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Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons

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SUMMARY

The TrkA/NGF receptor is essential for the survival and differentiation of sensory neurons. The molecular mechanisms regulating tissue and stage-specific expression of TrkA are largely unknown. The Brn3a POU-domain transcription factor has been implicated in the development of the PNS and proposed as a transcription regulator for TrkA. The molecular mechanisms underlying the regulation of TrkA by Brn3a is unclear. In this study, we provide genetic, transgenic and biochemical evidence that Brn3a binds to novel, specific sites in the 457 bp enhancer that regulates TrkA expression in embryonic sensory neurons. We employ *Bax*-knockout mice, in which sensory neurons no longer require neurotrophins for survival, to uncouple TrkA-dependent cell death from downregulation of *TrkA* expression. In addition, when mutagenized, the novel Brn3a-binding sites identified fail to drive appropriate reporter transgene expression in sensory neurons. Thus, *TrkA*, a gene that is crucial for the differentiation and survival of sensory nociceptive neurons, requires *Brn3a* to maintain normal transcriptional activity.

Key words: TrkA, Brn3a, Sensory neuron, Transcriptional regulation, Bax

INTRODUCTION

Neurotrophins and their Trk family receptor tyrosine kinases act on the development and function of the PNS. Numerous in vitro and in vivo studies have established the roles of each Trk receptor in neurite outgrowth, survival and differentiation (Bibel and Barde, 2000; Huang and Reichardt, 2001; Snider, 1994). The differential expression of Trk receptors in subsets of sensory neurons indicates that Trk receptor expression and sensory neuron differentiation are closely related processes. For example, regulation of the expression of Trk family receptor is fundamental to the appropriate appearance and survival of sensory neurons.

TrkA (*Ntrk1* – Mouse Genome Informatics), the prototype of the Trk gene family and the receptor for nerve growth factor (NGF), is required for the survival of nociceptive sensory and sympathetic neurons (Martin-Zanca et al., 1990; Smeyne et al., 1994). In the murine PNS, *TrkA* expression is confined to neural-crest-derived sensory neurons, including trigeminal and dorsal root ganglia, from early embryonic stages (E9.5) to adults. TrkA is also expressed in sympathetic neurons from E16.5 onwards (Martin-Zanca et al., 1990; Tessarollo et al., 1993). In the CNS, *TrkA* expression is described in only a subset of cholinergic neurons in the basal forebrain (Holtzman et al., 1992). Understanding the molecular mechanisms for TrkA expression is important to dissect the biological processes regulating the survival and differentiation of sensory and sympathetic neurons.

One approach to identify transcriptional regulators of TrkA is to search for known transcription factors that are expressed in sensory neurons during mouse embryonic development and whose loss-of-function lead to alterations of TrkA expression. The POU-homeodomain transcription factor Brn3a (Pou4f1 – Mouse Genome Informatics) (Gerrero et al., 1993; He et al., 1989; Ninkina et al., 1993; Xiang et al., 1993) stands out as an attractive candidate. Brn3a is expressed early in emerging sensory neurons (Artinger et al., 1998; Fedtsova and Turner, 1995) and mutation of this gene in mice leads to abnormal development and loss of neurons that express TrkA, TrkB (Ntrk2 – Mouse Genome Informatics) and TrkC (Ntrk3 – Mouse Genome Informatics) (Eng et al., 2001; Huang et al., 2001; Huang et al., 1999; McEvilly et al., 1996; Xiang et al., 1996). However, questions remain about why and how *TrkA* becomes downregulated in *Brn3a*-null sensory neurons. Whether Brn3a is a requisite transcriptional regulator of the TrkA enhancer or an indirect mediator of TrkA expression is unresolved.

Previously, we identified a *TrkA* minimal enhancer that confers specific expression of β-galactosidase transgenes in PNS sensory neurons (Ma et al., 2000). Through mutational analysis in transgenic mice, multiple important, cis-elements were identified. These results indicate that the tightly regulated

TrkA gene is controlled by multiple transcription factors acting in concert (Lei et al., 2001; Ma et al., 2000). However, these studies failed to identify canonical Brn3a-binding sequences in this enhancer. If Brn3a directly regulates TrkA transcription, the binding sites on the TrkA enhancer remain unidentified.

In the present study, we have used genetic, biochemical and transgenic approaches to examine whether Brn3a is a direct or indirect regulator of TrkA transcription.

MATERIALS AND METHODS

Mouse maintenance

Brn3a$^{+/-}$ mice and *Bax*$^{+/-}$ mice were crossed to generate nine different genetic combinations with mixed genetic background: *Brn3a*$^{+/+}$/*Bax*$^{+/+}$; *Brn3a*$^{+/+}$/*Bax*$^{+/-}$; *Brn3a*$^{+/+}$/*Bax*$^{-/-}$; *Brn3a*$^{+/-}$/*Bax*$^{+/+}$; *Brn3a*$^{+/-}$/*Bax*$^{+/-}$; *Brn3a*$^{+/-}$/*Bax*$^{-/-}$; *Brn3a*$^{-/-}$/*Bax*$^{+/+}$; *Brn3a*$^{-/-}$/*Bax*$^{+/-}$ and *Brn3a*$^{-/-}$/*Bax*$^{-/-}$. Four genotypes were used for experiments (see results). Mouse husbandry followed the guidelines of UTSW IACRAC.

Genotyping

We designed novel PCR primers to genotype *Brn3a*-mutant mice: wild-type 5′ primer, 5′-CTTGGCTTCCACTCAGCATCTGGAGC-3′; wild-type 3′ primer, 5′-CTGTATTCAGTGGAGAGAAGTGG-AAACGG-3′; NEO primer, 5′-GATTCGCAGCGCATCGCC-TTCTATCG-3′. PCR conditions were: 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute; 35 cycles. The size of the wild-type and mutant bands are ~600 bp and ~450 bp, respectively. The PCR primers and conditions for *Bax* genotyping were as described (Deckwerth et al., 1996): *Bax* exon primer 5′-TGATCAGAACCATCATG-3′; *Bax* intron primer 5′-GTTGACCAGAGTGGCGTAGG-3′; and *Bax* neo/pgk primer 5′-CCGCTTCCATTGCTCAGCGG-3′. PCR primers for β-galactosidase genotyping were: 5′-AACTGGAAGTCGCC-GCGCCACTGGTGTGGG-3′ and 5′-TGAACTGCCAGCTGG-CGCAGGTAGCAGAGC-3′. PCR conditions were: 94°C, 30 seconds; 65°C, 30 seconds; 72°C, 30 seconds; 31 cycles. Generation of transient transgenic embryos and X-gal staining of the transgenic embryos were performed as described (Ma et al., 2000).

