hindbrain region and spinal cord by in situ hybridization (Figs 2 and 3) did not indicate any obvious changes in the expression of neurotransmitters or transcription factors in the CNS of *Brn3a* knockout mice. However, in most areas of the CNS, Brn3a-expressing neurons have a scattered distribution, requiring methods of detection with cellular resolution to identify changes in target gene expression. For this reason we examined the CNS of embryos in more detail by immunohistochemistry for the increased gene products calretinin and somatostatin, and for the decreased gene products galanin and tyrosine hydroxylase.

In the midbrain and hindbrain, calretinin and Brn3a are expressed in adjacent but non-overlapping cell populations (Fig. 4A,B), while in the retina, a subset of neurons cells co-express these antigens (Fig. 4D,E). The expression of calretinin was not altered in either of these regions in the absence of Brn3a (Fig. 4C,F). Similarly, somatostatin was not ectopically expressed in the CNS of mice lacking Brn3a (Fig. 4G-K). Galanin and tyrosine hydroxylase were not co-expressed with Brn3a in the CNS as they are in the sensory system (Fig. 4L,M and data not shown), and thus could not be the targets of cell-autonomous regulation by Brn3a. Taken together, the in situ hybridization and immunohistochemical data for the targets of Brn3a regulation in the trigeminal ganglia demonstrate considerable conservation of the regulatory role of Brn3a in sensory neurons at different levels of the neural axis, but suggest a distinct role for Brn3a in the CNS.

Discussion

In this study, we have presented a systematic examination of the changes in gene expression resulting from the loss of a key regulator of sensory neurogenesis. Although the list of significantly changed genes is fairly extensive, the specificity of the developmental effects of Brn3a are underscored by several findings: a majority of the changed transcripts encode proteins with known or hypothesized roles in sensory neuron development or function, a far greater number of neuron-specific genes did not significantly change (see Supplemental Data), and there were almost no significant changes in the expression of ubiquitously expressed or ‘housekeeping’ genes.

Most of the genes with profoundly changed expression can be divided into three functional categories: neurotransmitter systems and ion channels, mediators of axonogenesis/synaptogenesis, and transcriptional regulators. Each of these classes of regulated transcripts may be related to the defects in axon growth and cell survival seen in *Brn3a* mutant mice, but

it is likely that these changes in gene expression synergise to produce the *Brn3a* knockout phenotype, and that no single target gene is sufficient to account for the observed defects.

Beyond explaining the sensory phenotype of *Brn3a* knockout mice, two interesting generalizations may be made which encompass many of the genes with altered expression. First, in the absence of Brn3a, trigeminal development is retarded, in the sense that the expression of numerous markers of a mature sensory phenotype are reduced, and the developmental expression of factors that play a transient role in the early phases of differentiation is abnormally prolonged. Second, several transcription factors are expressed outside their normal axial level in the sensory ganglia, suggesting that Brn3a acts to spatially restrict their expression.

Neurotransmitter systems and channels

The array results clearly demonstrate that Brn3a has a major role in determining the neurotransmitter phenotype of the developing trigeminal ganglia. Expression of the neuropeptides PACAP and galanin and the NPY1 receptor are highly dependent on Brn3a, and the rate-limiting enzyme of catecholamine synthesis, tyrosine hydroxylase, is also significantly reduced in *Brn3a* knockouts. In contrast, the expression of somatostatin and the 5HT3A receptor are

