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Regulation of Target Gene Expression in Sensory Neurons by Brn3a

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Jason James Lanier

Committee in charge:

Professor Eric Turner, Chair
Professor Jerold Chun
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2007

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ACKNOWLEDGEMENT

First of all, I would like to thank my advisor, Dr. Eric Turner for being an exceptional mentor and devoting enormous amounts of time and effort to my training. Thanks for all of your guidance and patience and for always having my best interest at heart. I would also like to thank the members of the Turner lab that I have had the privilege to work with: Natasha, Raisa, Lely, Eric Cox, Iain, and Amanda. You have all helped to create a great working environment in the lab. I have enjoyed getting to know all of you and appreciate your enormous contributions to my thesis project. Finally, I would like to thank the members of my thesis committee for helpful comments and advice along the way.

The text in Chapter Two is a reprint of the material as it appears in Development, 2003, Eng SR, Lanier J, Fedtsova N, Turner EE. I was a secondary author and participated in the research that forms the basis of this chapter. The text of Chapter Three is a reprint of the material as it appears in Developmental Biology, 2007, Lanier J, Quina LA, Eng SR, Cox E, Turner EE. I was the primary researcher, and the co-authors contributed to the research that forms the basis of this chapter. The text of Chapter Four is a reprint of the material as it appears in Neural Development, 2007, Eng SR, Dykes I, Lanier J, Fedtsova N, and Turner EE. I was a secondary author and participated in the research that forms the basis of this chapter.

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ABSTRACT OF THE DISSERTATION

Regulation of Target Gene Expression in Sensory Neurons by Brn3a

by

Jason James Lanier

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2007

Professor Eric Turner, Chair

During the development of the vertebrate nervous system, cellular proliferation and differentiation result in the formation of a large number of specialized physical structures composed of many different types of cells. The phenotypic properties of a cell are largely controlled by the complement of proteins that the cell expresses. Thus, the formation of properly functioning neuronal circuitry requires precisely coordinated regulation of gene expression. Tissue-specific transcription factors play a critical role in the process of development by exerting spatiotemporal control over the expression of their specific target genes. Hundreds of transcription factors have been identified

which exhibit tissue-specific expression in the nervous system. Many of these factors are critical for proper neural development, with their disruption leading to severe phenotypic abnormalities.

Brn3a (Pou4f1) is a POU domain transcription factor with a highly specific and complex pattern of expression in the developing nervous system. Disruption of Brn3a function results in severe developmental abnormalities and neonatal lethality. We have identified programs of gene expression controlled by Brn3a in both the trigeminal ganglia (TG) and dorsal root ganglia (DRG) using microarray analysis of dissected tissue from wild-type and Brn3a null embryos in midgestation. These experiments indicate that Brn3a regulates similar, but distinct complements of transcripts in sensory ganglia at different axial levels. We then show, using in vivo chromatin immunoprecipitation (ChIP) assays, that Brn3a directly represses the expression of the neurogenic transcription factors NeuroD1 and NeuroD4 in the developing TG. Finally, we provide evidence that epigenetic modifications of chromatin play an integral role in determining the regulatory targets of Brn3a and contribute to target gene differences between the TG and DRG. The work presented in this dissertation has provided insight into the role of Brn3a in the developing sensory nervous system. It also demonstrates a clear role for chromatin modification in transcription factor target gene selection which may be relevant for many tissue-specific transcription factors.

I

Introduction

Induction and development of the nervous system

The induction and development of the vertebrate nervous system comprise an extraordinarily complex series of events resulting in the generation of a multitude of distinct cell types and structures (Kandel, Schwartz et al. 2000). During early mammalian embryonic development, prior to the induction of the nervous system, the embryo consists of three main cell layers. The innermost cell layer, the endoderm, consists of cells that eventually give rise to the gut and internal organs. The vascular system, musculature, and connective tissues are derived from the middle cell layer, the mesoderm. The major tissues of the central and peripheral nervous systems are generated from the ectoderm, located on the surface of the early embryo.

At the gastrula stage of development, a sheet of cells located at the dorsal midline of the embryo, begins to acquire neural properties and forms a structure called the neural plate, which is the source of both neural and glial cells. In a process called neurulation, the neural plate folds into a structure called the neural tube, which eventually gives rise to the central nervous system. The adult spinal cord and brain are derived from the posterior and anterior regions of the neural tube, respectively (Schoenwolf, Bortier et al. 1989; Eagleson and Harris 1990).

Many of the cells comprising the peripheral sensory nervous system are derived from a group of migratory cells called the neural crest (Selleck and Bronner-Fraser 1996). Prior to neurulation, precursors of this specialized group of cells can be identified at the border of the neural plate and the non-neural ectoderm (Huang and Saint-Jeannet 2004). Around the time of neural tube closure, neural crest cells migrate ventrally throughout the embryo, differentiating into a wide range of both neuronal and nonneuronal cells, including a majority of sensory neural progenitors. The remainder of the cells in the peripheral sensory nervous system originate in ectodermal cellular structures called neurogenic placodes (Barlow 2002).

The patterning and differentiation of cells in the nervous system is ultimately controlled by signaling molecules called inducing factors which are secreted from cells in a particular location within an embryo and influence the physiology of surrounding cells. Secretion of an inducing factor from a localized region within an embryo creates a signaling gradient which can determine the arrangement and fate of responding cells according to the concentration of the factor perceived by each cell (Gurdon and Bourillot 2001). Cells occupying different positions within a developing embryo are exposed to different inducing factors. Thus the position that a cell occupies early in development has a direct influence over its ultimate fate. Examples of inducing factors involved in neurogenesis include the Bone Morphogenetic Proteins (BMPs), members of the transforming growth factor β (TGFβ) superfamily of signaling molecules, and the glycoprotein Sonic Hedgehog (Shh).

Tissue-specific transcription factors in neural development

Activation of extracellular receptors by secreted inducing factors results in the subsequent activation or repression of transcription factors within the cell which, in turn, control expression of the genes that mediate specific functions of the cell. At the most basic level, the identity and functionality of a cell is determined by the complement of proteins it expresses. Because transcription factors regulate the expression of specific genes, the complement of transcription factors activated by a cell plays a major role in determining the cell's identity and functionality. For example, TGFβ superfamily signaling is mediated by a family of serine/threonine receptor kinases. Activation of these receptors by BMPs results in phosphorylation of intracellular molecules known as Smads. Once phosphorylated, Smads translocate to the nucleus where they associate with DNA-binding transcription factors in order to activate transcription of specific target genes (Baker and Harland 1997). In the dorsal neural tube graded BMP signaling is required for the formation distinct subtypes of dorsal interneurons which are defined by differential expression of specific basic helix-loop-helix (bHLH) and homeodomain HD transcription factors (Helms and Johnson 2003).

In the ventral neural tube, graded Shh signaling establishes progenitor cell identities by controlling the expression of specific transcription factors. A series of homeodomain (HD) transcription factors are involved in mediating the inductive effects of Shh in the ventral spinal cord. These factors can be divided into two classes based on their response to Shh signaling. Class I factors are repressed by Shh, whereas

Class II factors are activated by Shh signaling. Each of these homeodomain transcription factors responds to a different threshold concentration of Shh. The result is that graded Shh signaling creates distinct progenitor cell populations by inducing distinct homeodomain transcription factor profiles (Shirasaki and Pfaff 2002).

In each case, depending upon a cell's position within the morphogen gradient, the cell expresses a specific combination of transcription factors. Differences in neuronal phenotypes occur, in large part, due to differential gene expression patterns between individual neurons. Several classes of tissue-specific transcription factors participate in this process by regulating the expression of specific target genes (Pfeffer, Bouchard et al. 2000; Bermingham, Shumas et al. 2002; Ebert, Timmer et al. 2003; Lee and Pfaff 2003; Scardigli, Baumer et al. 2003; Saba, Johnson et al. 2005). These transcription factors exhibit highly specific patterns of expression and control cellular morphology and functionality by mediating activation or repression of their target genes. A recent large-scale analysis identified over 300 transcription factors expressed in a tissue-specific manner in the mouse nervous system (Gray, Fu et al. 2004).

Regional and temporal control over the expression of many neural transcription factors is critical for orchestrating the morphogenesis and subsequent topographic mapping of the nervous system. Ablation or misexpression of developmentally critical transcription factors has been shown to result in abnormal specification of neuronal subtypes, failure of particular classes of neurons to develop, disruptions in topographic mapping and synapse formation, gross developmental

abnormalities, and embryonic lethality (Cai, Morrow et al. 2000; Shirasaki and Pfaff 2002; Ferland, Cherry et al. 2003; Helms and Johnson 2003; Marquardt 2003; Zaki, Quinn et al. 2003) Because the primary known role of transcription factors is to regulate the expression of target genes, the phenotypic abnormalities observed in transcription factor knockouts are likely to be mediated, in large part, by misexpression of target genes. In a few cases, the transcripts that become misexpressed in response to ablation of tissue-specific transcription factors have been identified through microarray analysis of mRNA from neural tissue of wild-type and knockout embryos (Livesey, Furukawa et al. 2000; Gold, Baek et al. 2003; Mu, Beremand et al. 2004). However, for the most part the downstream targets of developmentally critical neural transcription factors remain unknown.

Mechanisms of transcriptional regulation

The primary function of transcription factors is to either activate or repress the expression of target genes by interacting with sequence-specific DNA motifs located within cis-acting regulatory elements. Regulatory elements may be located within the gene promoter, directly adjacent to the promoter region, or at a distance from the transcriptional start site of the gene. Upon DNA binding, tissue-specific transcription factors regulate gene expression by interacting directly with the basal transcriptional machinery to modulate its activity or by recruiting additional regulatory factors which induce modifications of the underlying chromatin structure (Latchman 2004).

In eukaryotic cells, DNA is associated with highly basic histone proteins and packaged into a compact structure known as chromatin. The basic unit of chromatin, called the nucleosome, consists of 147 base pairs of DNA wrapped 1.75 times around a core of eight histone molecules (Margueron, Trojer et al. 2005). Regions of DNA that are never transcribed in a cell are often found to exist in a compacted structure called constitutive heterochromatin which prevents access of the transcriptional machinery (Dillon 2004; Craig 2005). In contrast, actively transcribed regions of DNA have been shown to assume a more loosely-associated, structurally accessible conformation, termed euchromatin. Non-transcribed genomic regions that are capable of undergoing transcription in certain circumstances and may be stably repressed in others are referred to as facultative heterochromatin.

Many transcription factors modulate target gene expression through the recruitment of transcriptional cofactors which, in turn, modify the local chromatin structure (Xu, Glass et al. 1999; Kishimoto, Fujiki et al. 2006). These cofactors can be divided into two broad categories. The first category consists of proteins that catalyze covalent modification of histone tails, including histone deacetylases (HDAC) and histone acetyl transferases (HAT). Histone modifications have profound influence over the conformation and accessibility of chromatin. The second class includes ATP-dependent chromatin remodeling factors which alter the structure of the nucleosome (Rosenfeld, Lunyak et al. 2006). ATP-dependent chromatin remodeling factors actively displace histones from DNA, allowing greater access of the transcriptional machinery (Johnson, Adkins et al. 2005).

Transcription factor target gene specificity

Transcription factors are usually modular in structure and contain conserved DNA binding domains which confer affinity for specific DNA sequences (Nelson 1995). The sequence to which a transcription factor binds with highest affinity is known as its consensus binding site, and typically consists of 5-15 unique base pairs (Remenyi, Scholer et al. 2004). The ability of a transcription factor to interact with a target gene is determined by the presence of its specific binding site within the regulatory regions of the target gene. In addition to their consensus binding sites, transcription factors also bind with moderately reduced affinity to DNA sites containing slight variants of the consensus sequence (Gruber, Rhee et al. 1997; Rhee, Gruber et al. 1998; Rhee, Trieu et al. 2001; Bulyk 2003). The range of preferred binding sites for a given factor is often summarized as a "position weight matrix" consisting of the probability of finding a given base at a specific position of the binding site. However, DNA sequence alone is insufficient to account for transcription factor specificity. The consensus binding sequences of most mammalian transcription factors are likely to occur hundreds of thousands of times in the genome, yet the few transcription factors with known sets of target genes in the developing nervous system regulate on the order of 100 target genes in a given tissue (Livesey, Furukawa et al. 2000; Gold, Baek et al. 2003; Mu, Beremand et al. 2004).

Evidence suggests that in many cases, target gene regulation is dependent upon concurrent binding of multiple transcription factors, providing an additional layer of

target gene specificity (Davidson, McClay et al. 2003; Remenyi, Scholer et al. 2004). In such cases, target gene expression is controlled by cis-regulatory modules that contain multiple transcription factor binding sites. These cis-regulatory modules are postulated to integrate information from multiple transcription factors, performing "logic-functions" in order to control the expression of target genes (Istrail and Davidson 2005). In such a model, transcriptional output can be finely-tuned based on the levels of occupancy of the individual binding sites contained within a cis-regulatory module. Cis-regulatory modules containing multiple transcription factor binding sites have been shown to mediate the spatiotemporal expression patterns of members of the Hox family and other developmentally regulated genes (Kirchhamer, Yuh et al. 1996).

An important question that remains unanswered for most tissue-specific transcription factors is whether they regulate the same or different sets of target genes in the different tissues in which they are expressed. Many transcription factors exhibit spatiotemporally specific patterns of expression and are often expressed in functionally and morphologically diverse tissues. For example, the LIM-HD transcription factor Islet I (Isl1) is expressed in a highly specific, but extremely diverse array of tissues and cell types. Isl1 expression has been reported in the trigeminal and dorsal root ganglia, the sensory and neuronal lineages of the inner ear, motor neurons in the ventral spinal cord, in a cardiac progenitor cell population that gives rise to the majority of myocardial cells in the developing heart, pancreatic islet cells and a variety of other polypeptide producing endocrine cells (Thor, Ericson et al. 1991; Pfaff,

Mendelsohn et al. 1996; Radde-Gallwitz, Pan et al. 2004; Lin, Bu et al. 2006). Expression of Isl-1 is critical for the development of each of these tissues and its genetic ablation has been shown to cause arrest of pancreas development, and preclude the differentiation of motor neurons as well as the migration, proliferation, and survival of cardiac progenitors (Habener, Kemp et al. 2005). Because transcription factors have been shown to play integral roles in the determination of cell identity and functionality by controlling entire programs of gene expression, it is somewhat surprising that certain transcription factors are expressed in such diverse tissues. This suggests the possibility that, although the regulatory DNA elements mediating transcription factor target gene expression are identical in every cell of an organism, tissue-specific transcription factors may regulate distinct sets of target genes in different tissues.

The requirement for concurrent binding of multiple transcription factors within a cis-regulatory module represents a possible mechanism by which a transcription factor may regulate different target genes in different tissues. Due to differential exposure to signaling molecules, cells with different developmental histories are likely to express different complements of proteins. Thus, the presence of distinct combinations (and concentrations) of transcription factors in various tissues may account for differences in transcriptional regulatory targets. An additional mechanism for tissue-specific target gene regulation involves the distinct expression of specific required cofactors in different tissues. If specific components of a cofactor complex

are not be sufficiently expressed in a given cell type, transcription factor binding may not be translated into modulation of target gene expression.

Epigenetic influence on transcription factor binding

It is clear that, in a given cell, many transcription factor binding sites in the genome are not utilized for the regulation of transcription. An important question addressed by this dissertation is whether transcription factors occupy all of their potential binding sites in the genome, yet only regulate transcription from a subset of the sites; or whether only a fraction of the potential binding sites are occupied. We have discussed mechanisms by which a transcription factor may bind to DNA without actively regulating transcription. Recent studies have also shown that tissue-specific transcription factor activity may also be regulated at the level of DNA binding. For example, GATA-1 is a bHLH transcription factor which directly activates expression of β-globin genes in mouse hematopoietic precursor cells. ChIP analysis has demonstrated that GATA-1 occupies a small fraction of the conserved GATA motifs within the β-globin locus (Im, Grass et al. 2005).

Epigenetic factors may influence target gene regulation by modulating the accessibility of specific regions of chromatin. During development cells are exposed to external and internal signals which lead to DNA methylation and covalent modifications of histones. Many of these modifications are maintained after cell divisions such that the developmental history of a cell is epigenetically encoded into its genome. Because progenitors of different tissues are exposed to distinct sets of

signals, chromatin modifications accumulate in a cell type specific manner. The state of chromatin at a particular locus exerts profound influence over the ability of the basal transcriptional machinery to access and transcribe DNA. As a result, in a given cell at a particular stage of development, specific regions of chromatin become inaccessible to general transcription factors, and thus transcriptionally silenced, due to the accumulation of repressive chromatin modifications.