Purification of GST-Brn3a fusion protein

Escherichia coli BL21 competent cells were transformed with an expression plasmid for GST-Brn3a POU-domain fusion protein (Xiang et al., 1995). 1 liter cell culture at OD_{600}=0.6 was induced to express the fusion protein with 1 mM IPTG for 4 hours. The cell pellet was resuspended in lysis buffer and sonicated five times on ice for 10 seconds. The supernatant was purified with Glutathone-Sepharose 4B beads (Pharmacia). Cells transformed with the control plasmid expressing only GST were processed simultaneously.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described (Gruber et al., 1997) with minor modifications. Briefly, 10,000 to 40,000 cpm of ^{32}P-labeled oligonucleotide probe was mixed with 1 μl (0.3 μg) purified GST-Brn3a fusion protein, 5 × gel-shift buffer and 1 μg poly(dI-dC) (Boehringer). Finally, water was added to a total volume of 20 μl. The mix was incubated at 25°C for 30 minutes. For competition assays, different amounts of cold oligonucleotides were mixed with radiolabeled probes. Electrophoresis was done using 5% polyacrylamide gels in 0.5×TBE buffer at 25°C for 3 hours. Gels were then dried and exposed with Kodak X-ray film.

DNase I footprint assay

DNase I footprint assays were performed as described (Brenowitz et al., 1995) with minor modifications. Either the 5′ or 3′ end of the *TrkA* minimal-enhancer fragment was labeled with [γ-^{32}P]-ATP. The labeled fragment was purified with phenol/chloroform extraction and

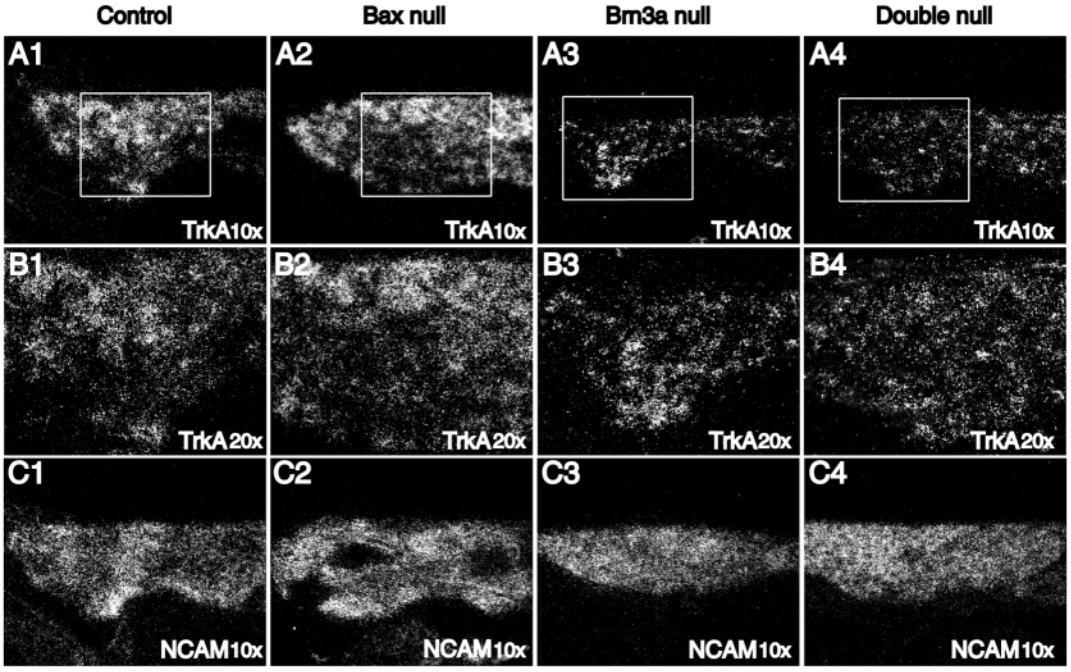

Fig. 1. Reduced *TrkA* mRNA in P0 *Brn3a*-null or double-null trigeminal ganglia. Low (A1-A4) and high (B1-B4) magnification pictures of *TrkA* mRNA in situ hybridization signal, showing that *Brn3a*-null (A3,B3) and double-null (A4,B4) ganglia have reduced *TrkA* mRNA compared with control (A1,B1) and *Bax*-null ganglia (A2,B2), respectively. As a control, (C1-C4) *NCAM* mRNA signal is similar in all four genotypes.