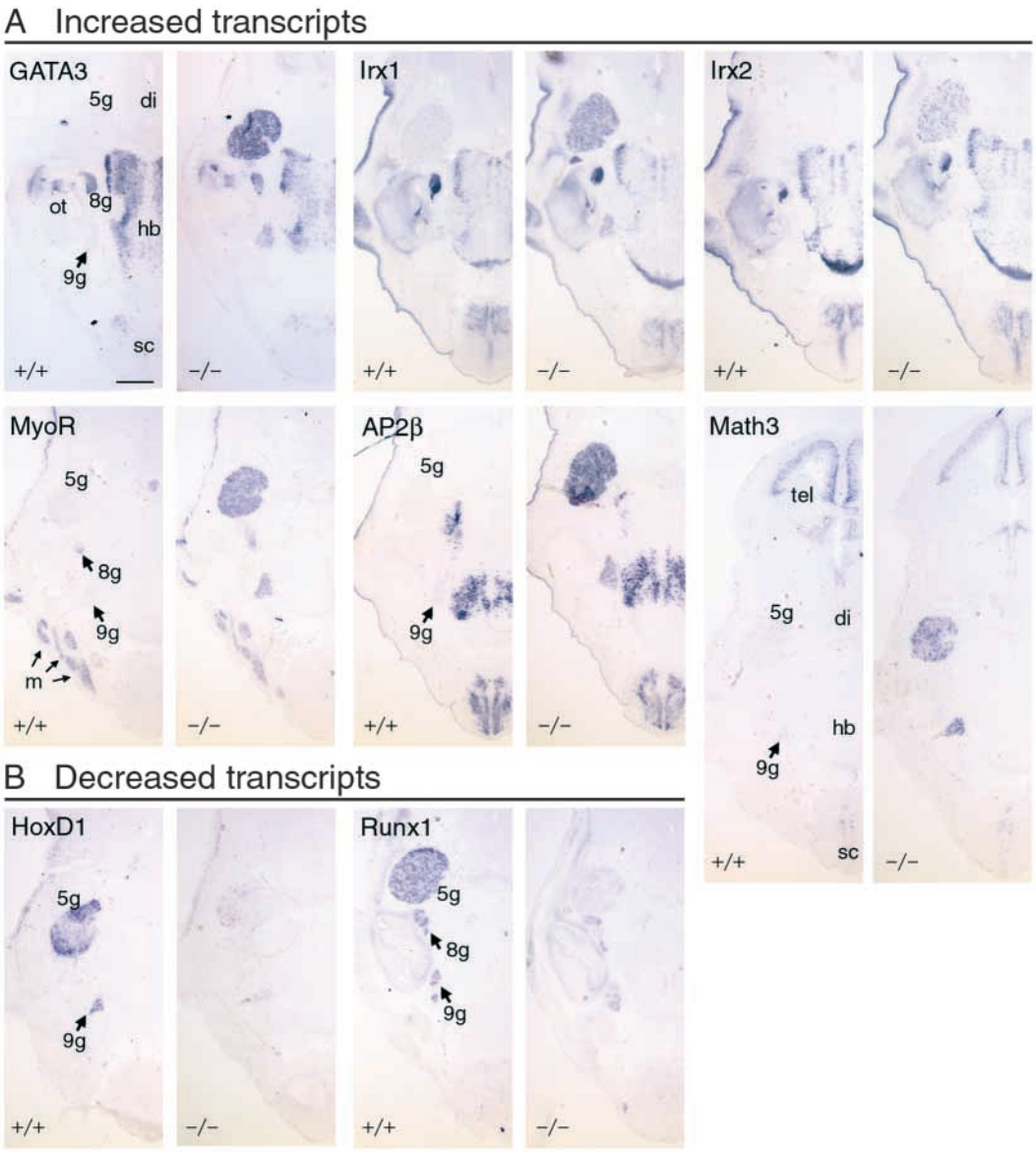

Fig. 3. Coordinated regulation of transcription factor expression in sensory ganglia by Brn3a. (A) In situ hybridization analysis of E13.5 embryos for expression of the transcription factors GATA3, Irx1, Irx2, MyoR, Ap2β and Math3, all of which exhibited increased expression in the microarray analysis in *Brn3a* knockout ganglia. (B) Expression of mRNA for the decreased transcription factors HoxD1 and Runx1. In addition to the cranial sensory ganglia, each of these factors also exhibited previously known patterns of expression in the CNS, and in the case of MyoR, in developing cranial musculature. The plane of section used in all views is shown in Fig. 2A. 5g, trigeminal ganglion; 8g, vestibulocochlear ganglion; 9g, IX/X ganglion complex; di, diencephalon; hb, hindbrain; m, differentiating occipital musculature; ot, otic region; tel, telencephalon; sc, spinal cord.