The conformational state of chromatin is influenced by DNA methylation, covalent modification of histones, and remodeling or displacement of nucleosomes. The N-terminal tails of chromatin histone molecules are capable of undergoing several types of covalent modifications including acetylation, methylation, and phosphorylation(Jenuwein and Allis 2001; Turner 2002). Accumulation of specific histone modifications results in the condensation or relaxation of chromatin, depending upon the type of modification and the protein residue that becomes modified. For example, hyperacetylation of histone H3 at lysine residues 9 and 14 (H3K9/K14) increases the equilibrium accessibility of nucleosomal DNA and is a hallmark of structurally open euchromatin (Anderson, Lowary et al. 2001). In contrast, methylation of H3K9 is a modification commonly associated with tightly condensed, transcriptionally repressive heterochromatin (Dillon 2004; Craig 2005).

The conformational state of chromatin plays a well characterized role in the regulation of transcription, with active transcription requiring an accessible DNA template characterized by specific histone modifications. The role of chromatin conformation in regulating tissue-specific transcription factor activity is less

understood. However, recent evidence suggests that the mechanisms regulating accessibility of the basal transcriptional machinery may also regulate the binding of tissue-specific transcription factors. For example, sites of active transcription are often characterized by nucleosome depletion, a modification that increases the accessibility of the DNA template such that the transcriptional machinery is able to associate with and transcribe DNA. This increased accessibility also causes nucleosome depleted regions to be hypersensitive to DNAse I digestion. Analysis of the β-globin locus has shown that, in addition to regions of active transcription, important distal regulatory elements are DNAse I hypersensitive (Bulger, Sawado et al. 2002). Furthermore, GATA-1 preferentially occupies conserved GATA motifs that occur in regions of DNAse I hypersensitivity at the β-globin locus (Im, Grass et al. 2005). Specific histone modifications are also likely to play a role in regulating the accessibility of tissue-specific transcription factor binding sites (Barrera and Ren 2006). Sites of DNAse I hypersensitivity at the β-globin locus colocalize with specific histone modifications such as hyperacetylation of H3. A recent study which mapped specific chromatin modifications over 1% of the human genome showed that distal enhancers as well as promoters are marked by distinct chromatin signatures. Both enhancers and promoters were marked by nucleosome depletion, DNAse I hypersensitivity, acetylated H3K9/K14, and dimethylated H3K4 (Heintzman, Stuart et al. 2007). These examples suggest that chromatin modification is likely to be a common mechanism for regulating the activity of tissue-specific transcription factors.

Brn3a is a critical regulator of sensory neural development

The work described in this dissertation is focused on understanding the function of Brn3a (Pou4f1), a POU domain transcription factor. The POU family of transcription factors is named after three molecules; the mammalian Pit-1 and Oct-1, and nematode Unc-86 (Clerc, Corcoran et al. 1988; Herr, Sturm et al. 1988; Phillips and Luisi 2000). Pit-1 plays an important role in pituitary-specific gene expression, and its inactivation leads to failure of pituitary gland development (Andersen and Rosenfeld 1994). Unc-86 encodes a transcription factor required for the development of specific neuronal cell types in nematodes (Finney, Ruvkun et al. 1988). Each of these molecules contains a two-part DNA binding domain consisting of a POU homeodomain and a POU-specific domain (Sturm and Herr 1988). The combination of these two DNA binding motifs confers POU transcription factors with highly specific DNA binding properties.

The vertebrate Brn3 (Pou4) class includes the highly homologous proteins Brn3a, Brn3b (Pou4f2), and Brn3c (Pou4f3) which exhibit similar DNA binding properties. Brn3a has been shown, using random oligonucleotide selection experiments, to bind with highest affinity to the nucleotide sequence ATAATTAAT, with single A or T substitutions at position 3, 5, or 7 resulting in relatively little reduction in affinity (Gruber, Rhee et al. 1997; Rhee, Gruber et al. 1998; Phillips and Luisi 2000). These factors have highly specific, partially overlapping patterns of expression in the vertebrate nervous system and each has a significant loss-of-function phenotype in mice. Expression of Brn3b in the retinal ganglion cells of midgestation

embryos is required for proper development of the eye and disruption of Brn3b expression leads to a 60-70% reduction in the number of retinal ganglion cells in mature mice (Erkman, McEvilly et al. 1996; Gan, Xiang et al. 1996; Erkman, Yates et al. 2000). Brn3c protein is highly expressed in the auditory and vestibular hair cells of the inner ear. Brn3c mutant mice exhibit a complete loss of sensory hair cells in the inner ear leading to deafness and impaired balance (Xiang, Gan et al. 1997).

Brn3a is highly expressed in terminally differentiating neurons throughout the peripheral sensory nervous system and in discrete locations within the central nervous system (Xiang, Gan et al. 1996; Quina, Pak et al. 2005). Ablation of Brn3a function leads to loss of specific populations of cells in the CNS as well as widespread sensory neural death and neonatal lethality(McEvilly, Erkman et al. 1996; Xiang, Gan et al. 1996). Prior to the onset of neuronal death in Brn3a knockout mice, sensory neurons are characterized by abnormally branching, defasciculated axons which fail to reach their target fields (Eng, Gratwick et al. 2001). Although Brn3a is clearly required for the proper development and survival of neurons in the sensory nervous system, prior to this study, the downstream target genes mediating the Brn3a knockout phenotype were unknown.

Previous experiments have demonstrated using transgenic misexpression experiments that Brn3a directly attenuates its own expression in the sensory nervous system (Trieu, Rhee et al. 1999; Trieu, Ma et al. 2003). Brn3a protein binds to a cluster of near consensus binding sites located approximately 5 kb upstream of its transcriptional start site, repressing its own transcription. This autoregulation provides

a means for gene-dosage compensation, such that the Brn3a mRNA level is nearly equal in the sensory neurons of wild type and Brn3a heterozygote embryos. Consistent with this finding, Brn3a heterozygote embryos have no detectable phenotypic differences from wild-type embryos.

This dissertation consists of a body of work that has been published in three separate research articles. These articles have been reformatted to appear in the following three chapters. Chapter two describes experiments that have elucidated many genes comprising the program of gene expression regulated by Brn3a in the trigeminal ganglion. These experiments show that expression of Brn3a is required for the proper regulation of several genes including neurotransmitters and neurotransmitter receptors, mediators of axon growth and pathfinding, and components of cellular signaling systems.

The experiments in chapter three provide an experimental framework for distinguishing between direct and indirect targets of tissue-specific transcription factors, demonstrating additional evidence for the direct nature of Brn3a autoregulation, and identifying NeuroD1 and NeuroD4 as direct Brn3a target genes. We also show, using ChIP assays with antibodies recognizing specifically modified histones, that epigenetic modifications of chromatin play a role in regulating the access of Brn3a to its potential binding sites in the genome.

In chapter four, we show that the trigeminal and dorsal root ganglia have extremely similar patterns of gene expression, despite their distinct embryological origins. Loss of Brn3a generates many changes in gene expression that are common to

both tissues. However, a few genes are specifically activated in the trigeminal but not the dorsal root ganglia of Brn3a null mice. The loci of the differentially regulated genes are characterized by acetylation H3K9/K14 in the TG and deacetylation in the DRG suggesting that Brn3a repression of these genes in the DRG may be redundant, due to an existing repressive conformation of chromatin. These data demonstrate that epigenetic mechanisms contribute to tissue-specific differences in target gene regulation by Brn3a.

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II

Coordinated Regulation of Gene Expression by Brn3a in the Developing Trigeminal Ganglion

ABSTRACT

Mice lacking the POU-domain transcription factor Brn3a exhibit marked defects in sensory axon growth and abnormal sensory apoptosis. Here 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 which express this factor.

INTRODUCTION

Studies of the developing vertebrate nervous system have revealed a large number of transcription factors which 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, due to 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, 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 neural 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, immunohistochemistry. To generate tissue for microarray analysis, timed matings of Brn3a heterozygote animals were performed, and the embryos harvested at E13.5. Only embryos corresponding to 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 5μg 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 online Supplemental Material. Immunofluorescence for Brn3a was performed with rabbit polyclonal antisera as previously 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, heterozygote, and knockout mice at embryonic day 13.5 (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 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 effects due to cell loss should be minimized at this stage.

Although no differences in the phenotype of Brn3a wild-type and heterozygote 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 5,993 known genes and 6,429 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, 4,885 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 which 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 2-fold 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 appears in the online Supplemental Materials, Figure S1.

Figure 2.1A 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 two-fold change in expression. The expression values for significantly changed transcripts are located off the central axis, 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 which met the criteria for significantly increased expression appears in Table 2.1, and the significantly decreased genes are shown in Table 2.2. 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 the online Supplemental Materials (Table S1).

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 2.3. Several of the ESTs confirmed results obtained for the known transcripts, including GATA3, AP2-β, 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 heterozygote 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 wild type, 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 and decreased transcripts, the target gene expression levels in wild-type and heterozygote ganglia showed similar differences from the knockout (Figure 2.1B). 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 heterozygote 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 2.4). 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, 1998a,b). In recent work, we have shown that mRNA levels for Bcl2 are unaltered in Brn3a knockout mice just prior to the onset of cell death (Eng et al., 2003), suggesting that loss of Bcl-2 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 are 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), are 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 (Figure 2.2B). 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.

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 (Figure 2.2C), and the cervical DRG (not shown). Conversely, the regulator of G-protein signaling RGS10 exhibited strong expression in the trigeminal ganglion, IX/X ganglion (Figure 2.2D), and dorsal root ganglia (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, nor 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. 2.2E,F), and the direction and approximate extent of the expression changes in each of these proteins was entirely consistent with the array results (Fig. 2.2G). 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 TH 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 (Figure 2.3A), and NeuroD1 (not shown), and for the decreased transcripts HoxD1 and Runx1 (Figure 2.3B), 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 (Figure 2.3A). GATA3, MyoR and NeuroD1, but not the Irx transcripts, were also increased in the DRG (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 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 (Figures 2.2 and 2.3) did not exhibit 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 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 (Figure 2.4A,B), while in the retina, a subset of neurons cells co-express these antigens (Figure 2.4D,E). The expression of calretinin was not altered in either of these regions in the absence of Brn3a (Figure 2.4C,F). Similarly, somatostatin was not ectopically expressed in the CNS of mice lacking Brn3a (Figure 2.4G-K). Galanin and tyrosine hydroxylase were not co-expressed with Brn3a in the CNS as they are in the sensory system (Figure 2.4L,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 the fact that a majority of the changed transcripts encode proteins with known or hypothesized roles in sensory neuron development or function, that a far greater number of neuron-specific genes did not significantly change (online Supplemental Material), and that 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 synergize 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 which 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 NPY-1 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 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 (online Table S1). Remarkably, these changes affect only those sodium channels which 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 factors 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 in 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 (IGF-1) and insulin-like growth factor binding protein-5 (IGFBP-5). Mice lacking IGF-1 have abnormalities in sensory neurons (Gao et al., 1999), and show defective cortical dendritic growth (Cheng et al.,

2003). IGFBP-5 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 IGF-1, 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, and 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 which 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. Normally expressed in the hindbrain and spinal cord, little is known about the role of AP2β 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 which 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), and 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 likely 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 which are regulated directly, and regulated indirectly by the several other transcription factors which 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.

FIGURES

Figure 2.1. 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 heterozygote 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 2-fold 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 of knockout vs. wild-type, knockout vs. heterozygote, and wild-type vs. heterozygote ganglia for the 41 increased and 62 decreased transcripts from the U74Av2 array, Experiment 1. Wild-type and heterozygote expression levels showed a similar mean fold increase or decrease when compared to knockout. However, comparison of heterozygote to wild-type expression levels revealed no significant difference (fold change ~1, red horizontal line) for the 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 ($0.003 < \text{change-p} < 0.997$, listed in online Supplemental Material).

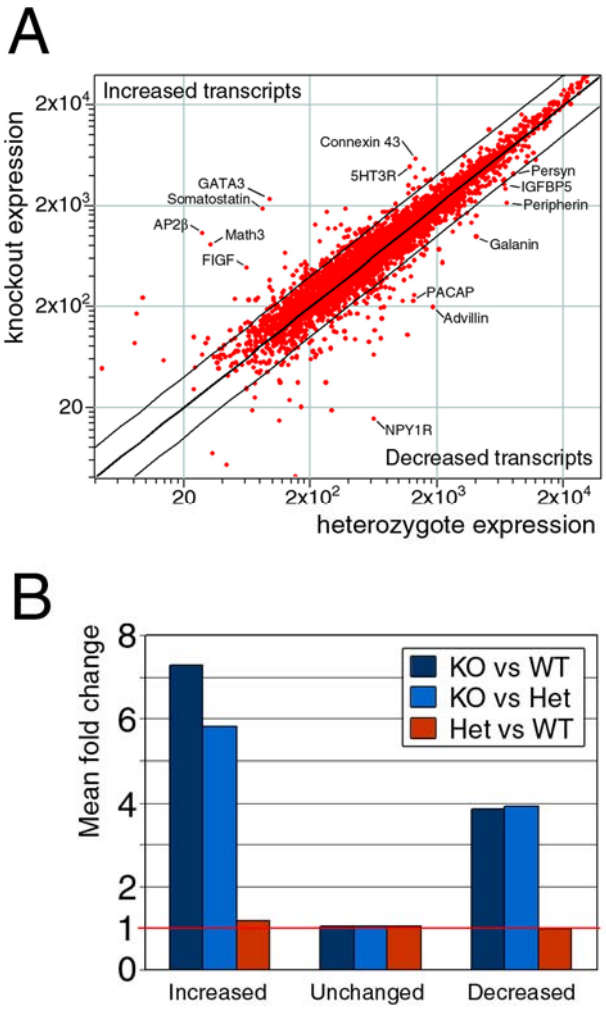

A
Increased transcripts
Decreased transcripts
knockout expression
heterozygote expression
2x10^4
2x10^3
2x10^2
20
Connexin 43
5HT3R
GATA3
Somatostatin
AP2β
Math3
FIGF
Persyn
IGFBP5
Peripherin
Galanin
PACAP
Advillin
NPY1R
B
Mean fold change
8
6
4
2
1
0
KO vs WT
KO vs Het
Het vs WT
Increased
Unchanged
Decreased

Figure 2.2. Brn3a regulates sensory neurotransmitter systems. The cranial sensory ganglia of control (+/+) and knockout (-/-) 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 βgalactosidase 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 expressed in a more limited subset of trigeminal neurons, most of which also express Brn3a. (F) A comparison of trigeminal ganglia from control mice and Brn3a knockouts, showing that, as predicted from the microarray studies, calretinin and somatostatin immunoreactivity is markedly increased in the absence of Brn3a, whilst galanin is reduced to below the threshold of detection, and tyrosine hydroxylase 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 bar: B, 400μM, D,E, 25 μm; F, 200 μm
.