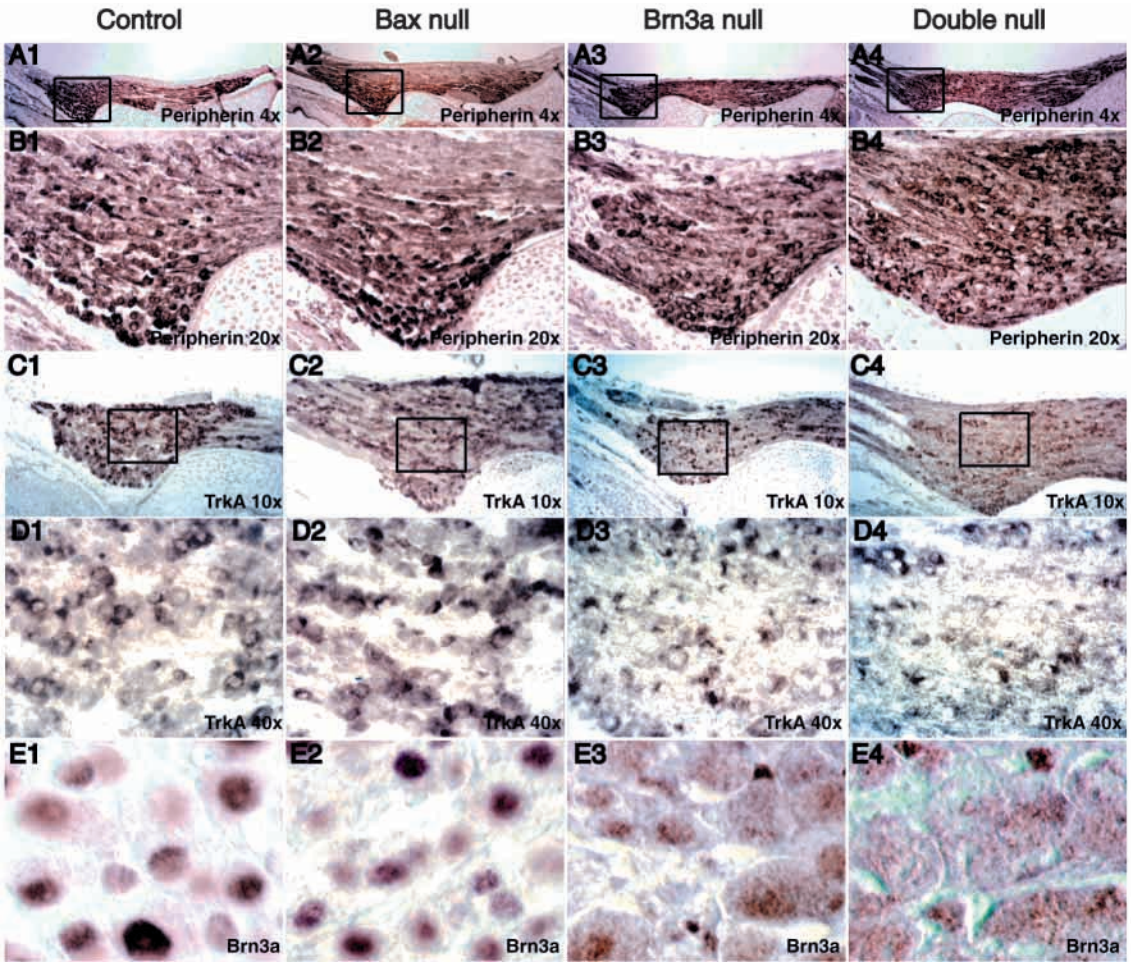

Fig. 2. Reduced TrkA protein in P0 *Brn3a*-null or double-null trigeminal ganglia. Low (A1-A4) and high (B1-B4) magnification pictures of peripherin immunostaining, showing that all four genotypes have similar levels of peripherin in trigeminal ganglia. Low (C1-C4) and high (D1-D4) magnification pictures of TrkA immunostaining, showing that *Brn3a*-null (C3,D3) and double-null (C4,D4) ganglia have reduced TrkA protein compared with control (C1,D1) and *Bax*-null (C2,D2) ganglia, respectively. (E1-E4) Brn3a immunostaining showing that *Brn3a*-null (E3) and double-null (E4) ganglia lack nuclear Brn3a protein compared with control (E1) and *Bax*-null (E2) ganglia. Double-null ganglia are always larger and have more neurons than *Brn3a*-null ganglia.

ethanol precipitation. Different amounts of either purified, GST-Brn3a fusion protein or GST control protein was mixed with the labeled fragment and treated with graduated amounts of DNase I for 2 minutes at 25°C. The reaction was stopped by adding three volumes of cold ethanol, precipitated and run on sequencing gel. Sequencing reactions primed from the starting position of the labeled end were loaded onto the same gel to identify the protected sequences.

In situ hybridization

In situ hybridization was performed as described (Lei et al., 2001). The templates for making antisense in situ probes were TrkA (454 bp, extracellular domain) and NCAM (a 420 bp coding sequence). Sections were processed simultaneously for comparison.

Immunohistochemistry

Immunohistochemistry was performed as described (Ma et al., 2002) using the following antibodies and dilutions: monoclonal NeuN (1:500) (Chemicon); rabbit anti-NF200 (1:1000) (Sigma); rabbit anti peripherin (1:1000) (Chemicon); and rabbit anti TrkA (1:1000) (Advanced Targeting System). Antigen retrieval was performed as described by the manufacturer (BioGenex, CA) to detect TrkA signal. Sections were processed simultaneously with the same antibody for comparison.

Affymetrix microchip assay and analysis

Affymetrix microchip assay and analysis followed the manufacturers instructions (Affymetrix, CA). The source of labeled probe was trigeminal ganglia prepared from E13.5 *Brn3a*-null, heterozygous and wild-type littermates.

RESULTS

Brn3a is required for normal *TrkA* expression

Brn3a-knockout mice exhibit extensive sensory neuron death in late gestation. During this process, expression of Trk family genes is noted to decrease (Huang et al., 1999; McEvilly et al., 1996). To determine whether *Brn3a*-mediated effects on *TrkA* expression are direct or secondary to unhealthy neurons that are in the process of perishing, we sought to uncouple neuronal survival from Trk-receptor expression and neurotrophin requirement.

The proapoptotic gene *Bax* is required to mediate apoptosis in sensory neurons (Deckwerth et al., 1996). Although the

Table 1. Specific downregulation of TrkA transcripts in *Brn3a*-null trigeminal ganglia at E13.5

Description	GenBank Accession Number	Experiment 1 Wild type	Heterozygote	Knockout	KO×HT change *P*	Experiment 2 Wild type	heterozygote	Knockout	KO×HT change *P*	Expn
Neurotrophin receptor TrkA	AW124632	2335	2667	1417	0.9999+	n.d.	3252	1528	0.999+	0.54
Housekeeping genes										
Creatine kinase, brain	X04591	8716	10366	9254	0.5	5613	6415	8772	0.031	1.16
Cytochrome c oxidase, subunit VIIa3	AF037371	5021	4704	4102	0.5	5228	5303	5409	0.5	0.94
Heat shock protein, 60 kDa, HSP60	X53584	6906	9422	6643	0.5	8075	7540	8458	0.5	0.95
Mitochondrial H+-ATP synthase, subunit g	Y17223	6795	6541	6609	0.5	5524	6680	6601	0.5	1.03
Phosphoglycerate kinase 1	M15668	1864	2147	2155	0.773	2659	2986	2641	0.5	0.99
Ribosomal protein L3	Y00225	6702	8446	7500	0.5	8540	8874	9275	0.294	1.03
Ribosomal protein S3	X76772	9473	11577	9801	0.5	8908	9193	10301	0.5	1.03
Neural genes										
BDNF	X55573	240	183	255	0.5	408	327	433	0.095	1.19
Doublecortin	AB011678	4753	5892	6581	0.5	5428	5796	6696	0.5	1.21
Ephreceptor B4	U06834	2255	2283	2218	0.5	2566	2604	2532	0.5	0.98
Ephrin A2	U14941	417	542	532	0.933	466	431	474	0.5	1.08
Microtubule-binding protein tau, probe set 1	M18775	7669	9582	9007	0.5	7994	8485	9590	0.148	1.1
Microtubule-binding protein tau, probe set 2	M18776	5063	5771	4866	0.666	4279	4606	4741	0.5	0.97
Neural cell-adhesion molecule 1 (Ncam1)	X15052	9356	9637	10969	0.5	7575	8431	9197	0.5	1.15
Opioidreceptor, sigma 1	AF004927	1231	1164	1095	0.5	868	994	925	0.5	0.95
Presenilin I	L42177	1551	1467	1435	0.5	1462	1298	1618	0.5	1.06
Synapsin II	AF096867	764	916	823	0.589	717	738	804	0.5	1.04