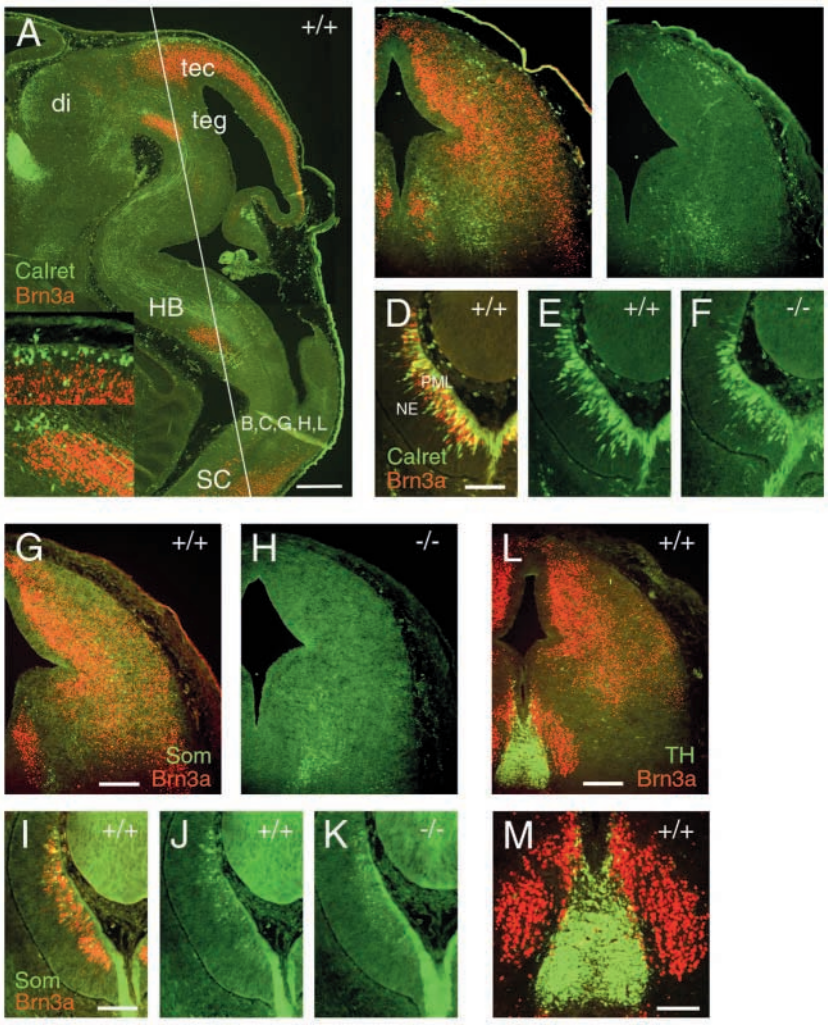

Fig. 4. Cellular expression of Brn3a target genes in the CNS. The brain and retina of E13.5 embryos were examined for alterations in Brn3a target genes identified in the trigeminal ganglion. (A) Calretinin and Brn3a characterize distinct populations of developing neurons in the E13.5 developing thalamus, midbrain, and hindbrain, shown in sagittal section, and are not co-expressed (inset). The diagonal line indicates the plane of section used in the midbrain views (B,C,G,H,L). (B) Control midbrain, showing distinct expression of Brn3a and calretinin. (C) Unchanged expression of calretinin in the *Brn3a* knockout midbrain. (D,E) Control retina, showing calretinin and Brn3a expression in overlapping populations of neurons. (F) *Brn3a* knockout retina showing no apparent increase in calretinin immunoreactivity. (G,H) Distinct patterns of somatostatin and Brn3a immunoreactivity in the midbrain, which are not changed in the *Brn3a* knockout. (I,J) Retinal expression of somatostatin, probably co-localized with Brn3a in a subset of ganglion cells, although the axonal distribution of somatostatin immunoreactivity makes precise cellular co-localization difficult to ascertain. (K) Retinal expression of somatostatin also appears unaltered in the absence of Brn3a. (L,M) Tyrosine hydroxylase and Brn3a identify entirely distinct populations of developing neurons in the ventral tegmental area (VTA) and the tegmentum (nuclei stained red), respectively. Scale bars: A, 400 μm; B,D,G, 100 μm; I, 100 μm; L, 200 μm; M, 50 μm.

markedly increased. Studies in the developing rat have shown that somatostatin is strongly expressed throughout the sensory ganglia soon after neurogenesis, but by mid-gestation its expression is restricted to a relatively small subset of sensory neurons (Katz et al., 1992). Thus the increased expression of somatostatin at E13.5 is very likely to represent a failure in the normal developmental attenuation of this gene, consistent with the idea that *Brn3a* knockout ganglia exhibit a pervasive maturation defect.