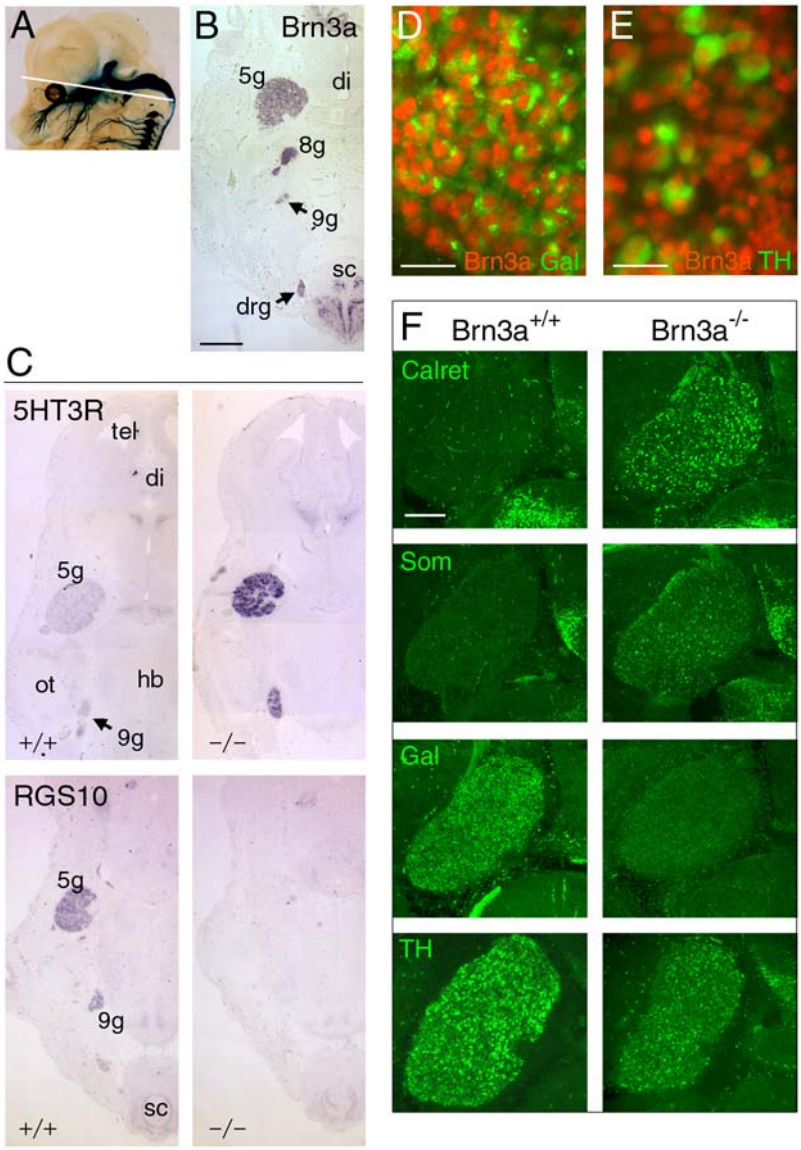

A
B
Brn3a
5g
di
8g
9g
sc
drg
C
5HT3R
tel
di
5g
ot
hb
+/+
9g
−/−
RGS10
5g
9g
sc
+/+
−/−
D
Brn3a Gal
E
Brn3a TH
F
Brn3a+/+
Brn3a-/-
Calret
Som
Gal
TH

Figure 2.3. 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 exhibiting 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 Figure 2.2A. 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 codr

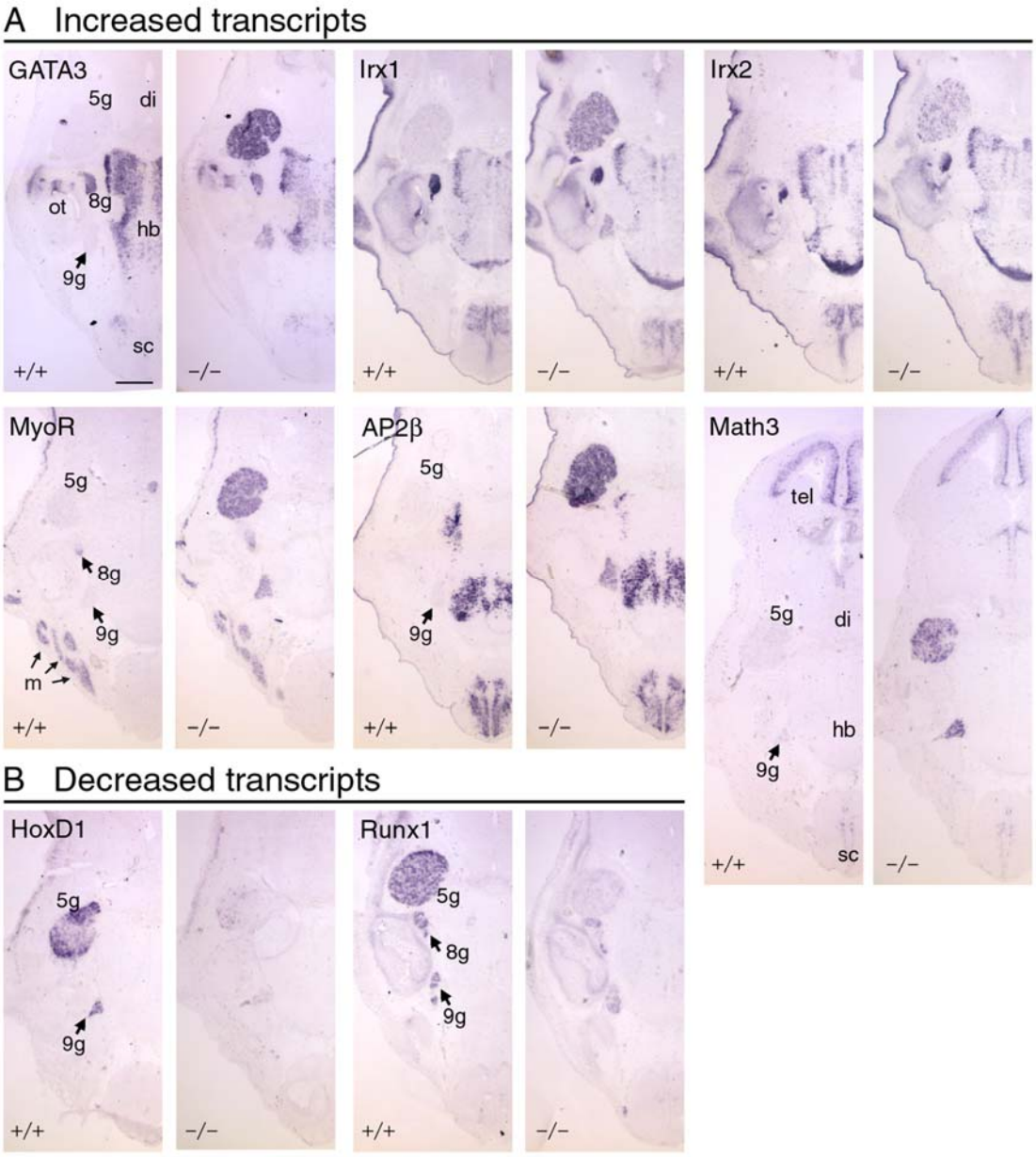
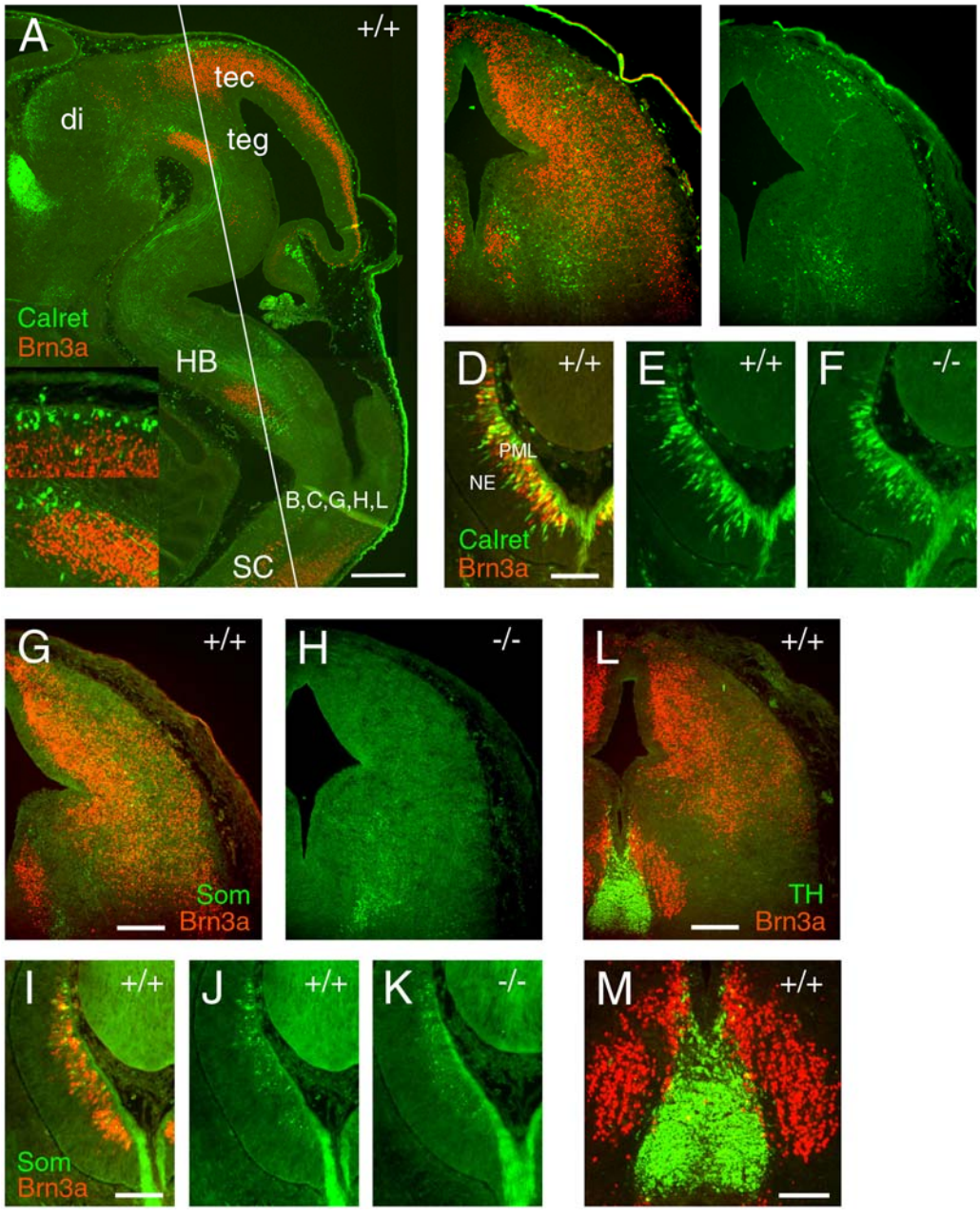

A Increased transcripts
GATA3
5g
di
ot
8g
hb
9g
sc
+/+
−/−
Irx1
+/+
−/−
Irx2
+/+
−/−
MyoR
5g
8g
9g
m
+/+
−/−
AP2β
5g
9g
+/+
−/−
Math3
tel
5g
di
hb
9g
sc
+/+
−/−
B Decreased transcripts
HoxD1
5g
9g
+/+
−/−
Runx1
5g
8g
9g
+/+
−/−

Figure 2.4. 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 sagital section, and are not co-expressed (inset views). 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 VTA and the red nucleus of the tegmentum, respectively. Scale bar: A, 400 μm; B,D,G, 100 μm; I, 100 μm; L, 200 μm; M, 50 μm.


A
+/+
tec
di
teg
Calret
Brn3a
HB
B,C,G,H,L
SC
D
+/+
PML
NE
Calret
Brn3a
E
+/+
F
-/-
G
+/+
Som
Brn3a
H
-/-
L
+/+
TH
Brn3a
I
+/+
Som
Brn3a
J
+/+
K
-/-
M
+/+

TABLES

Table 2.1. Increased transcripts in the trigeminal ganglion of Brn3a mutant mouse embryos. This table and Table 2.2 show expression data for all transcripts from among the 5,993 known genes represented on the U74Av2 chip which 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 ($p\Delta < 0.003$ or >0.997) in both experiments 3) A minimum of a two-fold 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 which exhibited changed expression but did not meet these criteria appears in the online Supplemental Material. 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.

Description	Genbank	Experiment 1			Experiment 2			Fold
Increased transcripts		**WT**	**HT**	**KO**	**WT**	**HT**	**KO**	**KO/ cont**
Cardiac responsive adriamycin protein	AF041847	4	4	170	11	10	244	34.3
GATA3	X55123	61	61	2524	85	96	2236	33.0
AP-2 beta	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

Table 2.2. Decreased transcripts in the trigeminal ganglion of Brn3a mutant mouse embryos. Transcripts were selected from among 5,993 known genes on the U74aV2 chip according to the criteria described in Table 2.1. The threshold change-p value for decreased transcripts is pΔ >0.997. The fold change is calculated as the ratio of control/knockout expression.

Description	Genbank	Experiment 1			Experiment 2			Fold
Decreased transcripts		**WT**	**HT**	**KO**	**WT**	**HT**	**KO**	**cont/ KO**
NPY-1 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

Table 2.3. Significantly changed ESTs in Brn3a mutant mice. Data are given for the relative expression of selected transcripts from amongst approximately 18,000 ESTs represented on the U74Av2 and U74Bv2 arrays.

[a]Affymetrix U74A chip
[b]affymetrix U74B chip
[c]Also appears as a known transcript on U74Av2 array
[d]Calculated for increased and decreased transcripts as described in Table 2.1 and 2.2 respectively

Description	Genbank	Mean Ratio[d]
Increased transcripts		
Transcription factor GATA-3[c]	AA717155[b]	52.1
Transcription factor AP2-Beta[c]	AI021690[b]	18.6
Homeobox protein Iriquois 2	AI851291[b]	18.4
Homeobox protein Iriquois 1 (2 probe sets)	AA028275[b]	6.5
LIM only protein Testin 2	AI845609[b]	6.1
HLH transcription factor NeuroD1[c]	AI848674[b]	3.5
Solute carrier family 1, member 3	AW121315[b]	3.4
Neuropilin 2	AW046112[b]	3.1
Protein Kinase C-binding protein NEL	AI838010[b]	2.9
Thymocyte selection-associated HMG box gene	AA688946[b]	2.9
Bruno-like 4 (2)	AI839880[a]	2.5
Dual specificity phosphatase 6 Dusp6 (=MKP3)	AI845584[a]	2.2
Catenin beta interacting protein 1	AI842284[b]	2.1
Regulator of G-protein signaling 4	AI854153[b]	2.1
Decreased transcripts		
Regulator of G-protein signalling 10 (2)	AI847399[a]	13.6
Na^+ channel Scn9a[c]	AI593229[b]	8.6
Ras family GTPase RAP	AA920095[b]	6.9
Runt-related transcription factor Runx1[c]	AI643935[b]	5.4
Downstream of tyrosine kinase 4 Dok4 (3)	AA982457[b]	4.8
Protein Tyrosine Phosphatase H	AI851090[b]	4.7
Insulin-like growth factor 1 (2)	AA544955[b]	4.6
Synaptotagmin-like 2	AW124071[b]	4.1
Doublecortin-like kinase	AI853072[b]	3.8
G-protein coupled receptor 64	AI132005[b]	3.6

Table 2.3. Significantly changed ESTs in Brn3a mutant mice (Continued).

Src homology 2 containing protein C1 Shc1	AI050321[a]	3.4
Ataxin-2 binding protein A2bp1 (2)	AW047913[b]	3.4
Wnt1 responsive Cdc42 homolog Wrch1	AV246963[b]	3.3
CRB1 isoform II precursor	AW046426 [b]	3.1
Microsomal glutathione S-transferase 3	AI843448 [a]	2.9
Oxysterol binding protein-like 3	AI591488[b]	2.9
Bicarbonate transporter SLC4A4[c] (2)	AW124701[b]	2.8
Nerve growth factor receptor p75	AA050723[b]	2.8
FXYD domain ion transport regulator 7	AI844246[a]	2.7
Intermediate filament protein Desmuslin (3)	AI852401[b]	2.7
ELKL motif serine-threonine protein kinase 3	AI430097[b]	2.6
Actin-binding double zinc finger protein	AI840403[b]	2.6
Growth factor receptor bound protein 14 Grb14	AW124221[b]	2.6
N-chimaerin (2)	AI848376[b]	2.5
Calcium channel, Cacna2d2	AA008996[b]	2.4
Synaptotagmyn 1[c] (2)	AW125093[b]	2.4
Axonin, (TAG1, contactin 2)	AW049675[b]	2.3
Calsyntenin 2	AI842881[b]	2.3
Neuritin	AI839544[b]	2.2

Table 2.4. Relative expression of previously reported Brn3a target genes in the trigeminal ganglia of Brn3a mutant embryos. 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 experments as Tables 2.1-2.3. Only one experiment is shown, similar results were obtained in both experiments, and complete data appear in the online Supplemental Materials, Table S1.

[a]Change call values: I, increased, change-p < 0.003; NC, no change, 0.003 < change p < 0.997; D, decreased, change p > 0.997

[b]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 not conclusive.

[c]U74B chip EST. All others are known transcripts from the U74Av2 array.

[d]Not represented on array

[e]Measured in late gestation, and may represent selective cell death.

Description	Probe set accession	Reference	WT	HT	KO	KO x HT Change Call[a]	KO/HT ratio
Cell transfection studies:							
alpha-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
Bcl-2	L31532	Smith et al., 1998	578[b]	612[b]	466[b]	NC	0.76
p53	AB021961	Budhram-Mahadeo et al., 2002	533	578	641	NC	1.11
Nicotinic AchRα3	(d)	Milton, et al., 1996	--	--	--	--	--
Knockout studies:							
trkA[c]	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[c]	AA050723	McEvilly, et al., 1996	2745	3655	1107	D	0.30
Parvalbumin[e]	X59382	Ichikawa, et al., 2002b	1412[b]	1808[b]	1330[b]	NC	0.74
Calbindin[e]	D26352	Ichikawa, et al., 2002b	177	196	302	NC	1.54
Vanilloid-like receptor 1[e]	AB021665	Ichikawa, et al., 2002a	582	645	490	NC	0.76

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ACKNOWLEDGEMENTS

We would like to acknowledge Bob Stuart, Bill Wachsman and Lutfunnessa Shireen for assistance with microarray technology. We would also like to thank the following investigators and their associates for in situ hybridization probes: Reinhard Buettner, Peter Gruss, Alar Karis, Allan Basbaum, Quifu Ma, Ryoichiro Kageyama, Denis Duboule, Stephen Gold, and Eric Olson. In addition, we thank John Kelsoe for collaborative use of Celera Genomics database, and Ying Liu, Jane Johnson and William Snider for helpful comments on the manuscript. Supported in part by Department of Veterans Affairs MERIT funding and VISN 22 MIRECC (E.E.T.), and NIH awards HD33442 and MH065496. EET and NF are a NARSAD Investigators.