The values indicate the expression ratio (Expn), which is the average of the knockout values for the two experiments, divided by the average of the wild-type and heterozygote values. The KO × HT change *P* is the statistical *P* value of the difference of the target mRNA level between Brn3a knockout and heterozygous animals. Change $P \leq 0.003$ (incresed in knockout) or ≥ 0.997 (decreased in knockout) is considered evidence for significant change. 12,422 transcripts were assayed on the u74A chip. Of these, 4125 were positive in all three genotypes in Experiment 1, 321 were changed by these criteria. Of these, only 55 exhibited a greater than twofold change, i.e. a greater change than TrkA. Results were similar for Experiment 2.

nociceptive sensory neurons are normally absent in the *TrkA*$^{-/-}$ mice at birth, these neurons survive in TrkA$^{-/-}$/Bax$^{-/-}$ mice (Patel et al., 2000). We reasoned that if we placed the *Brn3a* mutation (Xiang et al., 1996) in the *Bax*-null background, sensory neurons would be rescued, thus permitting analysis of *TrkA* expression in the absence of cell death. The genetic cross between *Brn3a*-null and *Bax*-null mice yields nine different genetic combinations (see Materials and Methods). To simplify our studies, we used the following four genotypes in our experiments: *Brn3a*$^{+/-}$/*Bax*$^{+/-}$ (control); *Brn3a*$^{-/-}$/*Bax*$^{+/-}$ (Brn3a null); *Brn3a*$^{+/-}$/*Bax*$^{-/-}$(Bax null); and *Brn3a*$^{-/-}$/*Bax*$^{-/-}$ (double null). The *Brn3a*$^{+/-}$/*Bax*$^{+/-}$ mice used as controls appear to be phenotypically normal with respect to sensory function, fertility and longevity.

To examine *TrkA* expression in trigeminal ganglia, we first performed in situ hybridization using a *TrkA*-specific probe with each of the four analyzed genotypes at postnatal day 0 (P0) (Lei et al., 2001; Martin-Zanca et al., 1990). As indicated in Fig. 1A1-B4, *TrkA* transcripts were reduced in the two genotypes that lack *Brn3a* (*Brn3a*-null and double-null pups). Thus, loss of *Brn3a* results in the downregulation of *TrkA* expression, which is unrelated to TrkA functional (trophic) dependence (n=3 for all genotypes). As shown in Fig. 1C1-C4, *NCAM* mRNA is present at similar level in all four genotypes.

We next examined trigeminal ganglia from P0 pups of the four genotypes using Nissl staining and neuron-specific-antibody immunostaining (peripherin, NeuN and NF-200; Fig. 2A1-B4 and data not shown). No apparent difference in staining intensity was observed between the four genotypes, indicating the presence of apparently differentiated sensory neurons (n=3 for all genotypes). As anticipated, *Bax*-null mice have increased numbers of sensory neurons (Lonze et al., 2002; Patel et al., 2000) and *Brn3a*-null mice have decreased numbers of sensory neurons (Huang et al., 1999). Assessment of TrkA protein was consistent with the data obtained for mRNA expression. Control and *Bax*-null trigeminal ganglia have similar TrkA staining intensity (Fig. 2C1,C2,D1,D2) whereas both *Brn3a*-null and double-null ganglia exhibit reduced TrkA-specific staining (Fig. 2C3,C4,D3,D4). We confirmed the specificity of the TrkA antibody by performing immunostaining using sections of dorsal root ganglion from control and TrkA-null animals. Only weak background signals were detected in *TrkA*-null sections whereas strong, specific TrkA signals were observed in control sections (data not shown). We also stained trigeminal neurons from these mice with a Brn3a polyclonal antibody (Fedtsova and Turner, 1995). Both *Brn3a*-null and double-null neurons lost the Brn3a signal in the nucleus whereas control and *Bax*-null neurons maintained specific, nuclear staining (Fig. 2E1-E4). This confirms the results of in situ hybridization studies (Fig. 1): that loss of *Brn3a* contributes to the loss of *TrkA* expression in the absence of cell death.

Another independent genetic method to assess whether *TrkA* expression was dependent on Brn3a function was employed by crossing transgenic mice containing a β-galactosidase gene driven specifically by the *TrkA* minimal enhancer in nociceptive neurons (Ma et al., 2000) with control, *Bax*-null, *Brn3a*-null, and double-null mice. In the absence of Brn3a, sensory neurons in trigeminal ganglia have apparently reduced