Also notable are changes in the expression of sodium channels, including Scn6 and Scn9, which are markedly decreased in *Brn3a* knockout ganglia, and Scn10, which is moderately decreased (Table S2, http://dev.biologists.org.supplemental/). Remarkably, these changes affect only those sodium channels that appear to have specific expression in the sensory nervous system (Goldin, 1999; Waxman et al., 1999), suggesting that Brn3a directly or indirectly coordinates expression of these channels. In contrast, expression levels of most neurotransmitter receptors, such as the GABA and glutamate receptors, and several classes of ion channels with wide expression in the CNS and PNS, are unchanged. Two other markedly changed genes, calretinin and the regulator of G-protein signaling RGS10, have putative roles in the modulation of neurotransmitter signals mediated by Ca^{2+}-dependent and G-protein pathways, respectively. Altered expression of these genes may represent primary changes, or they may occur in an attempt to compensate homeostatically for other changes in neurotransmitter systems.

Changes in expression of genes related to axon growth

Mice lacking Brn3a have marked defects in sensory axon growth, including defasciculation of axon bundles and failure to innervate peripheral and central targets (Eng et al., 2001; Trieu et al., 2003). The transcripts for several proteins known to be involved in axon growth and synaptogenesis were significantly decreased in *Brn3a* null mice. Among the proteins in this category is advillin (pervin), an actin-binding protein with specific expression in sensory and sympathetic ganglia, which increases neurite outgrowth in cultured dorsal root ganglia (Ravenall et al., 2002). Apolipoprotein E knockout mice exhibit anatomical and functional defects in unmyelinated nerve fibers (Fullerton et al., 1998). Although this has been attributed to loss of ApoE expressed in associated glia, our results suggest that the defect may be intrinsic to sensory neurons.

Also decreased in *Brn3a* knockout ganglia were transcripts for the functionally interrelated proteins insulin-like growth

factor 1 (IGF1) and insulin-like growth factor binding protein 5 (IGFBP5). Mice lacking IGF1 have abnormalities in sensory neurons (Gao et al., 1999), and show defective cortical dendritic growth (Cheng et al., 2003). IGFBP5 is a widely expressed protein whose role in vivo has not been clearly defined. However, it is highly expressed in the axon terminals of developing sensory neurons (Cheng et al., 1996), where it is frequently co-localized with IGF1, suggesting that it also has a role in axon growth. Because these proteins are known to interact, relative deficiencies in their expression may have a synergistic effect.

Another group of Brn3a-regulated proteins likely to have a role in axon growth are those involved in cell signaling and intracellular signal transduction. Transcripts with significantly changed expression include N-chimaerin, downstream of tyrosine kinase 4 (Dok4), the low affinity neurotrophin receptor p75, the small GTPases RAP (Ras family) and WRCH1 (Rho family), and Dusp6/MKP3. The expression and potential role of some of these factors in the sensory nervous system has been described; in other cases, the function of related proteins suggest that they may have significant and synergistic effects on axon growth.

Transcription factors

Loss of Brn3a results in profound changes in the expression of several transcriptional regulators of various types, suggesting a web of cross-regulation between genes involved in sensory neurogenesis. The expression of a few transcription factors expressed late in sensory development, such as Runx1, were decreased in the absence of Brn3a, but the majority of the changes were increases, suggesting that Brn3a functions as a repressor of transcription factors that would be temporally or spatially inappropriate in the maturing trigeminal ganglion.