The text in Chapter Two is a reprint of the material as it appears in Development, 2003, Eng SR, Lanier J, Fedtsova N, Turner EE. I was a secondary author and participated in the research that forms the basis of this chapter. My contributions to the work included analysis of microarray data.

III

Brn3a Target Gene Recognition in Embryonic Sensory Neurons

ABSTRACT

Numerous transcription factors have been identified which have profound effects on developing neurons. A fundamental problem is to identify genes downstream of these factors and order them in developmental pathways. We have previously identified 85 genes with changed expression in the trigeminal ganglia of mice lacking Brn3a, a transcription factor encoded by the *Pou4f1* gene. Here we use locus-wide chromatin immunoprecipitation in embryonic trigeminal neurons to show that Brn3a is a direct repressor of two of these downstream genes, *NeuroD1* and *NeuroD4*, and also directly modulates its own expression. Comparison of Brn3a binding to the *Pou4f1* locus in vitro and in vivo reveals that not all high affinity sites are occupied, and several Brn3a binding sites identified in the promoters of genes that are silent in sensory ganglia are also not occupied in vivo. Site occupancy by Brn3a can be correlated with evolutionary conservation of the genomic regions containing the recognition sites and also with histone modifications found in regions of chromatin active in transcription and gene regulation, suggesting that Brn3a binding is highly context-dependent.

INTRODUCTION

The development of any organ system requires the coordinated regulation of gene expression to generate increasingly specialized cell types. Tissue-specific gene expression is regulated primarily at the transcriptional level, by DNA-binding transcription factors that are expressed at key decision points in developmental processes. No tissue presents a more complex problem in the developmental regulation of gene expression than the vertebrate nervous system, where transcription factors of several structural families have been shown to have profound effects on the development of the spinal cord, brainstem, forebrain, retina, and peripheral sensory and autonomic ganglia (Helms and Johnson, 2003; Marquardt, 2003; Shirasaki and Pfaff, 2002; Zaki et al., 2003). In each of these areas, loss of regulatory function may lead to the agenesis of a class of neurons, respecification of neuronal phenotypes, aberrant cell migration or axon growth, or abnormal cell death.

Although the importance of neuron-specific transcriptional regulation is clear, in most cases the downstream targets of transcription factors with established roles in neural development remain unknown. Recent studies using expression arrays have been successful in defining multiple regulatory targets for a few of these factors in the developing retina, cerebellum, and sensory system (Eng et al., 2004; Gold et al., 2003; Livesey et al., 2000; Mu et al., 2004). However, in each of these studies several of the regulated genes also encode transcription factors, leading to significant uncertainty about which of the downstream genes are directly regulated and which are secondary targets.

The direct target genes of a developmental transcription factor are expected to contain binding sites for that factor within cis-acting regulatory domains, typically but not necessarily located in upstream or intronic sequences near the transcriptional start site of that gene. However, the short recognition sequences of monomeric or dimeric transcription factors occur frequently in the genome, and are not in themselves sufficient to restrict transcriptional regulation to a small number of tissue-specific genes. Therefore some mechanism for the further restriction of transcriptional activity in vivo is required. Numerous examples are known in which transcription factors act at their recognition sites only when they occur in a specific context, and/or in the presence of other cell-specific DNA-binding factors, a concept which has been referred to as a "combinatorial code" for cell-specific transcription. However, at present there are no general predictive algorithms for recognizing functional binding sites in vertebrates, and these relationships must be determined empirically.

We have been engaged in studies of the Pou4 or "Brn3" class of transcription factors, consisting in vertebrates of Brn3a (Pou4f1, Brn3.0), Brn3b (Pou4f2, Brn3.2), and Brn3c (Pou4f3, Brn3.1). Brn3a is expressed throughout the peripheral sensory nervous system and at multiple levels of the CNS, and targeted disruption of the *Pou4f1* locus results in excessive apoptosis in several regions of the embryonic brain, marked defects in sensory axon growth, and neonatal lethality (Eng et al., 2001; McEvilly et al., 1996; Xiang et al., 1996). Brn3b and Brn3c are more restricted in their expression, and null mutations of these genes result in viable mice with more specific

defects in the development of the retina and the inner ear, respectively (Erkman et al., 2000; Gan et al., 1996; Xiang et al., 1997).

The POU transcription factor class is characterized by a bipartite DNA binding domain, consisting of a POU-specific domain and a POU-homeodomain, which together recognize an extended DNA site (Phillips and Luisi, 2000). Biochemical and transfection studies have shown that the vertebrate and invertebrate members of the Pou4-subclass bind to a consensus sequence consisting of ATAATTAAT and minor variants thereof (Gruber et al., 1997; Rhee et al., 1998). Genetic studies have demonstrated that Brn3a regulates its own expression in sensory neurons via a cluster of such sites residing approximately 5.5kb upstream from the *Pou4f1* transcriptional start site (Trieu et al., 2003; Trieu et al., 1999).

Two recent studies have begun to ascertain the transcriptional targets of the Pou4 factors in the developing nervous system, using expression arrays with partial genomic coverage to assay global gene expression in the sensory ganglia and retina of mice lacking Brn3a and Brn3b, respectively (Eng et al., 2004; Mu et al., 2004). In both cases, the majority of regulated transcripts belong to gene families with known or potential roles in neurodevelopment, although only a few shared targets were identified. Extrapolating these results to the entire transcriptome suggests that the Pou4 factors regulate on the order of 10^2 downstream transcripts in a single tissue type at a given developmental stage, including both direct and indirect targets.

In the present study we use locus-wide, real-time chromatin immunoprecipitation assays in embryonic sensory neurons to better understand the

relationship between Brn3a and its transcriptional targets. These experiments show that Brn3a is a direct repressor of the bHLH genes *NeuroD4* (Math3) and *NeuroD1*, via consensus Brn3a binding sites. Brn3a also interacts directly with its own autoregulatory enhancer via relatively weak sites. In all three loci, most occupied Brn3a recognition sites occur in regions of local conservation between the mouse and human genomes. Examination of histone H3 acetylation and H3-lys4 dimethylation across the target gene loci reveals that site occupancy by Brn3a also correlates with these markers of transcriptionally active chromatin. Brn3a binding sites in the promoters of genes which are not transcribed in sensory ganglia and are de-acetylated in vivo appear to be generally unoccupied by Brn3a. Together these findings begin to illuminate the genomic context in which Brn3a regulates its downstream targets in sensory neurons.

MATERIALS AND METHODS

Bioinformatic analysis. Interspecies conservation was determined by comparing mouse and human genomic sequences using the pairwise LAGAN function from the LAGAN Alignment Toolkit website (Brudno et al., 2003). Alignments were visualized using VISTA software (Mayor et al., 2000), and the percent conservation between the mouse and human sequences was mapped using a 100 base pair moving window.

Brn3a Chromatin Immunoprecipitation. Embryos for ChIP assays were generated from timed matings of ICR mice and staged according to the method of Theiler (Theiler, 1972). Trigeminal ganglia were dissected from E13.5 embryos and fixed in 4% paraformaldehyde for 30 minutes, then quenched with 150 mM glycine. The fixed tissue was washed with PBS and stored at -80°C until analysis.

Selection of Brn3a complexes from embryonic sensory ganglia was performed by a modification of a widely used procedure (Luo et al., 1998). For each analysis, 60 fixed ganglia were pooled and suspended in lysis buffer containing 50 mM Tris-HCl, pH 8.1, 1% Triton-X-100, 10 mM EDTA, 1 mM AEBSF and a proprietary protease inhibitor mix (1X Complete Mini, Roche, used according to instructions). An oligonucleotide containing a high affinity Brn3a binding site was added to a final concentration of 0.5 μM to suppress any artifactual Brn3a-chromatin interactions which may form during the homogenization process. The oligonucleotides GATCTCTCCTGCATAATTAATTACCCCCGGAT and GATCCGGGGGTAATTAATTATGCAGGAGAGAT were annealed to form the

double stranded oligonucleotide competitor which contains the previously described consensus Brn3a binding site ATAATTAAT (Gruber et al., 1997). Ganglia were incubated in lysis buffer at 4º on a rotating platform for 10 minutes. Chromatin was then fragmented to an average size of 500 base pairs by sonication, and insoluble cellular debris was removed by centrifugation. The supernatant containing fragmented chromatin was diluted in 15 mM Tris-HCl, pH 8.1 with 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, and protease inhibitor mix. An unselected ("input") sample of 10% of the total homogenate was removed prior to antibody selection.

Selection of Brn3a protein-containing complexes was performed using 50 μL of sheep anti-rabbit IgG magnetic beads (Dynal M-280), pre-loaded with 10 μg of rabbit anti-Brn3a antibody. In order to reduce non-specific background, the chromatin sample was pre-cleared using the magnetic beads with the secondary antibody alone. The sample was then incubated overnight with Brn3a antibody-coupled beads to select the Brn3a protein-containing chromatin complexes. The selected complexes were washed with 20 mM Tris-HCl, pH 8.1 with 150 mM NaCl, 2 mM EDTA, and 1% Triton-X-100, followed by two washes with 10 mM Tris-HCl, pH 8.1 with 0.25 M LiCl, 1% IGEPAL, 1% Deoxycholate, and 1 mM EDTA. The final wash was performed in 20 mM Tris-HCl, pH 8.1 with 150 mM NaCl and 2 mM EDTA. Each wash was performed for 5 minutes at room temperature. DNA was extracted from both the antibody-chromatin complexes and input sample by heating in 0.1 M $NaHCO_3$ with 200 mM NaCl and 1% SDS at 65º for 4 hours with constant shaking.

The input and selected samples were then digested with proteinase K, extracted with phenol/chloroform, and precipitated with ethanol.

Histone H3 Chromatin Immunoprecipitation. Modified histone H3 ChIP experiments were performed using rabbit anti-acetylated histone H3 antibody (Upstate Biotechnology, Inc., catalog no. 06-599), which recognizes histone H3 acetylated at lys9 and lys14, and rabbit anti-dimethyl-histone H3-Lys4 (H3-K4, catalog no. 07-030). The anti-Brn3a ChIP protocol described above was slightly altered for use with antibodies against modified histones. For each selection, 50 μg of anti-histone antibody was coupled to 250 μL of anti-rabbit IgG magnetic beads, and the lysis buffer contained 50 mM Tris-HCl, pH 8.1 with 10 mM EDTA and 1% SDS. The selected complexes were washed for five minutes at room temperature with each of the following solutions: 20 mM Tris-HCl, pH 8.1 with 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, and 1% Triton-X-100; 20 mM Tris-HCl, pH 8.1 with 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton-X-100; 10 mM Tris-HCl, pH 8.1 with 1 mM EDTA, 0.25 M LiCl, 1% NP-40, and 1% deoxycholate. Salt and detergent were removed by washing twice with TE, pH 8.

Real-time locus-wide PCR analysis. Chromatin fragments recovered from the immunoprecipitated and input samples were then assayed by real-time PCR using an ABI 7300 thermocycler and SYBR Green fluorimetric detection. Selected and unselected samples were run in parallel in a 96-well plate format, and the enrichment of immunoprecipitated chromatin fragments was assayed by the cycle-threshold difference method (Livak and Schmittgen, 2001). To screen an entire gene locus,

oligonucleotide pairs were designed at 500-1000bp intervals throughout the region. Because the selected chromatin was sheared to an average size of approximately 500bp, it was not necessary for the ChIP oligonucleotide pairs to encompass the actual binding site to give a positive signal, and as expected the ChIP signal decayed over about 1kb in either direction from the site. When enrichment was seen in a region with the screening set of oligonucleotides, further independent oligonucleotide pairs were designed in the interval to confirm the result in subsequent assays.

Real-time PCR signals were measured using the "cycle threshold", or Ct parameter, which is the number of cycles required for the amplification product to reach an arbitrary level of fluorescence intensity (threshold). The threshold is set in the log-linear range of the product generation curve, assuring that the quantitation of the PCR products occurs in the logarithmic phase of amplification. A further advantage of the method is that the small amplicons used for real-time PCR amplify much more reliably than products which are designed to be quantitated by gel electrophoresis. The Ct value is related logarithmically to the initial abundance of the target sequence in the sample in that more abundant targets will reach the threshold faster, and yield a lower Ct value. Because product formation approximately doubles with each cycle in the linear range of amplification, a Ct difference of one cycle represents a two-fold difference in starting template.

For each PCR amplicon, signals from the unselected ("input") and antibody-selected DNA samples were then compared using the cycle threshold difference (ΔCt) method. A ΔCt value was calculated for each amplicon by subtracting the $Ct_{selected}$

from the Ct_{input} signal ($\Delta Ct=Ct_{input} - Ct_{selected}$). A significant advantage of this method is that for each primer pair, a selected sample is compared directly to its unselected control, which differs only by the antibody selection process. Potentially confounding factors such as small differences in the PCR amplification efficiency of different primer pairs are eliminated in this comparison.

The ΔCt assays for each pool of anti-Brn3a selected material were normalized to a baseline (1-fold enrichment) determined using the average ΔCt value ($\Delta Ct_{control}$) for ten primer pairs spanning the *Alb-1* (albumin) gene, which was chosen for this purpose because is not transcribed in the nervous system. Little variation was observed across flanking, promoter, intronic and translated sequences in ChIP selection assays of the *Alb-1* locus (online Supplementary Information, Figure S1A). Anti-histone selected assays were normalized to the average ΔCt value of two amplicons in the *Alb-1* promoter region which showed enrichment values representative of the whole locus (Figure S1B). A second method of normalizing the ΔCt values, using primer pairs to promoter regions of *Mapt* (microtubule-associated protein tau) and *Eno2* (neuron specific enolase), which exhibit tissue specific expression in the nervous system, and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), which is expressed ubiquitously, gave very similar results.

Fold enrichment values for target sequences bound by the selecting antibodies, corresponding to the y-axis of the locus-ChIP plots, was calculated using the following equation: $E=2^{(\Delta Ct-\Delta Ct_{control})}$. The statistical significance (p-value) for ChIP selection at a given Brn3a binding site was calculated using fold enrichment values for all

primer pairs located within 500 base pairs of the binding site, compared to the enrichment values for the Alb-1 locus, using two-sample, unequal variance t-tests. Very similar results were obtained when the peaks of ChIP enrichment were compared to the intergenic baseline flanking the same gene.

Electrophoretic Mobility Shift Assay (EMSA). Electrophoretic mobility shift assays were performed using probes generated by PCR using radiolabeled primers, or with 32 base pair double-stranded oligonucleotides containing Brn3a recognition sites (online Table S1), as previously described (Gruber et al., 1997; Trieu et al., 1999). Full-coding Brn3a protein was expressed in sf9 cells using a baculovirus expression system. Use of alternate translation initiation sites resulted in some size heterogeneity of Brn3a/DNA complexes in EMSA assays, but did not affect the C-terminal DNA-binding domain of Brn3a.

Dissociation rates for Brn3a-DNA complexes were estimated by EMSA, providing a rapid method for comparing the stability of Brn3a association with a large number of binding sites. EMSAs were performed a 50µl volume containing 20 mM Tris (pH 8.0), 100 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 100µg/ml of poly(dI-dC), 100 µg/ml BSA, 10% glycerol, and 1 mM dithiothreitol. In these assays, Brn3a protein was incubated with a radiolabeled DNA probe in EMSA binding cocktail for 20 minutes at 20°. Then, at 5 or 20 minutes before the start of electrophoresis, 1 X 10^{-11} mol of an unlabeled high affinity Brn3a oligonucleotide competitor, containing the consensus Brn3a binding site ATAATTAAT, was added to the binding assay (consensus, Table S2, Gruber et al., 1997). The relative quantities of Brn3a complexes

remaining after 5 and 20 minute incubation with the oligonucleotide competitor were determined from scanned autoradiograms using Image J software. The half-lives of Brn3a complexes were determined based on first order kinetics.