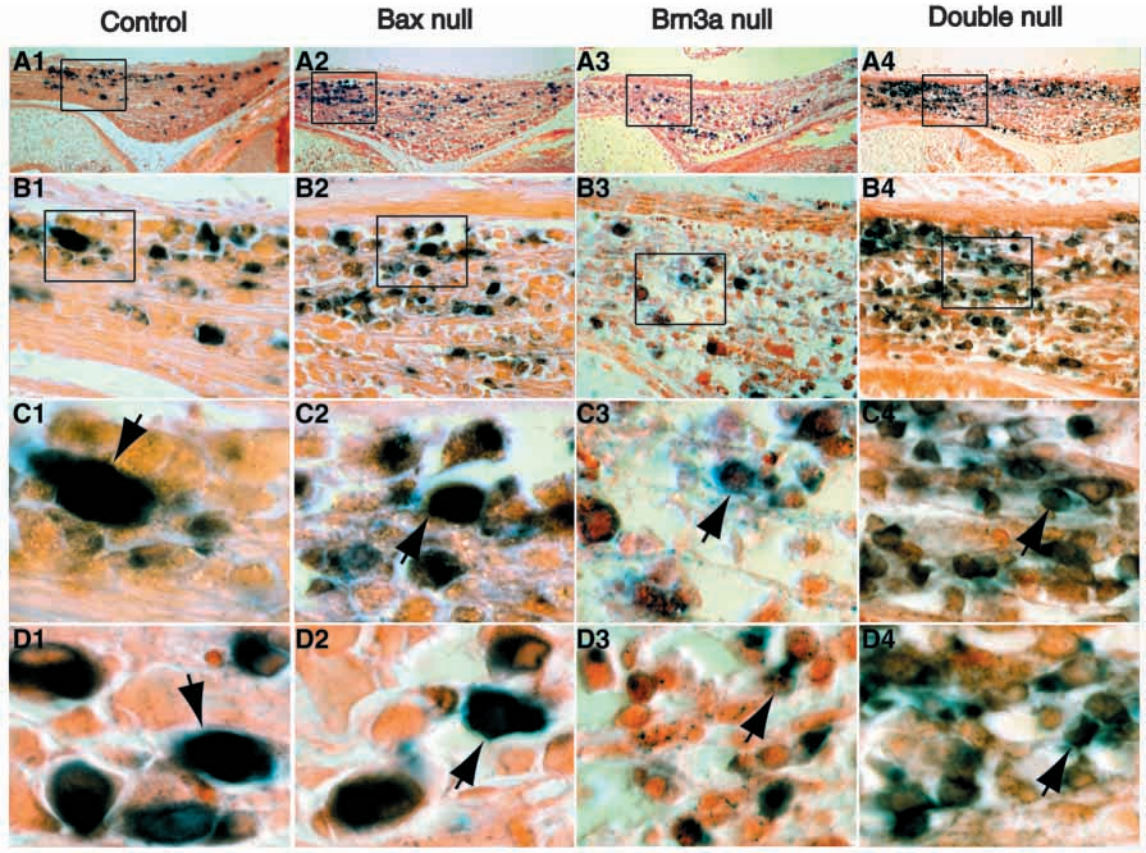

Fig. 3. Reduced *TrkA* enhancer activity in P0 *Brn3a*-null or double-null trigeminal ganglia. Low (A1-A4) and high (B1-B4) magnification pictures of β-galactosidase activity driven by the *TrkA* minimal enhancer, showing that *Brn3*-null (A3,B3) and double-null (A4,B4) ganglia have reduced β-galactosidase activity compared with control (A1,B1) and *Bax*-null (A2,B2) ganglia, respectively. (C1-C4, arrows) Individual β-galactosidase positive cells from each genotype show apparently reduced β-galactosidase activity in cell bodies of *Brn3a*-null (C3) and double-null (C4) ganglia, but normal activity in control (C1) and *Bax*-null (C2) ganglia. (D1-D4, arrows) A second set of β-galactosidase-positive trigeminal ganglia neurons from an independent group of mice, showing that reduced *TrkA* enhancer activity in *Brn3a*-null (D3) and double-null (D4) ganglia is consistent, compared with control (D1) and *Bax*-null (D2) ganglia.

β-galactosidase signal compared to either control or *Bax*-null ganglia. These results are consistent with the immunohistochemistry and in situ hybridization experiments (Fig. 3). Thus, in vivo, the *TrkA* minimal enhancer is significantly attenuated in the absence of functional Brn3a, as assayed by β-galactosidase staining.

In situ hybridization and immunohistochemical approaches discern the qualitative loss of *TrkA* expression in *Brn3a*-null sensory neurons, which is most apparent around P0 (Figs 1, 2) (Huang et al., 1999). To obtain more quantitative information and gain better insight into the timing of *Brn3a* requirement for *TrkA* expression, we analyzed the gene-expression profile of E13.5 trigeminal ganglia from wild-type, heterozygous and *Brn3a*-null mice using Affymetrix gene-expression assays (Table 1). Consistent with preceding data at P0 and the following transgenic analysis (see below; Figs 5, 6), *TrkA* mRNA was already downregulated two-fold in E13.5 *Brn3a*-null trigeminal ganglia compared to controls (Table 1). By contrast, the expression of multiple housekeeping and nervous-system-specific genes was unchanged in *Brn3a*-null embryos at E13.5 (Table 1). Therefore, as early as E13.5, before the onset of sensory apoptosis in *Brn3a*-null mice, loss of *Brn3a* attenuates *TrkA* expression.

Collectively, these results demonstrate that *TrkA* expression is reduced but not absent in the context of *Brn3a* mutation, regardless of whether the neurons are dying (*Brn3a*-null) or alive (double-null). In addition, the genetic data indicate that *Brn3a* acts upstream of *TrkA* and lead to the hypothesis of a direct transcriptional control of the *TrkA* minimal enhancer.

Brn3a binds the *TrkA* minimal enhancer

To address whether the genetic interaction between *Brn3a* and *TrkA* is direct, we searched the minimal enhancer sequence of *TrkA* for consensus Brn3a-binding sites, either GCAT(A/T)A(T/A)T(A/T)AT (Gruber et al., 1997) or (A/G)CTCATTAA(T/C) (Xiang et al., 1995). No canonical Brn3a-binding sites could be identified. We next scrutinized the full, 2 kb promoter/enhancer region upstream of the *TrkA* gene and found no consensus Brn3a-binding sequences (data not shown).

We then turned to an empirical approach using purified GST-Brn3a DNA-binding-domain-fusion proteins (Xiang et al., 1995) to perform gel-shift assays with six, overlapping, 100 bp DNA fragments that cover the entire 457 bp minimal enhancer (Fig. 4A). Only the two, outlying, DNA fragments at the 5′ and 3′ ends of the minimal enhancer were shifted by the GST-Brn3a