The clearest example of the role of Brn3a in restricting the spatial expression of other transcription factors is the ectopic expression of GATA3, Irx1, Irx2, NeuroD1 and MyoR/musculin in *Brn3a* knockout mice. These factors are all expressed in the developing vestibulocochlear ganglion in control embryos, and in the absence of Brn3a are markedly increased in the trigeminal and IX/X ganglia, demonstrating an expansion of the expression domain of these genes in both directions of the rostrocaudal axis. It is likely that some of the downstream changes in gene expression in *Brn3a* knockout ganglia are mediated by these factors, but current knowledge of their role in neural development is not sufficient to predict the effect of their mis-expression in the trigeminal ganglion. GATA3 has a known role in the development of motor neurons originating in rhombomere 4, which innervate the inner ear, and the inner ear itself (Karis et al., 2001). NeuroD1 is also required for normal development of the sensory neurons of the inner ear (Liu et al., 2000), and may have a cross-regulatory relationship with GATA3 (Lawoko-Kerali et al., 2004). Although the role of the Irx genes in sensory development has not been described in mice, the zebrafish protein iro7, a possible paralogue of Irx1, is required for trigeminal placode development in fish (Itoh et al., 2002). The bHLH factor MyoR (musculin) is normally expressed in the developing facial muscles of the first branchial arch, which are innervated by trigeminal neurons, but not in the trigeminal ganglion itself (Lu et al., 2002). Our observation that MyoR is expressed in the developing auditory system is the first report of the sensory expression of this gene, and its role in neurogenesis is unknown.

Although it was not detected in the vestibulocochlear ganglion at this stage, AP2β showed a similar pattern of ectopic expression in the trigeminal and IX cranial ganglia in E13.5 *Brn3a* knockout embryos. AP2β is normally expressed in the embryonic hindbrain and spinal cord, but little is known about its role in neural development. The nervous system of *AP2β* mutant mice, which die from polycystic kidney disease, has no obvious abnormalities (Moser et al., 1997). However, mice lacking the related factor AP2α, which is highly expressed in migrating neural crest and in the developing sensory ganglia, exhibit extensive cranial abnormalities and dysgenesis of the cranial ganglia (Schorle et al., 1996). There is some evidence that AP2β is a weak transcriptional activator, and may oppose gene activation by AP2α (Bosher et al., 1996). Thus the increased expression of AP2β observed here may mimic some aspects of the loss of AP2α.

The increased expression of Math3 and NeuroD1 in *Brn3a* knockout trigeminal ganglia, together with decreased expression of the inhibitor of bHLH function Id1, suggest a marked increase in bHLH activity in the absence of Brn3a. Math3 and NeuroD1 have been characterized in the early development of the trigeminal ganglion (E9.0), where both factors appear to be downstream of the neurogenic HLH factor Ngn1 (Ma et al., 1998). Thus the increased expression of bHLH factors in *Brn3a* knockout mice may reflect the abnormal persistence of genes normally down-regulated as sensory development progresses. Although the loss of NeuroD1 or Math3 alone does not have an obvious effect on neurogenesis in the trigeminal (Tomita et al., 2000), the increased expression of multiple bHLH genes may have a synergistic effect in *Brn3a* knockout mice.

Possible mechanisms of sensory cell death in mice lacking Brn3a

Embryonic day 13.5 was chosen for gene expression analysis because it precedes the extensive loss of sensory neurons observed at later stages in *Brn3a* knockout mice, and consistent with this, we did not observe altered expression of genes usually associated with cell death pathways, such as caspases or bcl2-family genes. Sensory cell death in mice lacking Brn3a occurs after these neurons normally become neurotrophin dependent, and the decreased expression of neurotrophins and their receptors in *Brn3a* knockout mice has been suggested as a cause of this mortality (Huang et al., 1999). We have previously reported that the expression of the TrkA neurotrophin receptor mRNA is moderately decreased in *Brn3a* knockout mice (Ma et al., 2003). This observation, and the decreased expression of the p75 low affinity NGF receptor shown here, are consistent with previous results (Huang et al., 1999; McEvilly et al., 1996). However, because the TrkA receptor is generally regarded as anti-apoptotic, and the p75 receptor as pro-apoptotic in sensory neurons (Huang and Reichardt, 2001), it is not obvious what net effect a moderate decrease in both receptors would have on cell survival. Given the severity of the axon growth defects in *Brn3a* knockout mice, another possibility is that excessive neural death occurs because of a failure to obtain target-derived neurotrophins, but this hypothesis has not been tested directly.