Complex stability screening. In order to identify the most stable (highest affinity) Brn3a binding sites within a large genomic region encompassing the *Pou4f1* locus, we adapted a protocol previously used to identify the Brn3a autoregulatory binding site cluster (Trieu et al., 1999). A BAC clone containing 188 kb of mouse genomic sequence surrounding the *Pou4f1* gene (Clone ID: RP23_360a14, BACPAC Resources Center) was sheared to an average size of 500 base pairs by sonication, and the ends of the DNA fragments were repaired for A-T cloning using mung bean nuclease and the addition of single 3'-dA residues using a commercial tailing kit (Novagen).

Selection of the sheared, tailed BAC DNA was performed using 15µg of rabbit anti-Brn3a antibody pre-incubated with 50µL paramagnetic beads conjugated to sheep anti-rabbit IgG (Dynal M-280). The coupled beads were then incubated with 20µL of sf9 cell lysate containing full-coding Brn3a protein (described above), rinsed, and incubated with 10µg of sheared BAC DNA. Binding reactions were performed in EMSA buffer as described above, except dI/dC was omitted. Following the initial binding reaction, the beads were resuspended in binding buffer containing 2µM oligonucleotide competitor containing a Brn3a consensus binding site, and incubated for 10 minutes before magnetic separation in order to block binding of unstable (low-affinity) Brn3a-DNA complexes. The beads were immediately washed two times with

binding buffer to remove the oligonucleotide competitor, and the remaining bound DNA fragments were eluted from the paramagnetic beads by incubation in TE buffer containing 1% SDS and 20μg glycogen at 60ºC for 15 minutes. DNA fragments were recovered by phenol/chloroform extraction followed by ethanol precipitation.

Selected BAC fragments were ligated into pSTBlue-1, and recombinant plasmids were used to transform E. coli using the Acceptor Vector Kit (Novagen). Positive colonies were isolated using blue/white selection and plasmid DNA was prepared for sequencing. Insert sequences were aligned with the BAC sequence using BLAST to determine the location of the selected fragments containing Brn3a binding sites.

In a prior study using CoSS screening (Trieu et al., 1999), we identified clustered Brn3a binding sites at -5633 relative to the start of transcription as the highest affinity sites within 11kb of the *Pou4f1* transcription start site. These sites, which deviate slightly from the optimal consensus, were not selected by the more stringent protocol described here. The principal difference in the method used in the prior study was the use of a Brn3a POU domain glutathione-S-transferase (GST) fusion protein for the selection process. Fusion to GST stabilizes the interaction between the POU domain and DNA, resulting in the retention of lower affinity complexes (Rhee et al., 2001). In the current study, full-coding Brn3a without a GST moiety was employed.

RESULTS

Brn3a is a direct repressor of bHLH transcription factors. In the absence of Brn3a, sensory neurons exhibit abnormal axon growth, fail to correctly innervate their target fields, and undergo abnormal cell death in late gestation. In the trigeminal ganglion these defects are accompanied by significant changes in the expression of multiple genes, including both increased and decreased transcripts (Eng et al., 2004). These changes in gene expression may be either mediated directly by Brn3a, or indirectly by several other transcription factors which exhibit altered expression in Brn3a knockout mice.

In order to better understand the relationship of Brn3a DNA binding to transcriptional regulation, we employed chromatin immunoprecipitation (ChIP) in embryonic sensory neurons to map the direct sites of Brn3a interaction with selected downstream genes. For this analysis, we chose microdissected embryonic day 13.5 (E13.5) trigeminal ganglia because at this stage the developing sensory neurons are relatively homogeneous with respect to the expression of Brn3a. At E13.5, a majority of cells in the trigeminal express Brn3a, and relatively few express the glial marker Sox10 (Figure 3.1), while at E16.5 and later stages, differentiating glia, which may contribute to background in this assay but not to Brn3a ChIP signal, are far more numerous (data not shown).

For screening assays of Brn3a binding to target gene loci, real-time PCR primer sets were designed at intervals of 0.5-1kb spanning a region of approximately 40kb for each gene, a strategy we refer to as locus-wide chromatin

immunoprecipitation (locus-ChIP). To verify the results of the initial locus-ChIP screens, multiple primer pairs were subsequently designed to confirm Brn3a binding at specific sites. For each target gene, the region examined spans the entire transcription unit, plus extended 5'- and 3'-flanking regions. The rationale for screening entire loci in this manner was two-fold. First, although the Brn3a consensus binding sequence has been determined in vitro, the extent to which the site may deviate from the consensus and still permit binding in vivo is unknown, and the locus-ChIP method is unbiased with respect to the sequence of the occupied site. Second, enhancer elements are often located at a considerable distance from the transcription units they regulate, and we did not want to arbitrarily restrict the analysis to the region immediately adjacent to the transcription start site.

Prior work has shown that three bHLH transcription factors exhibit significantly increased expression in the trigeminal ganglia of mid-gestation *Pou4f1* knockout embryos, including NeuroD4 (Math3, 8-fold increase) NeuroD1 (3-fold), and Msc (Musculin, MyoR, 7-fold) (Eng et al., 2004). For this reason, the locus-ChIP method was first applied to these gene loci, using pools of 60 microdissected E13.5 trigeminal ganglia as starting material for the assay. Figure 3.2 shows the result of locus-ChIP screening of the *NeuroD4* locus, revealing two positions in the locus occupied by Brn3a in vivo, in the 5'-flanking region at -12,996 relative to the start of transcription, and to a region within the first intron at +4,410. Sequence analysis of the regions identified by ChIP confirmed the presence of Brn3a consensus sites at both locations. Kinetic electrophoretic mobility shift assays (EMSAs, Materials and

Methods) were then performed to demonstrate that Brn3a forms stable complexes with each of these sites in vitro (Figure 3.2B).

Each of the binding sites identified in the *NeuroD4* locus contains a partial duplication of the Brn3a consensus sequence, indicating the possibility of multimeric Brn3a binding. In order to assay the stoichiometry of Brn3a association with each site, we compared the EMSA complexes formed by Brn3a bound to the *NeuroD4* sites to those formed with a consensus monomeric Brn3a binding sequence (b3s1, Gruber et al., 1997; Rhee et al., 1998). Each of the *NeuroD4* binding sites forms a highly stable complex with multiple Brn3a molecules (Figure 3.2C), consistent with prior evidence that Brn3a exhibits cooperative homodimerization on extended sites (Rhee et al., 1998). The identification of in vivo Brn3a binding sites at the *NeuroD4* locus, in conjunction with increased NeuroD4 expression in the absence of Brn3a, demonstrates that Brn3a is a direct repressor of NeuroD4 expression in the developing trigeminal ganglion.

Locus-ChIP screening of the *NeuroD1* locus revealed a broad area of enrichment beginning approximately 7 kb upstream from the start of transcription, and extending through the end of the transcribed region of the gene (Figure 3.3A). Five sites were identified in *NeuroD1* 5'-flanking region which contained a Brn3a consensus or variant consensus site. The maximum enrichment occurred in the 3'UTR of the *NeuroD1* gene, encompassing a Brn3a consensus site at position +2973. In contrast to *NeuroD4* and *NeuroD1*, a locus-ChIP survey of the *Msc* locus from –20kb to +20kb did not reveal any occupied Brn3a binding sites (Figure 3.3B). Thus it is

likely that increased expression of Musculin in the trigeminal ganglia of *Pou4f1* knockout mice is mediated by an indirect mechanism, although is also possible that Brn3a may regulate the expression of Musculin by direct interaction with binding sites outside the region examined.

Direct autoregulation by Brn3a. In prior work, we have shown genetic evidence that Brn3a attenuates its own expression by autoregulation, mediated by a cluster of Brn3a binding sites ~5kb upstream from the start of transcription (Trieu et al., 2003; Trieu et al., 1999). These binding sites reside within a well-characterized *Pou4f1* enhancer which regulates the expression of Brn3a in the sensory PNS (Eng et al., 2001), but the sequences targeting Brn3a expression to the CNS have not been identified. To screen for additional Brn3a binding sites in the extended *Pou4f1* locus that may mediate autoregulation in the CNS, we employed an in vitro immunoprecipitation method termed complex stability screening (CoSS, Trieu et al., 1999). To apply the CoSS method to the *Pou4f1* locus, a BAC clone containing 188kb of genomic DNA encompassing the *Pou4f1* gene and surrounding sequences was sheared into 200-500 base pair fragments by sonication, then incubated with recombinant Brn3a protein. Following this binding reaction, the Brn3a-DNA complexes were incubated briefly in the presence of excess competitor oligonucleotide containing a consensus Brn3a site, followed by separation of the remaining stable complexes using an immobilized anti-Brn3a antibody (Materials and Methods). Under these conditions, only fragments containing the highest affinity binding sites in the BAC clone are retained.

The CoSS-selected DNA fragments were ligated into a cloning vector for sequencing, and approximately 130 cloned fragments were mapped onto the complete sequence of the extended *Pou4f1* locus (Figure 3.4A). In nearly every case in which multiple selected fragments mapped to the same region of the locus, a Brn3a consensus binding site was identified in the region common to the fragments. However, this high-stringency screen did not select the autoregulatory sites approximately 5kb upstream from the *Pou4f1* transcriptional start site identified by lower stringency screening and genetic analysis in prior studies (Trieu et al., 2003; Trieu et al., 1999). To resolve this possible divergence in results, EMSAs were performed on the Brn3a sites identified in both screens in order to obtain a more quantitative estimate of the in vitro stability of Brn3a bound to each site (Figure 3.4B). These results confirm the presence of multiple stable Brn3a binding sites in the extended *Pou4f1* locus, and also show that the sites within the *Pou4f1*autoregulatory region (-5,633) have sub-optimal binding, suggesting that the in vitro affinity of Brn3a for its cognate sites may not be the best predictor of biological function.

In order to determine the relationship between in vitro binding and in vivo occupancy of the sites identified within the *Pou4f1* locus, we next used locus-ChIP to screen the *Pou4f1* locus for Brn3a binding from –75 kb to +85 kb in E13.5 trigeminal ganglia (Figure 3.4C). A total of 103 primer pairs were used in the assay, including those designed to confirm areas of enrichment detected in the in vitro screen. These results demonstrate in vivo Brn3a binding to the autoregulatory enhancer at -5,633

relative to the transcriptional start site, and to multiple sites identified 3' to the Brn3a transcription unit.

Taken together, data from in vitro selection assays, in vivo ChIP assays, and EMSAs for Brn3a binding sites at the *Pou4f1* locus suggest that specificity conferred by the local DNA sequence is not sufficient to explain the interaction of Brn3a with chromatin in vivo. Instead, sites in the distal 5' region of the *Pou4f1* BAC appear to exhibit low occupancy relative to their in vitro binding, exemplified by the very stable site at -58,080 which was selected most frequently in vitro, but is weakly bound in vivo. Conversely, sites within the genomic region from approximately -10,000 to +3,000 relative to the start of transcription, encompassing the *Pou4f1* sensory enhancer, autoregulatory region, transcriptional start, 5' UTR, and first intron, are more highly occupied by Brn3a protein than would be predicted based on in vitro binding.

Brn3a binding to target gene loci in vivo correlates with interspecies conservation and specific histone modifications. In order to better understand the context-dependent binding of Brn3a, we examined the conservation of the identified binding sites between mouse and human genomic sequences and the specific modifications of histones across the gene loci of Brn3a and its downstream targets. Sequence conservation between species is a hallmark of functional regulatory regions, and local histone modifications have also been shown to play an important role in the regulation of gene expression (Margueron et al., 2005). Specifically, acetylation of the N-terminal tail of histone H3, associated with other modifications, is thought to induce

an open conformation of chromatin that is accessible to the ubiquitous transcriptional machinery (Eberharter and Becker, 2002; Struhl, 1998). Although histone acetylation is generally associated with the promoter regions of transcribed genes, several examples indicate that tissue-specific distal regulatory regions may also be acetylated (Bulger, 2005).

Comparison of the mouse and human genomic loci for the downstream bHLH targets of Brn3a regulation and the Pou4f1 locus itself reveal that nearly all of the occupied Brn3a binding sites reside in regions of highly conserved sequence (Figure 3.5B, 3.5C, and Figure 3.6). The peak of Brn3a ChIP selection in the *NeuroD1* locus (+2,973) resides in a highly conserved region of the 3' untranslated portion of this gene. Similarly, in the *NeuroD4* locus, the selected site in the first intron (+4,410) resides in a highly conserved region. However, no region of extended homology could be identified for the distal *NeuroD4* site (-12,996). Within the regions of extended conservation containing the Brn3a binding sites at the *NeuroD1* and *NeuroD4* loci, the specific sequence of the consensus Brn3a binding sites are also fully conserved (online Figure S3).

Occupied Brn3a binding sites within the *Pou4f1* locus showed a similar degree of interspecies conservation. The Brn3a autoregulatory enhancer region (-5,633) has been previously shown to encompass a region of 244bp which is 100% conserved between mouse and human (Trieu et al., 2003), including five variant Brn3a binding sites. Alignment of mouse and human genomic sequences show that the occupied Brn3a binding sites in the 3' flanking region of the *Pou4f1* locus (+7,093, +43,473,

+51,848, +78,456) also reside in islands of highly conserved sequence, and exhibit conservation of the Brn3a binding sites within these regions (Figure 3.5B, 3.5C, and S3).

To assay chromatin modification in sensory neurons, we performed ChIP analysis of the *Pou4f1* locus and target gene loci using an antibody recognizing acetylated histone H3 (Materials and Methods). As described for locus-ChIP assays using anti-Brn3a antibodies, these experiments were performed using microdissected E13.5 trigeminal ganglia as starting material, and were analyzed using PCR amplicons targeting the locus from -75 kb to +85 kb relative to the transcription start site (Figure 3.5A).

Enrichment values for most of the region examined approached background, indicating that chromatin is not hyperacetylated over most of the extended *Pou4f1* locus. However, a major area of increased H3-acetylation extends from approximately -8,000 to +10,000 relative to the start of transcription (Figure 3.5B). Examination of this region at higher resolution (Figure 3.5B) reveals discrete peaks of acetylation at the *Pou4f1* autoregulatory region (-5.5kb) and at the transcription start site, while the intervening region from -4kb to -2kb is much less acetylated. Significant acetylation also occurs 3' to the transcription unit, encompassing an occupied Brn3a binding site identified at +7,093. In the far distal part of the locus, a relatively weak but significant area of acetylation was identified encompassing an occupied Brn3a binding site located at +78,456 (Figure 3.5C). Finally, a hyperacetylated region was identified at -53,620 relative to the *Pou4f1* transcription start site, which corresponds precisely to

the transcription start site of an adjacent gene of unknown function, 2610206B13Rik. This peak of acetylation does not coincide with the identified Brn3a binding site at –58,080, which exhibits very stable binding in vitro, but exhibits low occupancy in vivo.

We then examined histone H3 acetylation at the genomic loci of the bHLH factors directly regulated by Brn3a (Figure 3.6). The *NeuroD4* locus is characterized by increased acetylation from approximately -2,000 to +5,000, with maximal enrichment observed near the transcription start site. A distinct peak of acetylation encompasses the in vivo Brn3a binding site located within the *NeuroD4* first intron at +4,410. An additional minor acetylation peak occurs approximately 13 kb upstream of the transcription start site, corresponding with another in vivo Brn3a binding site at -12,995.

Histone H3 acetylation of the *NeuroD1* locus extends from approximately -5,000 to +6,000 relative to the transcriptional start site. H3 acetylation at the *NeuroD1* locus exceeded that at the *NeuroD4* locus, consistent with the higher expression of *NeuroD1* in wildtype ganglia (Eng et al., 2004). The region of acetylation corresponds with the area of enrichment observed in locus-ChIP analysis using α-Brn3a antibody, and maximal acetylation was observed near the transcription start site and at the identified Brn3a binding site at +2,973.

In addition to acetylation of histone H3, dimethylation of H3 lysine 4 (K4) has been shown to mark actively and potentially transcribed genes in yeast and higher eukaryotes (Santos-Rosa et al., 2002; Schneider et al., 2004; Schubeler et al., 2004).

Therefore, in order to further define the relationship between markers of chromatin structure and Brn3a binding, we performed dimethyl H3-K4 locus-ChIP analysis of the relevant genes in embryonic trigeminal ganglia. At the *Pou4f1* locus (Figure 3.5D), the pattern of H3 K4 dimethylation is remarkably similar to that of H3 acetylation, including significant enrichment in the autoregulatory enhancer region, the transcription start site, and an occupied Brn3a binding site identified at +7,093. The *NeuroD4* and *NeuroD1* genes also reveal patterns of H3 K4 dimethylation which closely parallel H3 acetylation at these loci (online Figure S2), leading to the conclusion that these marks reflect a similar state of chromatin, associated with Brn3a binding.