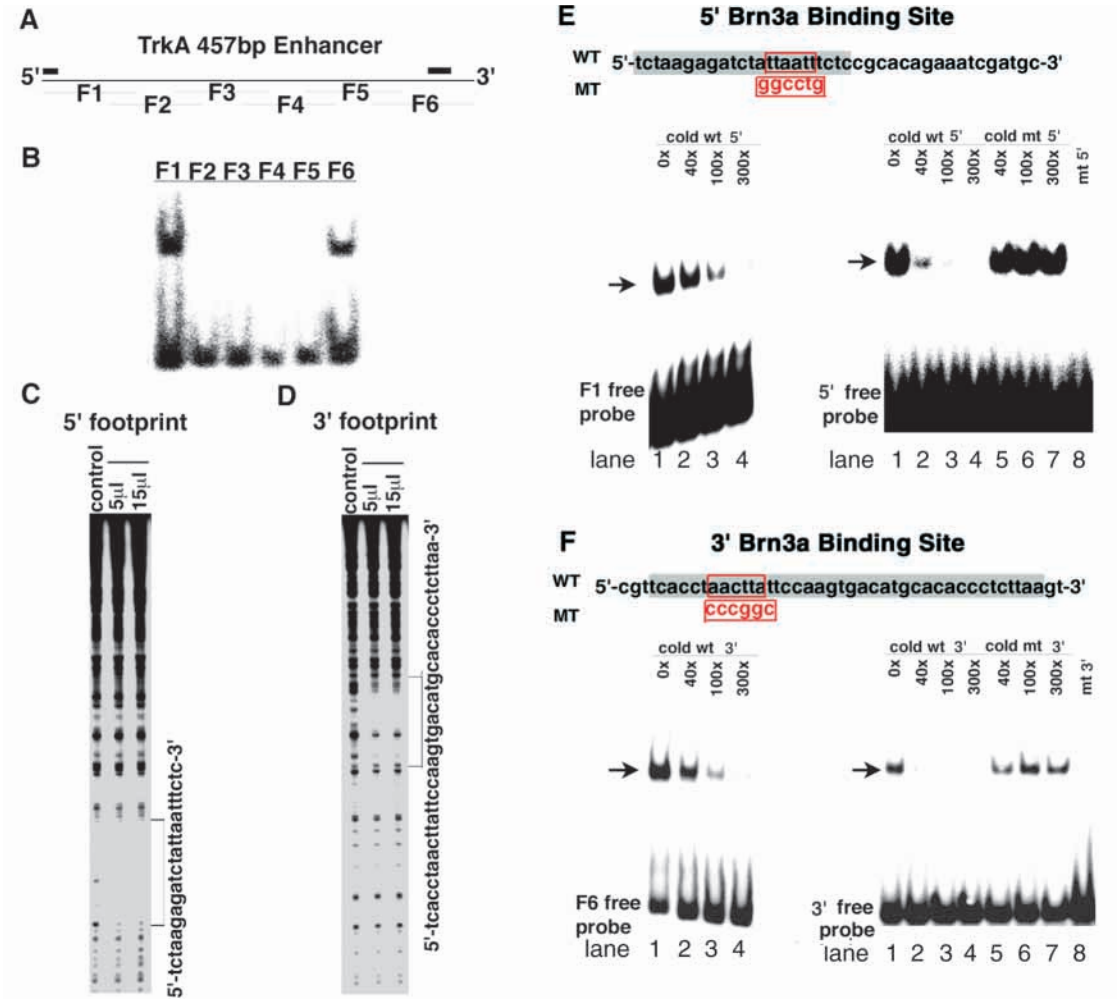

Fig. 4. Gel-shift and footprint assays define two Brn3a-binding sites in the *TrkA* minimal enhancer. (A) Illustration of six, overlapping, 100 bp fragments covering *TrkA* minimal enhancer and (B) gel-shift using a GST-Brn3a POU-domain fusion protein indicating that only the first and sixth fragments can bind Brn3a. (C,D) DNAse I footprint assays to define the Brn3a-binding sequences in the *TrkA* minimal enhancer. The 5′ protected sequence is 5′-TCTAAGAGATCTATTAATTTCTC-3′ (C) and the 3′ protected sequence is 5′-TCACCTAACTTATTCCAAGTGACATGCACACCCTCTTAA-3′ (D). (E,F, left panels) Gel-shift assays showing that the protected sequences in (C,D) compete with the first and sixth 100 bp fragments in (A,B) for GST-Brn3a POU-domain fusion-protein binding. (E,F, top panels) Protected sequences from (C,D) share (red boxes) an A/T-rich core sequence. The mutant sequences used in the competitive gel-shift assays (left panels) and the following transgenic analysis (Figs 5, 6) are shown in red. (E,F, right panels) Competitive gel-shift assays using either the wild-type or mutated Brn3a-binding sites, showing that mutated Brn3a-binding sites do not bind the GST-Brn3a POU-domain fusion protein (lane 8) and, unlike wild-type sites (lanes 1-4), do not compete with wild-type sites for binding (lanes 5-7). Arrows in E,F indicate the DNA-protein complexes.

fusion protein (Fig. 4B), indicating the presence of novel Brn3a-binding sites in these two regions. As controls, GST protein alone did not cause any shift whereas an anti-GST antibody disrupted the interaction of F1 and F6 with the GST-Brn3a protein (data not shown). To identify the core Brn3a-binding sequences in the two 100 bp fragments, we performed DNase I footprint analyses (Ausubel et al., 1995) with the entire 457 bp minimal enhancer using the GST-Brn3a fusion protein. As shown in Fig. 4C and D, sequences protected by the GST-Brn3a fusion protein were present in the 5′ and 3′ sequences that were included in the gel-shifted fragments (Fig. 4A,B). The length of both protected DNA sequences is <40 bp, providing more accurate information about the core binding sites of Brn3a. These two protected sequences compete successfully with the corresponding 100 bp sequences for GST-Brn3a binding in a gel shift assay (Fig. 4E,F, left panels), providing further evidence of Brn3a-specific DNA sequences in the *TrkA* minimal enhancer.

To pinpoint the Brn3a core-binding sequences in the minimal enhancer, we compared the two protected sequences. A similar A/T-rich sequence, T(A/T)ATT, that resembles the consensus Brn3a core binding site was found in both fragments (Fig. 4E,F, red shadows, top panels). To determine the importance of the core T(A/T)ATT sequence for Brn3a binding, we mutated these sequences in both protected oligonucleotides (Fig. 4E,F, red, top panels) and tested them in gel-shift assays (Fig. 4E,F, lower panels). Although wild type oligonucleotides successfully competed for the binding of

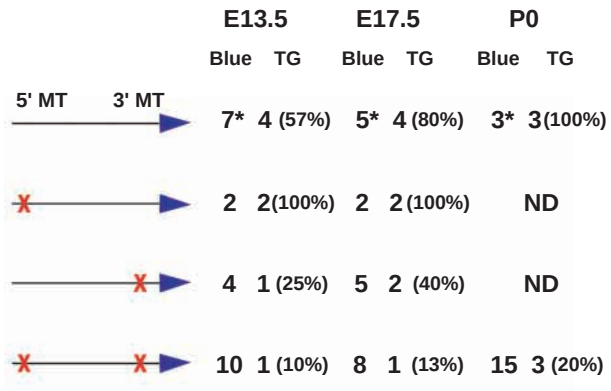

Fig. 5. Summary of transgenic analysis of the wild-type *TrkA* minimal enhancer and *TrkA* minimal enhancers with one or both Brn3a-binding-site mutations. Note that the minimal enhancer that contains both Brn3a-binding-site mutations has apparently reduced activity in trigeminal ganglia. *The reported numbers include results from three stable *TrkA*-minimal-enhancer transgenic lines.

GST-Brn3a fusion proteins (Fig. 4E,F, lanes 1-4), the mutant oligonucleotides lost the ability to compete with wild-type sequences or to bind the GST-Brn3a fusion protein (Fig. 4E,F, lanes 5-8). These data indicate the presence of two novel, specific Brn3a-binding sites in the *TrkA* minimal enhancer that could mediate the effect of *Brn3a* on *TrkA* transcription.