Tissue specificity of gene regulation

In the present study we have defined a set of genes regulated by Brn3a in sensory ganglia. This represents one of the first comprehensive descriptions of the in vivo regulatory targets for any factor regulating vertebrate neurogenesis. Like many developmental regulators, Brn3a is expressed in a highly specific, yet diverse set of neurons, including those of the retina, diencephalon, midbrain, spinal cord and sensory system, leading to the question of whether Brn3a regulates a common set of targets in these distinct locations. In the present study we have found little evidence that the targets of Brn3a regulation in the trigeminal ganglia are also regulated in the CNS or in the retina. A recent analysis of the regulatory targets of the closely related POU-factor Brn3b in the retina revealed few changed transcripts in common with the present study, despite the fact that the retinal ganglion cells in *Brn3b* knockout mice show a secondary loss of Brn3a (Mu et al., 2004); it also did not detect changes in the retinal target genes in sensory ganglia.

Even within the peripheral sensory system, Brn3a targets appear to be distinctly regulated in the vestibulocochlear ganglion when compared to the coordinated changes in expression in the trigeminal, IX, and dorsal root ganglia. The lack of change in trigeminal target genes in the vestibulocochlear ganglion cannot be attributed to functional redundancy of Brn3 genes. Although Brn3b is also expressed in the vestibulocochlear system, the loss of Brn3a expression in the vestibulocochlear ganglion also leads to diminished expression of Brn3b, and results in significant defects in cochlear innervation (Huang et al., 2001). Thus it appears probable that Brn3a will have at least a partially distinct set of regulatory targets in the auditory system.

The genes downstream from Brn3a in the sensory ganglia are very likely to include targets that are regulated directly, and regulated indirectly by the several other transcription factors that change expression in the absence of Brn3a. One of the surprising features of the current study is the large number of markedly increased transcripts in the knockout ganglia, implying direct or indirect transcriptional repression by Brn3a. Although nearly all prior studies of the transcriptional activity of Brn3a have proceeded from the assumption that it is a positive regulator of gene expression, we have recently shown that Brn3a is a direct repressor of its own expression in the trigeminal ganglion in vivo (Trieu et al., 2003). The recent study of the target genes of Brn3b in the retina showed mainly decreased expression of downstream transcripts (Mu et al., 2004), but this study was conducted with a retina-specific cDNA array, which would be unlikely to include strongly increased transcripts which have low levels of expression in the normal retina. Thus it is plausible that Brn3a, and perhaps other factors in this class, exert their direct effects by transcriptional repression.

Identifying the regulatory targets of neural transcription factors is an essential component of understanding developmental pathways in the nervous system. Here we have demonstrated an extensive program of gene regulation mediated by one such factor. Future studies of this kind will be greatly facilitated by the availability of more complete gene expression arrays based on genomic sequences rather than cDNA libraries. Additional data about the location of the transcription units in the mouse genome, and better information about the DNA recognition properties of the various transcription factor classes, will help to distinguish direct from secondary targets. In addition, the confirmation of direct regulation by chromatin immunoprecipitation may be facilitated by combining this method with array technology or other high throughput methods (Ren et al., 2002). These anticipated technical advances should in principle allow the identification of a complete set of regulatory targets for any transcription factor in any tissue.

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References

Birren, S. J., Lo, L. and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development* **119**, 597-610.

Bosher, J. M., Totty, N. F., Hsuan, J. J., Williams, T. and Hurst, H. C. (1996). A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* **13**, 1701-1707.

Budhram-Mahadeo, V., Morris, P., Ndisang, D., Irshad, S., Lozano, G., Pedley, B. and Latchman, D. S. (2002). The Brn-3a POU family transcription factor stimulates p53 gene expression in human and mouse tumour cells. *Neurosci. Lett.* **334**, 1-4.

Budhram-Mahadeo, V., Morris, P. J., Lakin, N. D., Theil, T., Ching, G. Y., Lillycrop, K. A., Moroy, T., Liem, R. K. and Latchman, D. S. (1995). Activation of the alpha-internexin promoter by the Brn-3a transcription factor is dependent on the N-terminal region of the protein. *J. Biol. Chem.* **270**, 2853-2858.

Cheng, C. M., Mervis, R. F., Niu, S. L., Salem, N., Jr, Witters, L. A., Tseng, V., Reinhardt, R. and Bondy, C. A. (2003). Insulin-like growth factor 1 is essential for normal dendritic growth. *J. Neurosci. Res.* **73**, 1-9.