Brn3a is excluded from the regulatory regions of genes not expressed in sensory ganglia. Taken together, the locus-ChIP data for Brn3a and its direct targets suggest that in vivo binding of Brn3a is highly context-dependent, and that site occupancy is predicted by occurrence of the sites in conserved regulatory regions which are also H3-acetylated and K4-dimethylated in trigeminal neurons. This observation leads to the prediction that Brn3a binding sites in the regulatory regions of genes which are never expressed in developing sensory neurons, and thus reside in regions of silenced chromatin, will not be occupied in vivo regardless of affinity. To test this hypothesis, we identified potential Brn3a binding sites using a bioinformatic search of a mouse promoter database containing upstream sequences of 14,963 well-characterized transcripts (MM5 upstream2000, UCSC Genome Bioinformatics). We then used microarray expression data from E13.5 (Eng et al., 2004) and adult (NCBI

Gene Expression Omnibus) trigeminal ganglia to identify a subset of these genes that are not expressed in trigeminal neurons.

The bioinformatic search of the promoter database identified several hundred promoters containing variants of the Brn3a consensus sequence. From this set, 28 sites with high sequence similarity to the occupied sites in the *Pou4f1* and *NeuroD4* promoters were analyzed for Brn3a binding using kinetic EMSA assays. Using the dissociation half-lives of the complexes of these sites with Brn3a, they were categorized as exhibiting low, moderate, high, or very high stability. In these assays, the monomeric Brn3a consensus site exhibited moderate stability, and all of the sites with "high" or "very high" stability were characterized by the association of multiple Brn3a molecules with the site. Extensive prior studies using cell transfection and transgenic assays have demonstrated the ability of sites with these monomeric and dimeric motifs to mediate the transcriptional effects of Brn3a (Gruber et al., 1997; Rhee et al., 1998; Trieu et al., 2003; Trieu et al., 1999). Figure 3.7A shows representative EMSAs of oligonucleotides containing a consensus monomeric Brn3a binding site and binding sites from the promoters of two of the identified genes, *Sema3e* and *Ngp*.

Nine of the promoters which contain Brn3a binding sites, but are transcriptionally silent in the trigeminal ganglion, were selected for ChIP analysis of histone acetylation and in vivo Brn3a binding (Figure 3.7), using PCR amplicons near each of the identified binding sites. Figure 3.7B compares H3 acetylation at these sites with sites in regulatory regions within the *Pou4f1* locus. As predicted, H3 acetylation

at these non-expressed genes is very low in comparison with the promoter/enhancer regions of the *Pou4f1* locus and the other expressed genes examined in this study, and in most cases approaches the baseline set by amplicons in the albumin locus. ChIP assays for Brn3a binding at these sites were also uniformly negative (Figure 3.7C). Thus these sites are unoccupied in vivo despite the fact that their sequence and in vitro Brn3a binding characteristics are very similar to the occupied sites in the *Pou4f1* and *NeuroD4* regulatory regions, providing a striking confirmation of the context-dependent binding of Brn3a.

DISCUSSION

Brn3a is a direct repressor of bHLH genes and its own autoregulatory enhancer. In the present study we have used locus-wide ChIP assays to examine the direct interaction of Brn3a with four of its regulatory targets, the bHLH genes *NeuroD4*, *NeuroD1*, and *Msc*, and the *Pou4f1* gene itself. These bHLH transcription factors were selected from among the 85 transcripts we have previously shown to exhibit >2-fold changed expression in the developing trigeminal ganglia of *Pou4f1* null mice (Eng et al., 2004) because they are likely to be high in the regulatory hierarchy that leads to the Brn3a null phenotype. ChIP assays were performed with microdissected embryonic trigeminal ganglia, which provide a relatively homogenous source of post-mitotic Brn3a-expressing neurons. Although the results of ChIP assays may in principle be quantitated either with PCR-based methods or with microarrays (ChIP-on-chip, Blais et al., 2005; Carroll et al., 2005; Ren et al., 2002), here the analysis was limited to PCR-based methods, because the samples obtained from embryonic ganglia provide approximately 100-fold less starting material than that required by established ChIP-on-chip protocols.

Two refinements in PCR-quantitated ChIP were incorporated into the methods used in this study. First, real-time PCR with fluorimetric detection was employed, and the immunoprecipitated chromatin fragments were assayed by the cycle-threshold difference method (Ct difference, Livak and Schmittgen, 2001). This method allows each assay of ChIP selected DNA to be compared directly to an unselected sample using the same set of primer pairs, such that the observed enrichment for a given set of

primers is highly likely to be a specific effect of the antibody selection process. The second refinement was the application of many sets of PCR primers arrayed across an extended gene locus (locus-ChIP). Locus-ChIP has the advantage of rapidly identifying regions of transcription factor binding or chromatin modification, yielding a map similar to a tiled array (Ciccone et al., 2004; Litt et al., 2001). In addition, multiple primer pairs can be designed to a particular region of interest to independently replicate significant findings. Finally, unlike ChIP assays targeting specific predicted binding sites or promoter regions, locus-ChIP analysis is independent of any assumption about where protein-DNA interactions will occur.

The results of these experiments demonstrate that Brn3a is a direct repressor of *NeuroD4* and *NeuroD1* in the developing trigeminal ganglion. Combined with the altered expression of other transcripts in the sensory ganglia of *Pou4f1* null mice (Eng et al., 2004), these results suggest that a major function of Brn3a in postmitotic trigeminal neurons is to terminate the expression of genes characteristic of earlier developmental phases, and also to repress genes normally expressed in the hindbrain but not in the sensory ganglia. In contrast to *NeuroD4* and *NeuroD1*, no evidence could be found for Brn3a binding at the *Msc* locus. Despite these results, it is possible that Brn3a acts directly to repress the *Msc* locus at regulatory sites which lie outside the 40kb region examined by locus-ChIP, or the *Msc* gene may be activated indirectly in *Pou4f1* null mice by other transcription factors which show markedly increased expression in the absence of Brn3a (Eng et al., 2004).

Direct regulation of the *Pou4f1* autoregulatory enhancer was also verified by locus-ChIP, although the occupancy of the autoregulatory sites appears to be significantly less than those of the *NeuroD4* locus. Relatively weak repression of the autoregulatory enhancer is consistent with the quantitative expression of NeuroD4 and Brn3a mRNA in the E13.5 trigeminal ganglion, in that expression from the *NeuroD4* locus is repressed to almost undetectable levels by Brn3a (Eng et al., 2004) whereas expression from the *Pou4f1* locus clearly continues throughout development, and is only modulated by autoregulation via relatively weak binding sites (Trieu et al., 2003; Trieu et al., 1999). Although no definitive conclusion can be drawn from these examples, it is plausible that different binding site affinities and site occupancy in target gene loci may be a general mechanism for controlling the extent of repression by Brn3a.

Context-dependent DNA binding by Brn3a. In general, tissue-specific transcription factors appear to have many more potential genomic binding sites than the observed number of downstream genes in a specific cell type. Most monomeric transcription factors have DNA recognition sites with a complexity equivalent to 4-5 unique base pairs, leading to an expected occurrence in vertebrate genomes of $>10^6$. In many cases, binding specificity is increased by homo- or heterodimerization, and in the case of the POU-domain family this is achieved by the integration of two DNA binding domains into a single transcription unit (Phillips and Luisi, 2000). Dimeric transcription factors recognize extended sites, but given the inherent flexibility in the DNA recognition properties of most of these proteins, the specificity of binding rarely

exceeds approximately 8 unique bases, yielding 10^4-10^5 predicted recognition sites. Consistent with these general predictions, we performed a compete search of mouse chromosome 4, which contains 5.94% percent of the genome, for the consensus Brn3a binding site ATAA$^{A}/_{T}$TAAT (Gruber et al., 1997), revealing 10,738 occurrences of this motif. Extrapolating from this search to the entire genome suggests that there are greater than 10^5 potential genomic Brn3a binding sites, and given that some variation in the consensus is permitted, this is likely to be an underestimate (calculations appear in online Supplemental Methods).

In contrast, recent micro- and macroarray studies which have analyzed global changes in gene expression in mice with mutations of neural transcription factors have identified much smaller sets of regulated genes. In the developing retina, microarray analysis has revealed 16 targets of the homeodomain factor Crx (Livesey et al., 2000), and 87 transcripts regulated by Brn3b (Mu et al., 2004). In the embryonic cerebellum, analysis of mice with mutations of the orphan receptor RORa revealed 36 significantly changed transcripts, using somewhat less stringent criteria than the retinal studies (Gold et al., 2003). In the developing trigeminal ganglion, 85 transcripts have been identified which exhibit >2-fold change in the absence of Brn3a (Eng et al., 2004). Although none of these studies achieved genome-wide coverage of potentially regulated genes, it is clear that the number of transcripts with altered expression is far fewer than the number of potential recognition sites for the transcription factors examined.

These observations imply that the regulatory activity of neuron-specific transcription factors will be highly dependent on the genomic context of their binding sites. In principle, this could be achieved by either of two mechanisms. First, a transcription factor might be bound to all of its genomic sites in relation to their intrinsic affinity, but have context-dependent transcriptional activity. In this model, the transcriptional activity of the bound factor might require that its binding site be located proximal to an active promoter, or adjacent to the binding sites for other factors required to make up an active enhancer/repressor unit. Second, the activity of a factor might be restricted at the level of DNA binding, such that only a fraction of its potential sites are occupied in vivo. This could also be due to a requirement for adjacent sites for other regulators that modulate binding, or because of alterations in chromatin structure which either exclude or permit interaction of the factor with its target sites. The examples cited here strongly suggest that the context-dependent activity of Brn3a is regulated at the level of DNA binding. This is suggested by the variability of site occupancy across the Brn3a locus, and confirmed by the observation that sites identified in the promoter regions of genes which are silent in sensory ganglia, but matched for sequence and in vitro affinity to known Brn3a-occupied sites, are not occupied in vivo.

Several determinants are likely to affect context-dependent DNA binding by Brn3a. Two features which strongly correlate with site occupancy in Brn3a target gene loci are the presence of sites in non-coding regions which show high conservation between the mouse and human genomes, and the localization of sites in regions of H3-

acetylated and H3 K4-dimethylated chromatin. Interspecies conservation in non-coding sequence is a recognized hallmark of regulatory domains (Xie et al., 2005). The regions of mouse-human homology identified by Brn3a binding include conservation of the consensus Brn3a binding sites, but also extend into adjacent regions, consistent with Brn3a binding within cis-regulatory modules which integrate inputs from multiple cellular transcription factors (Howard and Davidson, 2004; Wenick and Hobert, 2004).

Although less well-recognized, histone modifications may also useful markers of functional regulatory domains. H3 acetylation and H3 K4 dimethylation are predictive of regions of open chromatin structure with active transcriptional or regulatory activity (Margueron et al., 2005). Although these histone modifications are best characterized near the promoters of transcribed genes, there are several precedents for histone acetylation of distal regulatory regions (Bulger, 2005). In the present study, Brn3a binding in the *Pou4f1* locus, and in the loci of Brn3a target genes, occurs consistently in regions of H3-acetylated and H3 K4-dimethylated histones. In contrast, high-affinity Brn3a sites in deacetylated regions of the Brn3a locus and in silent, de-acetylated gene promoters show low occupancy. Although these examples are correlative, they suggest that increased H3 acetylation and H3 K4 dimethylation may be useful general markers for the regions of chromatin where Brn3a may interact.

Because Brn3a binding in vivo is highly associated with local histone H3 acetylation and K4-dimethylation, it is logical to consider whether these modifications

are partly the result of Brn3a binding. If Brn3a acted as a transcriptional activator at target bHLH genes and its own locus, it might induce these modifications through the recruitment of histone acetyltransferases and methyltransferases. However, the action of Brn3a as a repressor at these loci strongly suggests that Brn3a binding occurs only in the context of a pre-existing permissive state of chromatin modification, generated and maintained by other factors.

The correlation of Brn3a binding with histone modification is consistent with recent findings for other transcription factors. The most complete studies of this kind in vertebrates have characterized in vivo CREB binding to its recognition element CRE. In one study, serial analysis of chromatin occupancy (SACO) was used to carry out a genome-wide scan for CREB binding sites in forskolin-stimulated PC12 cells. The sequences bound by CREB in vivo were highly associated with CpG islands (Impey et al., 2004). Further work from the same group demonstrated that CREB occupies CRE-containing promoters in a cell type specific manner, and promoters of four genes with high CREB occupancy in stimulated PC12 cells also showed high H3 K4-dimethylation even in the unstimulated state, but unoccupied promoters were K4-demethylated (Cha-Molstad et al., 2004). In another a large scale ChIP study, myc binding to identified E-box elements demonstrated that E-boxes are much more likely to be occupied within 2kb of promoters than in intergenic regions (Fernandez et al., 2003). In a cell line inducibly expressing high levels of myc, the E-boxes highly bound in vivo were associated with H3 acetylation even prior to myc induction. Myc induction the resulted in additional acetylation at these sites. Thus for both CREB and

myc, pre-existing chromatin modifications may influence recognition of target sequences in vivo.

Taken together, these results suggest two important elements of context-dependent Brn3a target gene recognition in sensory neurons. First, Brn3a binding occurs in regions of extended interspecies conservation, suggesting an interaction between Brn3a and other transcription factors at these sites. Second, histone modifications may play a role in the modulation of Brn3a binding. Unlike the primary genomic sequence, histone modifications may be cell-type specific, and influence the interaction of transcription factors with chromatin in a cell-specific manner. In future studies, mapping genomic loci for cell-specific histone modifications, in combination with analysis of interspecies conservation, may significantly improve the prediction of functional transcription factor binding sites.

FIGURES

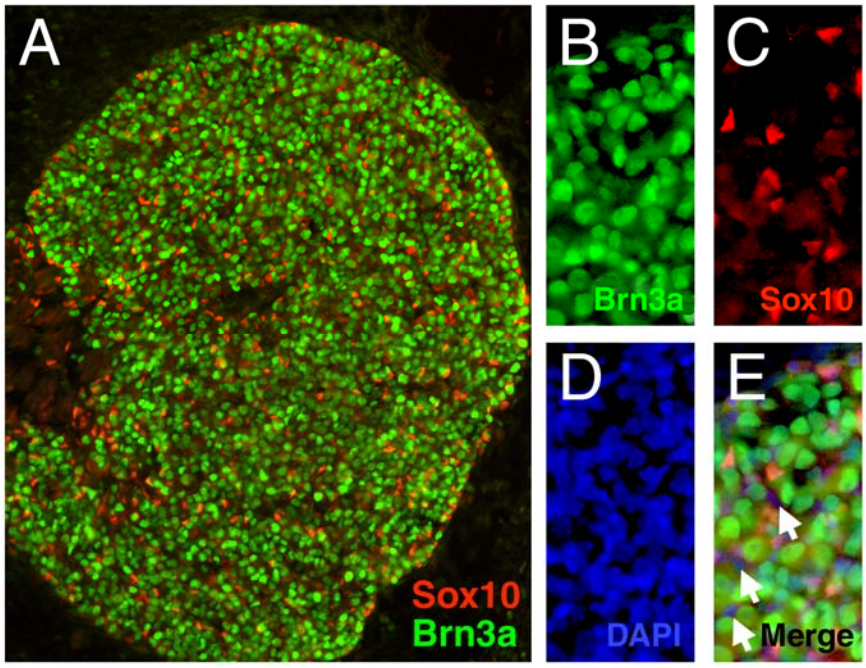


Figure 3.1. Cellular expression of Brn3a in the embryonic trigeminal ganglion. Guinea-pig anti-Sox10 (Maka et al., 2005) and rabbit anti-Brn3a were used to perform immunofluorescence as previously described (Fedtsova and Turner, 1995).
(A) In the E13.5 trigeminal, the majority of cells are Brn3a-expressing neurons. The relatively small population of differentiating glia present at this stage are identified by small nuclei and the expression of Sox10.
(B-E) An enlarged view shows mutually exclusive cellular expression of Brn3a and Sox10 (boxed area in A). Occasional cells express neither antigen (arrows, E), and may represent nucleated blood cells.