Functional analysis of Brn3a-binding sites

We have previously defined several cis elements in the *TrkA* minimal enhancer that are required for transcription through site-directed mutagenesis and transgenic assays (Ma et al., 2000). We therefore used similar methods to determine the importance of the newly defined Brn3-binding sites for in vivo enhancer function. The 5′ and 3′ core sequences defined in Fig, 4E,F (boxed sequences) were subjected to mutagenesis and then to transient transgenic analysis (Ma et al., 2000).

As previously described, the wild type *TrkA* minimal enhancer drives strong and specific lacZ expression in trigeminal ganglia in >50% of lacZ positive embryos at E13.5 (Fig. 5, E13.5 and Fig. 6A). The variability is related to transgene positional effects (Ma et al., 2000). When the minimal enhancer harbors the 5′ Brn3a-site mutation, two out of two (100%) blue E13.5 embryos exhibited normal expression in trigeninal ganglia (Fig. 5 and Fig. 6B). Mutation of the 3′ Brn3a site resulted in only one out of four blue embryos maintaining normal trigeminal expression (Fig. 5, Fig. 6C for normal expression, and data not shown).

We next tested the functional consequences of mutating both the 3′ and 5′ Brn3a-binding sites in the enhancer. Of ten blue E13.5 embryos, only one exhibited appreciable expression in the trigeminal ganglion (data not shown), the remaining nine embryos either had extremely weak, or undetectable trigeminal-ganglion expression (Fig. 5, Fig. 6D, and data not shown). These results are consistent with the microarray experiments indicating that by E13.5, mutation of both Brn3a sites significantly compromised the function of the *TrkA* minimal enhancer in trigeminal ganglia. Mutation of each site alone has either no detectable (5′ site) or weak (3′ site) effect on *TrkA*-enhancer function.

We extended our transient transgenic analysis to E17.5 with the minimal enhancer bearing mutations in either one or both Brn3a-binding sites. As predicted, the wild-type enhancer drives β-galactosidase expression strongly and specifically in nociceptive neurons in trigeminal ganglia at E17.5 (Fig. 5 and Fig. 6E) (Ma et al., 2000). Neither mutation alone (either 5′ or 3′) appreciably affected the activity of the *TrkA* minimal enhancer at E17.5 (Fig. 5; Fig. 6F,G). Finally, to test if these two Brn3a sites in the minimal enhancer are redundant for expression in the trigeminal ganglion at E17.5, transient transgenic embryos were generated with the minimal enhancer bearing both the 5′ and 3′ sites mutations. Of eight blue embryos generated, seven showed either greatly reduced or no detectable expression in trigeminal ganglia (Fig. 5; Fig. 6H,I, and data not shown). The remaining blue embryo expressed β-galactosidase comparably to wild type and single-mutant embryos (data not shown). We also generated *TrkA* minimal

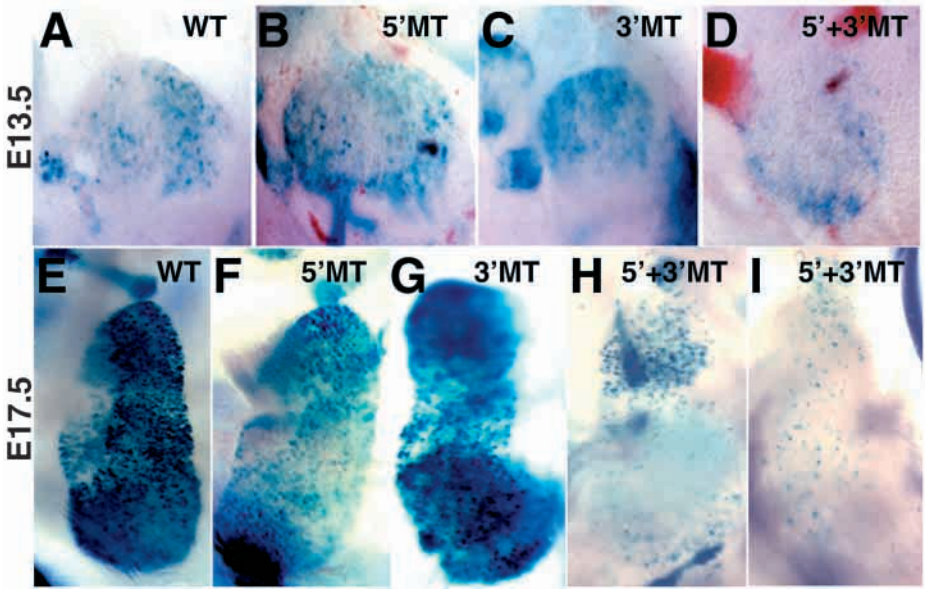

Fig. 6. The *TrkA* minimal enhancer containing both Brn3a-binding-site mutations has reduced activity in trigeminal ganglia. Representative trigeminal ganglia β-galactosidase activity driven by the wild-type *TrkA* minimal enhancer (A,E), by a *TrkA* minimal enhancer bearing a 5′ Brn3a-binding-site mutation (B,F), by a *TrkA* minimal enhancer bearing a 3′ Brn3a-binding-site mutation (C,G), and by a *TrkA* minimal enhancer bearing both Brn3a-site mutations (D,H,I) at E13.5 and E17.5. Only the *TrkA* minimal enhancer with both mutations caused severe reduction in enhancer activity at E13.5 and E17.5.

enhancer transgenic P0 pups bearing both Brn3a-binding-site mutations, with similar results. Double mutations clearly affected enhancer function in trigeminal ganglia (Fig. 5 and data not shown), with only three out of fifteen blue pups maintaining normal β-galactosidase expression in trigeminal ganglia. Taken together, our transgenic results indicate that these two Brn3a sites are important for maintaining *TrkA* enhancer function after E13.5, although they are functionally redundant.

DISCUSSION

The differentiation of sensory neurons has been closely linked to the specific expression of Trk family receptors in subtypes of sensory neurons (Liebl et al., 1997; Ma et al., 2000; Snider, 1994). The molecular programs that specify sensory neurons are largely unknown. Studies in other areas of the nervous system, such as motor neurons in spinal cord (Tanabe and Jessell, 1996), telencephalic neurons (Nieto et al., 2001; Schuurmans and Guillemot, 2002) and sympathetic neurons (Pattyn et al., 1997; Pattyn et al., 1999), show that spatial and stage-dependent interaction of multiple transcription factors is the driving force for the specification of neuronal subtypes. We undertook to understand the transcriptional regulation of Trk genes to derive clues about the molecular mechanisms of sensory-neuron specification.