Cheng, H. L., Sullivan, K. A. and Feldman, E. L. (1996). Immunohistochemical localization of insulin-like growth factor binding protein-5 in the developing rat nervous system. *Brain Res. Dev. Brain Res.* **92**, 211-218.

Eng, S., Gratwick, K., Rhee, J., Fedtsova, N., Gan, L. and Turner, E. (2001). Defects in sensory axon growth precede neuronal death in Brn3a-deficient mice. *J. Neurosci.* **21**, 541-549.

Eng, S. R., Kozlov, S. and Turner, E. E. (2003). Unaltered expression of Bcl-2 and TAG-1/axonin-1 precedes sensory apoptosis in Brn3a knockout mice. *Neuroreport* **14**, 173-176.

Fedtsova, N. and Turner, E. (1995). Brn-3.0 Expression identifies early post-mitotic CNS neurons and sensory neural precursors. *Mech. Dev.* **53**, 291-304.

Fullerton, S. M., Strittmatter, W. J. and Matthew, W. D. (1998). Peripheral sensory nerve defects in apolipoprotein E knockout mice. *Exp. Neurol.* **153**, 156-163.

Gao, W. Q., Shinsky, N., Ingle, G., Beck, K., Elias, K. A. and Powell-Braxton, L. (1999). IGF-I deficient mice show reduced peripheral nerve conduction velocities and decreased axonal diameters and respond to exogenous IGF-I treatment. *J. Neurobiol.* **39**, 142-152.

Goldin, A. L. (1999). Diversity of mammalian voltage-gated sodium channels. *Ann. NY Acad. Sci.* **868**, 38-50.

Huang, E., Zang, K., Schmidt, A., Saulys, A., Xiang, M. and Reichardt, L. (1999). POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. *Development* **126**, 2869-2882.

Huang, E. J., Liu, W., Fritzsch, B., Bianchi, L. M., Reichardt, L. F. and

Xiang, M. (2001). Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons. *Development* **128**, 2421-2432.

Huang, E. J. and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677-736.

Ichikawa, H., Mo, Z., Xiang, M. and Sugimoto, T. (2002a). Effect of Brn-3a deficiency on nociceptors and low-threshold mechanoreceptors in the trigeminal ganglion. *Brain Res. Mol. Brain Res.* **104**, 240-245.

Ichikawa, H., Yamaai, T., Jacobowitz, D. M., Mo, Z., Xiang, M. and Sugimoto, T. (2002b). Effect of Brn-3a deficiency on parvalbumin-, calbindin D-28k-, calretinin- and calcitonin gene-related peptide-immunoreactive primary sensory neurons in the trigeminal ganglion. *Neuroscience* **113**, 537-546.

Itoh, M., Kudoh, T., Dedekian, M., Kim, C. H. and Chitnis, A. B. (2002). A role for iro1 and iro7 in the establishment of an anteroposterior compartment of the ectoderm adjacent to the midbrain-hindbrain boundary. *Development* **129**, 2317-2327.

Karis, A., Pata, I., van Doorninck, J. H., Grosveld, F., de Zeeuw, C. I., de Caprona, D. and Fritzsch, B. (2001). Transcription factor GATA-3 alters pathway selection of olivocochlear neurons and affects morphogenesis of the ear. *J. Comp. Neurol.* **429**, 615-630.

Katz, D. M., He, H. and White, M. (1992). Transient expression of somatostatin peptide is a widespread feature of developing sensory and sympathetic neurons in the embryonic rat. *J. Neurobiol.* **23**, 855-870.

Lakin, N. D., Morris, P. J., Theil, T., Sato, T. N., Moroy, T., Wilson, M. C. and Latchman, D. S. (1995). Regulation of neurite outgrowth and SNAP-25 gene expression by the Brn-3a transcription factor. *J. Biol. Chem.* **270**, 15858-15863.

Lawoko-Kerali, G., Rivolta, M. N., Lawlor, P., Cacciabue-Rivolta, D. I., Langton-Hewer, C., Hikke van Doorninck, J. and Holley, M. C. (2004). GATA3 and NeuroD distinguish auditory and vestibular neurons during development of the mammalian inner ear. *Mech. Dev.* **121**, 287-299.