Figure 3.2. Brn3a is a direct regulator of the *NeuroD4* gene in the embryonic trigeminal ganglion.
(A) ChIP analysis of Brn3a binding to the *NeuroD4* locus in E13.5 ganglia. Sixty-three primer pairs were used in real-time PCR assays to screen the locus from -20kb to +20kb relative to the transcriptional start site (online Table S3). The fold enrichment of the selected versus unselected chromatin was normalized to a control value of one (C on Y-axis), based on the signal the *Alb-1* locus (Materials and Methods). The average enrichment from three selection experiments using independent pools of ganglia is shown for each primer pair. Sequence analysis of the regions of maximum ChIP selection revealed consensus Brn3a binding sites at –12996 and +4410 relative to the start of transcription (vertical red lines). Differences between the ChIP selection peaks at -12996 and +4410 and *Alb-1* locus control values were highly significant ($p=0.003$ and $p=0.002$, respectively, Materials and Methods).
(B) Kinetic EMSA assays were used to determine the relative stability of Brn3a binding to the sites identified by ChIP. In these assays, Brn3a protein was allowed to bind radiolabeled oligonucleotides, and the complexes were then incubated in the presence of an excess of specific competitor oligonucleotide (Materials and Methods). The amount of complex remaining after 5 and 20 minutes was used to determine the half-life of each complex. The relative first-order dissociation rates of the Brn3a-DNA complexes observed in these assays allow a rapid approximate comparison of relative site affinity (Gruber et al., 1997; Trieu et al., 1999). Multiple complexes were observed due to size heterogeneity of the expressed protein and multimeric binding to overlapping recognition sites (arrows). NP, no Brn3a protein; NC, no competitor; 5 min, 5 minute incubation with competitor, 20 min, 20 minute incubation with competitor.
(C) Oligonucleotide probes containing a previously determined optimal monomeric consensus binding site (Cons, Gruber et al., 1997) and the Brn3a recognition sequences in the *NeuroD4* locus were used to demonstrate the interaction of multimeric binding of Brn3a to the *NeuroD4* sites. Supershifts with Brn3a antibody (Ab) demonstrate that all major complexes contain Brn3a. NP, no Brn3a protein; B3, Brn3a protein containing cell lysate added; Ab, cell lysate plus Brn3a antiserum; Mono, monomeric Brn3a complexes; Multi, multimeric Brn3a complexes.

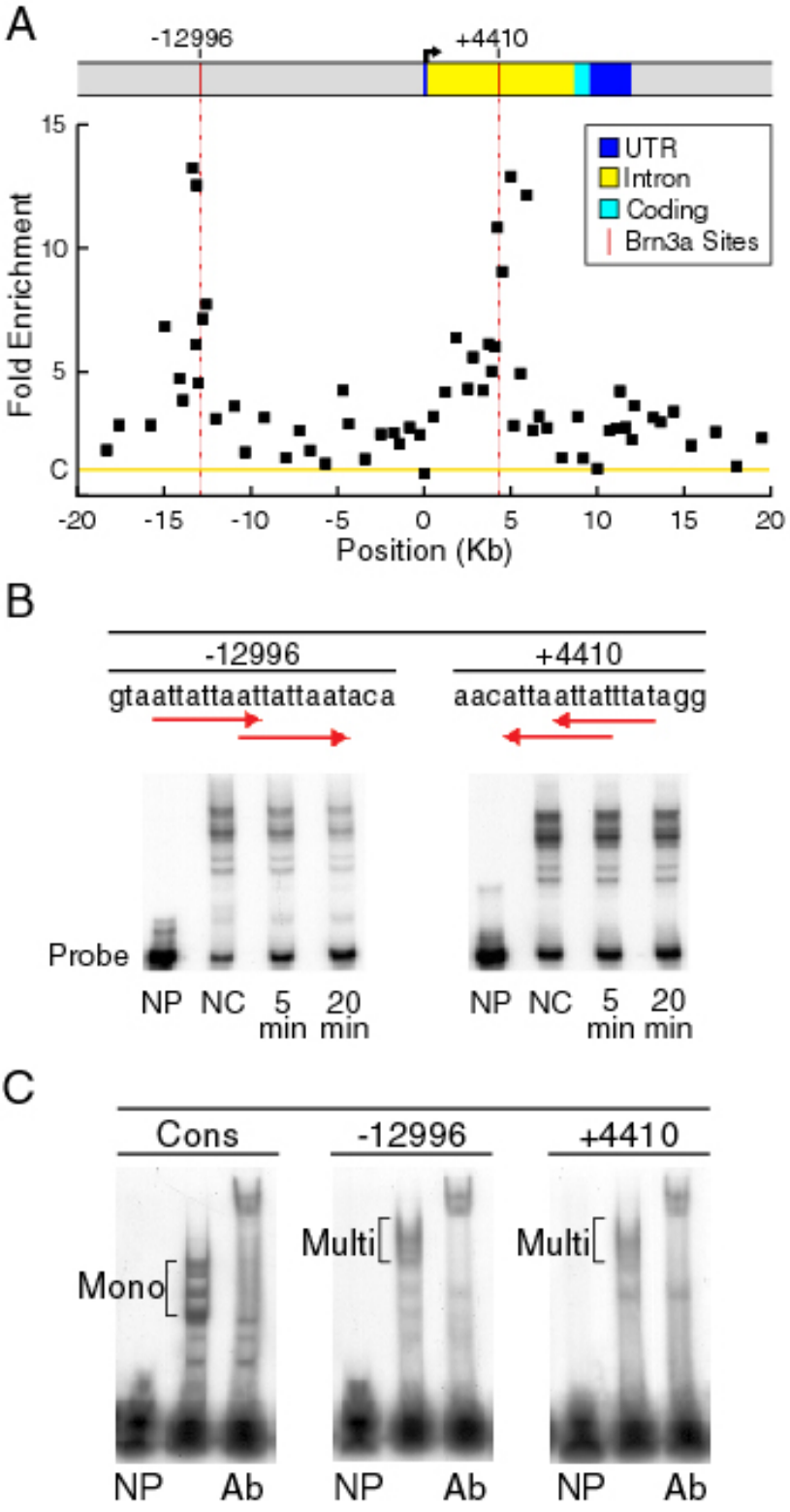

A
-12996
+4410
UTR
Intron
Coding
Brn3a Sites
Fold Enrichment
15
10
5
C
-20
-15
-10
-5
0
5
10
15
20
Position (Kb)
B
-12996
+4410
gtaattattaattattaataca
aacattaattatttatagg
Probe
NP
NC
5 min
20 min
NP
NC
5 min
20 min
C
Cons
-12996
+4410
Mono
Multi
Multi
NP
Ab
NP
Ab
NP
Ab

Figure 3.3. ChIP analysis of Brn3a binding to the *NeuroD1* and *Msc* loci in E13.5 trigeminal ganglia.
(A) In vivo binding of Brn3a to the *NeuroD1* locus. A peak of enrichment in a region of the 3' UTR conserved between the mouse and human genomes includes a Brn3a consensus site at +2973 (difference from controls, p=0.008). The region extending from –16 kb to –11 kb consists of repetitive sequence and was not included in the analysis. ChIP primer pairs used for the *NeuroD1* locus appear in online Table S4. (B) Brn3a ChIP analysis of the *Msc* locus from -20kb to +20 kb relative to the start of transcription reveals no significant in vivo binding of Brn3a. Average enrichment values from three independent ChIP assays are shown for both loci. Primer pairs used in ChIP assays of the *Msc* locus appear in online Table S5.

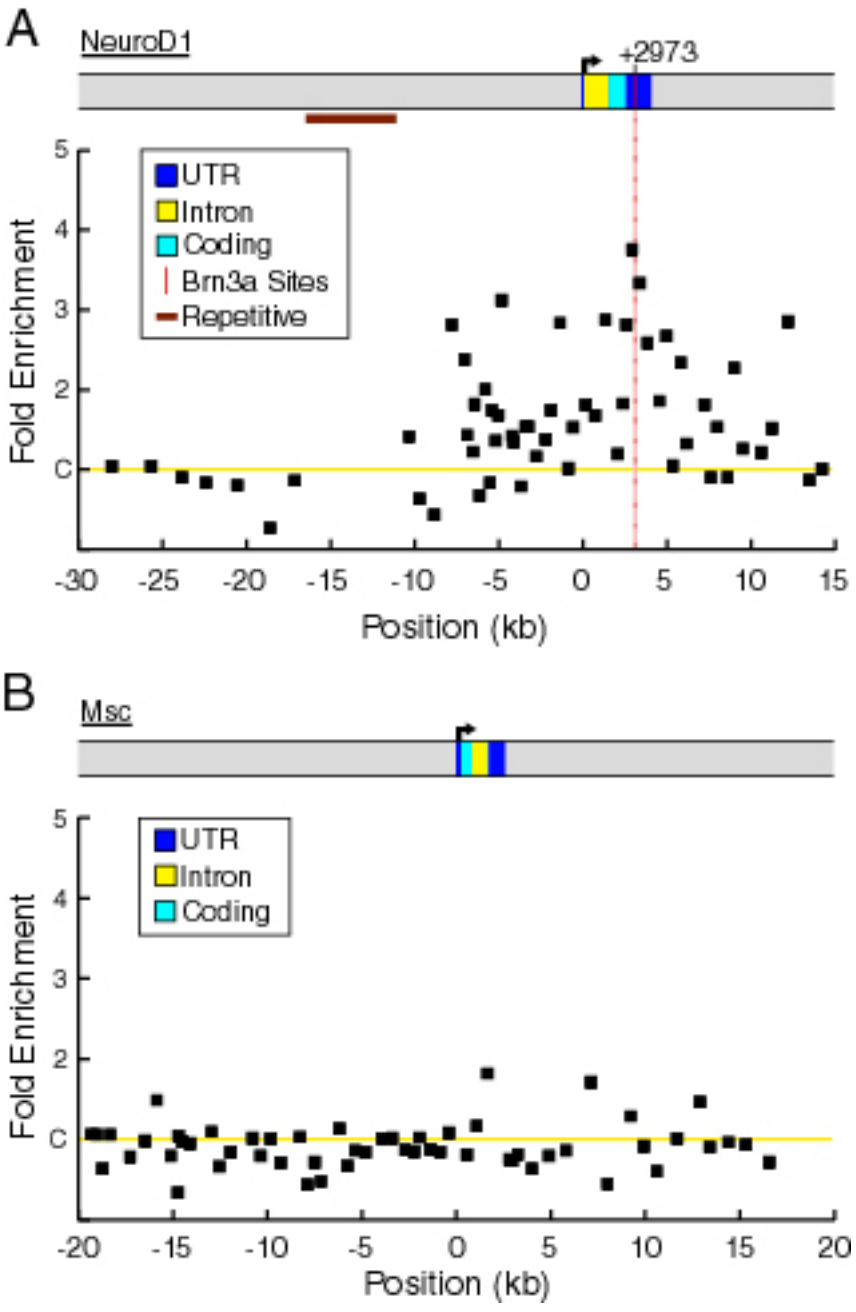

A
NeuroD1
+2973
UTR
Intron
Coding
Brn3a Sites
Repetitive
Fold Enrichment
Position (kb)
B
Msc
UTR
Intron
Coding
Fold Enrichment
Position (kb)

Figure 3.4. Brn3a binding to the *Pou4f1* locus in vitro and in embryonic trigeminal ganglia.
(A) Complex stability screening of a 188 kb BAC encompassing the *Pou4f1* locus. BAC DNA was sheared by sonication and selected using recombinant Brn3a protein (Materials and Methods). Fragments were ligated into a cloning vector, and 128 independent clones were sequenced. The histogram indicates the number of fragments identified within each 2000 base pair interval of the BAC. In nearly every case, BAC regions identified by multiple clones contained Brn3a consensus binding sites, as indicated on the map of the *Pou4f1* locus. In a prior study (Trieu, et al., 1999), lower stringency screening of a smaller region of the Pou4f1 locus identified Brn3a binding sites of moderate affinity in the autoregulatory enhancer region (-5,633). The higher stringency screening of a much larger genomic clone shown here did not identify these sites, but revealed several higher affinity sites in other regions of the locus.
Within the region encompassed by this BAC, two transcripts of unknown function have been identified, 2610206B13Rik and D130079A08Rik. These transcripts extend from -54,719 to -9,586 and +8,795 to +23,049 relative to the start of Brn3a transcription, respectively, and are transcribed on the same strand. D130079A08Rik was identified from ESTs cloned from mouse spinal ganglion, and it is unclear whether these sequences represent an independently transcribed gene, or alternate *Pou4f1* transcripts.
(B) Kinetic EMSA analysis of Brn3a binding to identified Brn3a binding sites. NP, no Brn3a protein; NC, no competitor; 5 min, 5 minute incubation with competitor; 20 min, 20 minute incubation with competitor.
(C) ChIP analysis of the *Pou4f1* locus using 103 primer pairs to screen a ~160 kb genomic region for in vivo binding of Brn3a. Multiple areas of enrichment were identified including the Brn3a autoregulatory site cluster at -5,633 (difference from controls, $p=0.003$) and four areas 3' to the transcription unit at +7,093 ($p=0.0007$), +43,473 ($p=0.007$), +51, 848 ($p<0.0001$) and +78,456 ($p=0.013$). Binding at -58,080 did not reach significance ($p>0.01$). Data are average values from three ChIP assays using independent pools of E13.5 trigeminal ganglia. Primer pairs used in ChIP assays of the *Pou4f1* locus appear in online Table S6.
(D) A comparison of in vitro affinity and in vivo binding suggests that Brn3a binding sites 5' to the transcription unit are occupied less often than expected based on affinity, sites in the vicinity of the autoregulatory enhancer are occupied more often than expected, and sites 3' to the transcription unit are occupied approximately in proportion to affinity.

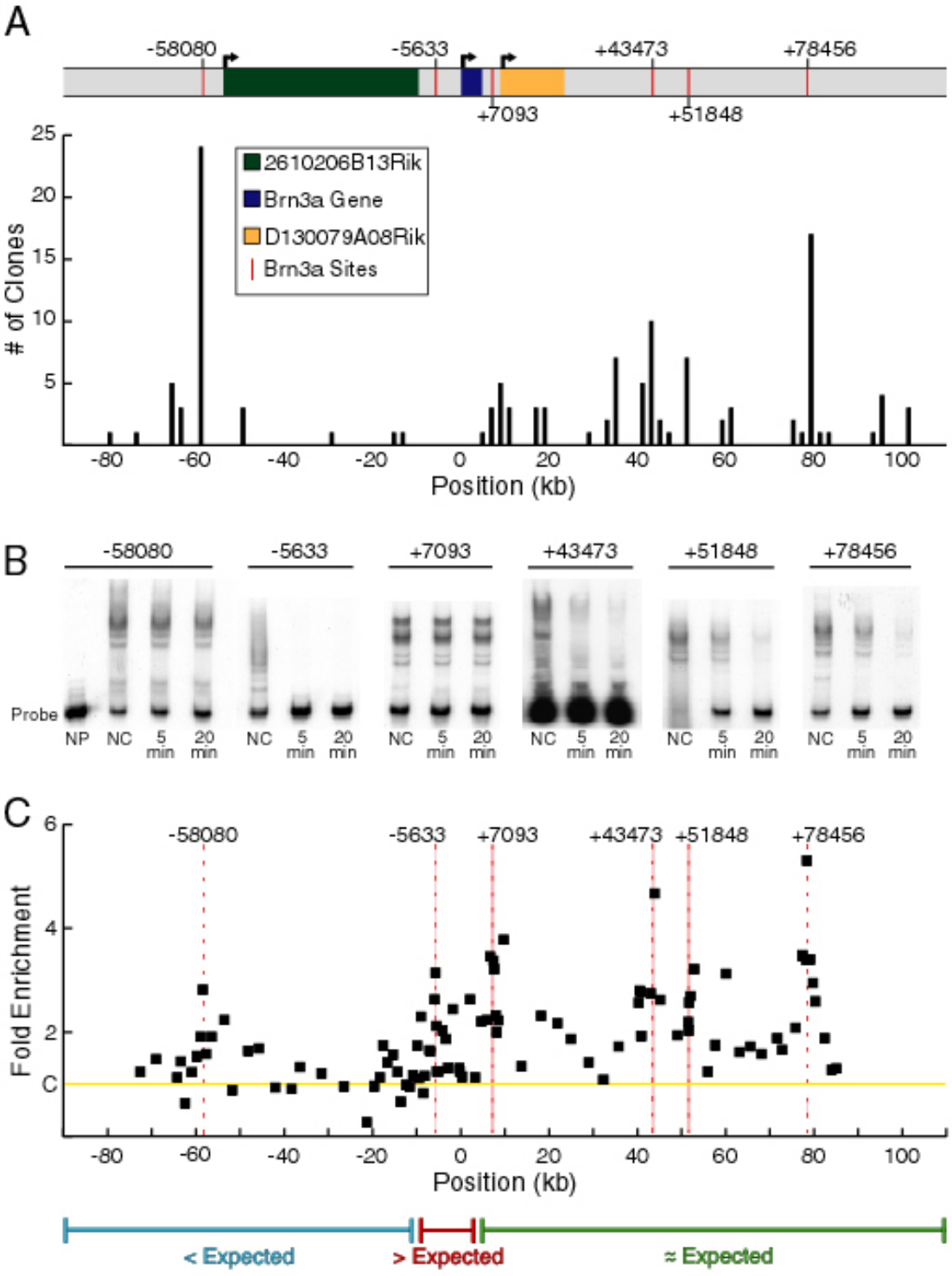

A
-58080
-5633
+43473
+78456
+7093
+51848
2610206B13Rik
Brn3a Gene
D130079A08Rik
Brn3a Sites
# of Clones
Position (kb)
B
-58080
-5633
+7093
+43473
+51848
+78456
Probe
NP
NC
5 min
20 min
C
Fold Enrichment
Position (kb)
< Expected
> Expected
≈ Expected

Figure 3.5. Markers of chromatin modification at the *Pou4f1* locus.
(A) Locus-ChIP assays were performed for acetylated histone H3 over a ~160 kb region encompassing the *Pou4f1* locus in E13.5 trigeminal ganglia. Data show average enrichment from two sets of assays using separate pools of ganglia. H3 acetylation is increased within a ~15 kb region including the autoregulatory domain, transcriptional start site and primary transcript. The area of enrichment at -53,620 is closely associated with the transcription start site of an adjacent gene, 2610206B13Rik, and does not coincide with the Brn3a binding site at -58,080 (vertical red bar, dashed line). The fold enrichment of the selected versus unselected chromatin was normalized to a control value of one (C on Y-axis), based on the signal from the *Alb-1* locus (Materials and Methods).
(B) Detailed map of histone H3 acetylation within a 25 kb region containing the *Pou4f1* transcription unit. A region of increased acetylation encompasses the Brn3a autoregulatory site cluster at –5,633, the transcription start site, and extends beyond the 3' end of the transcript to include the Brn3a binding site at +7,093. A VISTA plot of mouse-human homology within the locus indicates that regions of enhanced acetylation are correlated with areas of conserved sequence.
(C) Detailed map of histone-H3 acetylation from a 4 kb region containing the in vivo Brn3a binding site at +78,456. The region of moderately enhanced acetylation associated with this site also shows sequence conservation between the mouse and human genomes.
(D) Locus-ChIP of the *Pou4f1* locus using antibodies to dimethylated H3-K4. The patterns of H3 acetylation and H3-K4 dimethylation are highly congruent.