Several transcription factors have been shown to function in sensory and sympathetic gangliogenesis. For example, the bHLH transcription factors, neurogenin1, neurogenin2 and mash1, are required for normal development of sensory neurons (Anderson, 1999; Anderson et al., 1997). Because of their early expression, these factors are likely to affect neural-precursor proliferation rather than the differentiation and survival of postmitotic neurons (Ma et al., 1999). It is unclear whether these transcription factors participate in regulating expression of *TrkA*.

Pou-domain proteins exert crucial effects on neural development. In the PNS, Brn3a has received considerable attention because of its striking pattern of expression and the dramatic phenotypic consequences of *Brn3a* ablation in mice, which includes loss of sensory neurons (He et al., 1989; Huang et al., 1999; McEvilly et al., 1996; Xiang et al., 1996). A key, unresolved question has been the underlying mechanistic requirements for Brn3a by sensory neurons. Although putative Brn3a-target genes have been proposed (Smith et al., 1997a; Smith et al., 1998; Smith et al., 1999; Smith et al., 1997b), the relevance of these genes in vivo remains to be tested.

Brn3a-mutant sensory neurons from trigeminal ganglia have decreased expression of *TrkA*, beginning at E13.5. Because *Brn3a* expression appears at E9, the observed downregulation of *TrkA* at E13.5 and subsequent neuronal death in *Brn3a*-null mice could be caused by neuronal defects other than direct regulation of *TrkA* by Brn3a (Eng et al., 2001; Huang et al., 1999). Even if *TrkA* is regulated by Brn3a, it is unclear whether this is direct regulation, mediated by Brn3a binding to TrkA cis-regulatory sequences, or indirect regulation, mediated by other factors. To investigate these possibilities, we sought to dissociate neuronal survival from Trk-receptor expression and function.

The use of *Bax*-null mice permitted sensory neurons to survive in the absence of TrkA function because Bax is required for neurotrophin-deprived neuronal apoptosis (Deckwerth et al., 1996). Furthermore, the sensory neurons rescued from apoptosis by crossing into a *Bax*-null background are known to express normal sensory neuron markers and have been utilized to uncouple apoptosis from other biological events, such as peripheral axonal outgrowth and elaboration (Deckwerth et al., 1996; Lonze et al., 2002; Patel et al., 2000). Using a similar approach, we were able to discern the appearance of sensory neurons in the absence of functional Brn3a, which supports the model that *Brn3a* is not required for early specification and differentiation of sensory neurons. Instead, these neurons were present but had reduced expression of *TrkA* transcripts and proteins. These results are consistent with transcriptional regulation of *TrkA* expression by Brn3a.

The two Brn3a sites in the *TrkA* enhancer are not identical to binding sequences described previously (Gruber et al., 1997; Xiang et al., 1995). Analysis in vitro indicates that they have lower affinity than consensus Brn3a sites, although they are well conserved between mice and humans (data not shown) (Gruber et al., 1997). As expected, mutation of both sites did not completely ablate reporter-gene expression in sensory neurons. A reduced, but apparent, level of enhancer activity still exists. Considering possible enhancing and suppressing effects of genomic sequences surrounding the transgene-insertion sites, the mutagenized enhancers might exhibit either near normal or much weaker activity. However, the trend of reduced activity should be maintained if a significantly large number of transgenic embryos are analyzed. Our transient transgenic analysis fits this prediction nicely.

How does binding affinity in vitro relate to function in vivo? The physiological environment provided by chromatin and available cofactors in a given cell nucleus will impact on the accessibility of a transcription factor to its DNA-binding site and, thus, the physiological affinity. In *Caenorhabditis elegans*, *Drosophila* and mammals, there are many examples that low affinity sites in vitro are important for transcription in vivo. Among these are the FoxA transcription factor PHA-4 in *C. elegans* (Gaudet and Mango, 2002), bicoid and dorsal morphogen in *Drosophila* (Driever et al., 1989; Jiang and Levine, 1993), and HNF4 in the regulation of erythropoietin expression and Elf-1 in lymphoid-specific-gene expression in mammals (John et al., 1996; Makita et al., 2001). In the case of HNF4-mediated regulation of erythropoietin, transcriptional initiation is mediated by a high affinity interaction with the retinoid acid receptors, which is then supplanted by low affinity binding of HNF4 (Makita et al., 2001). Similarly, Brn3a is not required for initiation of *TrkA* transcription but is required for its maintenance, beginning at E13.5. Because TrkA is still expressed in *Brn3a*-null sensory neurons, although at a lower level, other transcription factors (Lei et al., 2001) may also function to regulate TrkA expression. These factors may interact with Brn3a to regulate TrkA expression in wild-type neurons.

Transcriptional cofactors also affect the activity of a transcription factor. UNC86, a Brn3a ortholog in *C. elegans*, interacts with the LIM-type homeodomain protein MEC-3 and the two proteins function synergistically to activate the *mec-3* promoter in vitro (Xue, 1992; Xue, 1993; Lichtsteiner, 1995). In addition, both proteins are required for the fate specification of touch cells in *C. elegans* through heterodimeric binding to

consensus cis-elements (Duggan et al., 1998). Recent experiments demonstrate further examples of how interactions between the Oct-1 POU-domain protein and SNAP190 cofactor affect DNA binding (Hovde et al., 2002). It remains interesting to identify Brn3a cofactors that are important for TrkA expression and the development of mammalian sensory neurons.

It is interesting to note that no significant differences in *TrkA* mRNA levels were detected in *Brn3a* wild-type and heterozygous embryos (Table 1). This finding is consistent with our recent observation that autoregulation of the *Brn3a* locus leads to suppression of haplotype insufficiency, and results in similar levels of *Brn3a* mRNA in trigeminal neurons that contain either one or two copies of the *Brn3a* gene (Trieu et al., 2003). It is also consistent with studies that show no detectable phenotype in *Brn3a* heterozygotes (Eng et al., 2001; Huang et al., 1999) and validates the use of heterozygotic controls in the present study.

Brn3a-null sensory neurons have aberrant axonal arborization at E13.5 (Eng et al., 2001). At this time TrkA protein levels are minimally reduced (Huang et al., 1999). Lack of TrkA/NGF signaling leads to defective innervation of peripheral targets by nociceptive neurons (Patel et al., 2000). Considering that Brn3a might regulate multiple target genes in sensory neurons, it is likely that the sensory axonal defects observed in *Brn3a*-null mice are the consequences of altered gene expression at many loci, including *TrkA*. Identification of additional *Brn3a* target genes should shed light on these complex neuronal phenotypes.

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