Liu, M., Pereira, F. A., Price, S. D., Chu, M. J., Shope, C., Himes, D., Eatock, R. A., Brownell, W. E., Lysakowski, A. and Tsai, M. J. (2000). Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems. *Genes Dev.* **14**, 2839-2854.

Lu, J. R., Bassel-Duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., Gan, L., Shelton, J. M., Richardson, J. A. and Olson, E. N. (2002). Control of facial muscle development by MyoR and capsulin. *Science* **298**, 2378-2381.

Ma, L., Lei, L., Eng, S. R., Turner, E. and Parada, L. F. (2003). Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons. *Development* **130**, 3525-3534.

Ma, Q., Chen, Z., Barrantes, I., de la Pompa, J. and Anderson, D. (1998). Neurogenin1 is essential for the determination of neuronal precursors for proximal sensory ganglia. *Neuron* **20**, 469-482.

McEvilly, R. J., Erkman, L., Luo, L., Sawchenko, P. E., Ryan, A. F. and Rosenfeld, M. G. (1996). Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons. *Nature* **384**, 574-577.

Milton, N. G., Bessis, A., Changeux, J. P. and Latchman, D. S. (1996). Differential regulation of neuronal nicotinic acetylcholine receptor subunit gene promoters by Brn-3 POU family transcription factors. *Biochem J.* **317**, 419-423.

Morris, P. J., Lakin, N. D., Dawson, S. J., Ryabinin, A. E., Kilimann, M. W., Wilson, M. C. and Latchman, D. S. (1996). Differential regulation of genes encoding synaptic proteins by members of the Brn-3 subfamily of POU transcription factors. *Brain Res. Mol. Brain Res.* **43**, 279-285.

Moser, M., Ruschoff, J. and Buettner, R. (1997). Comparative analysis of AP-2 alpha and AP-2 beta gene expression during murine embryogenesis. *Dev. Dyn.* **208**, 115-124.

Mu, X., Beremand, P. D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T. L. and Klein, W. H. (2004). Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. *Development* **131**, 1197-1210.

Ravenall, S. J., Gavazzi, I., Wood, J. N. and Akopian, A. N. (2002). A peripheral nervous system actin-binding protein regulates neurite outgrowth. *Eur. J. Neurosci.* **15**, 281-290.

Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A. and Dynlacht, B. D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. *Genes Dev.* **16**, 245-256.

Schorle, H., Meier, P., Buchert, M., Jaenisch, R. and Mitchell, P. J. (1996). Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* **381**, 235-238.

Smith, M. D., Morris, P. J., Dawson, S. J., Schwartz, M. L., Schlaepfer, W. W. and Latchman, D. S. (1997). Coordinate induction of the three neurofilament genes by the Brn-3a transcription factor. *J. Biol. Chem.* **272**, 21325-21333.

Smith, M. D., Ensor, E. A., Coffin, R. S., Boxer, L. M. and Latchman, D. S. (1998). Bcl-2 transcription from the proximal P2 promoter is activated in neuronal cells by the Brn-3a POU family transcription factor. *J. Biol. Chem.* **273**, 16715-16722.

Theiler, K. (1972). *The House Mouse; Development and Normal Stages from Fertilization To 4 Weeks of Age.* Berlin, New York: Springer-Verlag.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F. and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. *EMBO J.* **19**, 5460-5472.

Trieu, M., Ma, A., Eng, S. R., Fedtsova, N. and Turner, E. E. (2003). Direct autoregulation and gene dosage compensation by POU-domain transcription factor Brn3a. *Development* **130**, 111-121.

Trieu, M., Rhee, J., Fedtsova, N. and Turner, E. (1999). Autoregulatory sequences are revealed by complex stability screening of the mouse *brn-3.0* locus. *J. Neurosci.* **19**, 6549-6558.

Waxman, S. G., Cummins, T. R., Dib-Hajj, S., Fjell, J. and Black, J. A. (1999). Sodium channels, excitability of primary sensory neurons, and the molecular basis of pain. *Muscle Nerve* **22**, 1177-1187.

Xiang, M., Lin, G., Zhou, L., Klein, W. H. and Nathans, J. (1996). 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. *Proc. Natl. Acad. Sci. USA* **93**, 11950-11955.