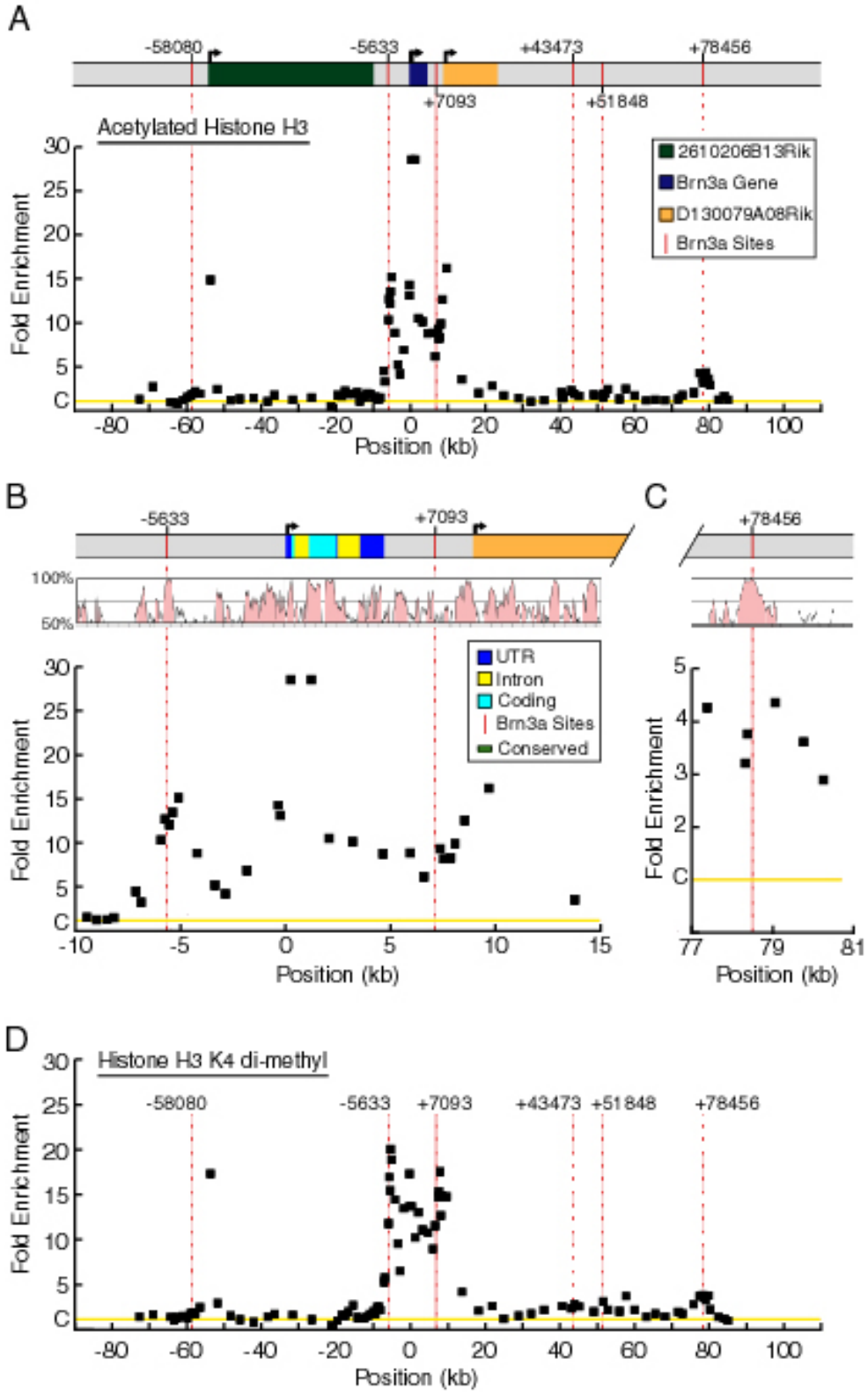

A
-58080
-5633
+43473
+78456
+7093
+51848
Acetylated Histone H3
2610206B13Rik
Brn3a Gene
D130079A08Rik
Brn3a Sites
Fold Enrichment
Position (kb)
B
-5633
+7093
100%
50%
UTR
Intron
Coding
Brn3a Sites
Conserved
C
+78456
D
Histone H3 K4 di-methyl

Figure 3.6. Acetylated histone H3 ChIP assays of the *NeuroD4* and *NeuroD1* loci. (A) Map of histone H3 acetylation of the *NeuroD4* locus from –20 kb to +20 kb relative to the start of transcription, revealing three peaks of acetylation which encompass the start of transcription and the two identified in vivo Brn3a binding sites. (B) Histone H3 acetylation of the *NeuroD1* locus. A broad peak of acetylation encompasses the *NeuroD1* transcription unit extending from approximately -5 kb to +7 kb relative to the transcription start site, including the in vivo Brn3a binding site at +2,973. (A) and (B) both show average enrichment values from two ChIP assays using separate pools of E13.5 trigeminal ganglia. In vivo Brn3a binding sites are represented by vertical red bars.

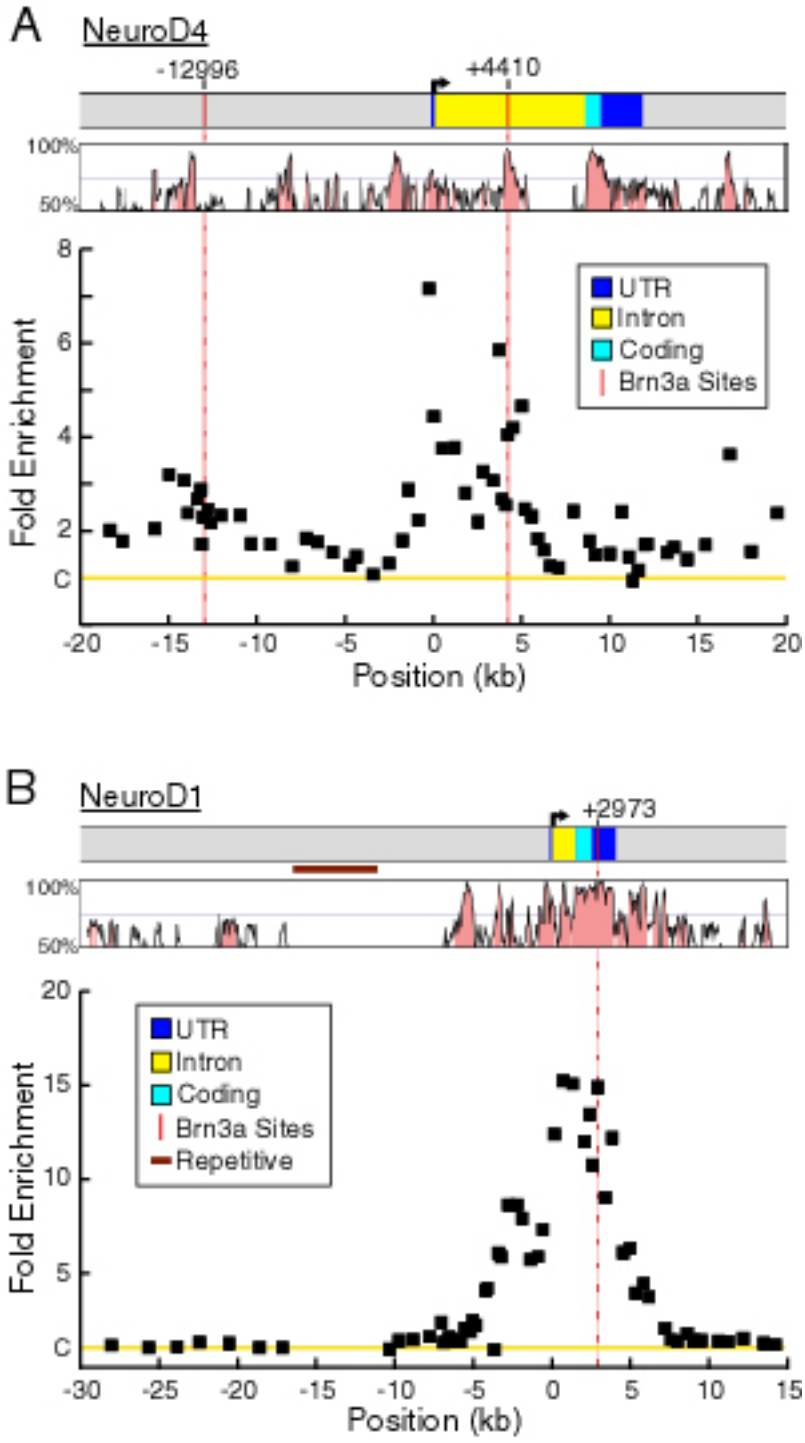
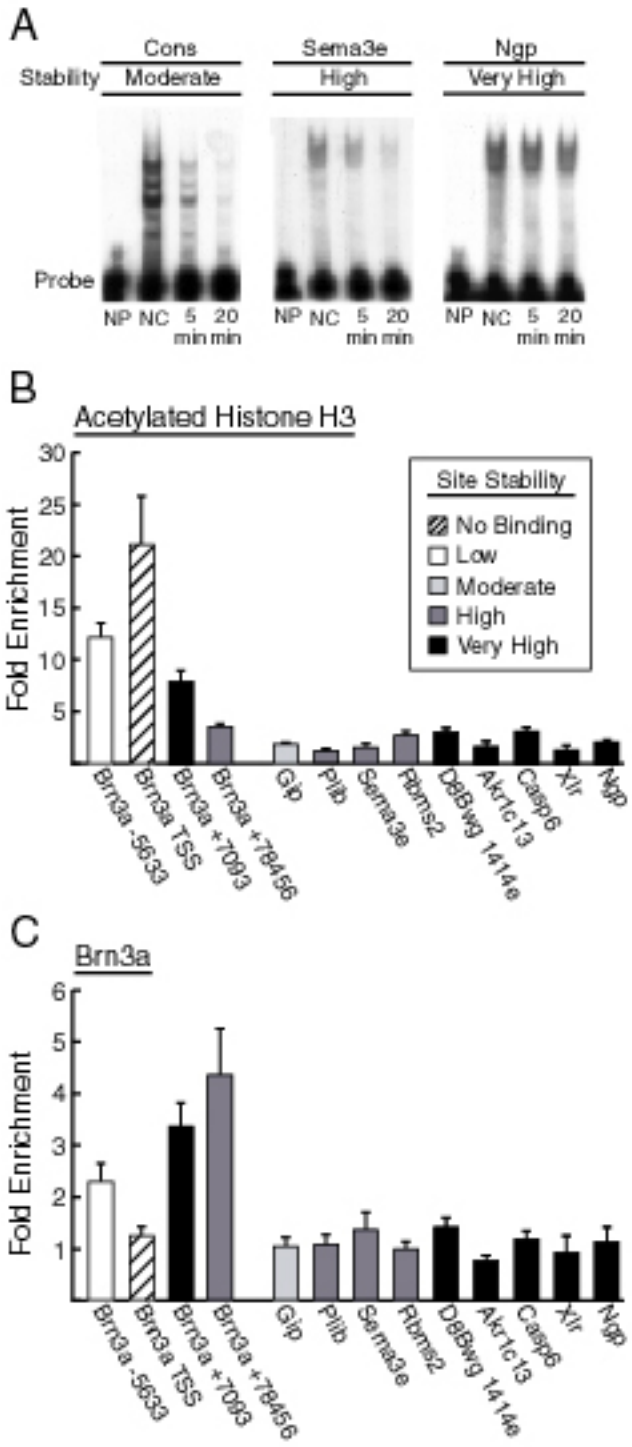

A
NeuroD4
-12996
+4410
100%
50%
Fold Enrichment
8
6
4
2
C
UTR
Intron
Coding
Brn3a Sites
-20
-15
-10
-5
0
5
10
15
20
Position (kb)
B
NeuroD1
+2973
100%
50%
Fold Enrichment
20
15
10
5
C
UTR
Intron
Coding
Brn3a Sites
Repetitive
-30
-25
-20
-15
-10
-5
0
5
10
15
Position (kb)

Figure 3.7. Analysis of Brn3a binding sites identified in the promoters of genes not expressed in the developing trigeminal ganglion.
(A) Representative kinetic EMSA results for a Brn3a consensus site, and Brn3a sites identified in the *Sema3e* and *Ngp* promoters. For all sites tested, dissociation half-lives of Brn3a-DNA complexes were estimated based on first order kinetics (Materials and Methods) and were categorized as low, $t_{1/2}$=0-2 minutes; moderate, $t_{1/2}$=2-4 minutes; high, $t_{1/2}$=4-15 minutes; and very high, $t_{1/2}$> 15 minutes. A single consensus Brn3a binding site exhibits moderate stability in this assay because the sites with the highest stability are characterized by multimeric binding. NP, no protein; NC, no competitor. Sites tested in (A-C) were identified within 2 kb of the transcriptional start of the following genes: gastric inhibitory polypeptide, *Gip*; placental lactogen-I beta, *Plib*; semaphorin 3E, *Sema3e*; RNA binding motif, single stranded interacting protein 2, *Rbms2*; transcript of unknown function, D8Bwg1414e; aldo-keto reductase family 1, member C13, *Akr1c13*; caspase 6, *Casp6*; x-linked lymphocyte-regulated complex, *Xlr*; and neutrophilic granule protein, *Ngp*. Gene map locations appear in online Table S1.
(B) ChIP analysis of histone H3 acetylation at silent promoters containing Brn3a binding sites. Fold enrichment for each promoter was determined using one or two real-time PCR primer pairs hybridizing near the binding site identified in the EMSA assays. Assays for sites in the *Pou4f1* locus (Figure 3.5A) are shown for comparison.
(C) ChIP analysis of Brn3a binding at silent promoters. Assays for sites in the *Pou4f1* locus (Figure 3.4C) are shown for comparison. No significant Brn3a binding was observed at any of the promoters of genes not expressed in the trigeminal. For (B) and (C) the enrichment values for three ChIP assays on independent samples were used for each primer pair. Enrichment values for multiple primer pairs targeting the same promoter were averaged, and bars show mean +SEM of three or six assays.


A
Cons
Sema3e
Ngp
Stability
Moderate
High
Very High
Probe
NP NC 5 min 20 min
B
Acetylated Histone H3
Fold Enrichment
Site Stability
No Binding
Low
Moderate
High
Very High
Brn3a -5693
Brn3a TSS
Brn3a +7093
Brn3a +78456
Gip
Plib
Sema3e
Rbms2
D8Bwg 1414e
Akr1c13
Casp6
Xlr
Ngp
C
Brn3a
Fold Enrichment

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ACKNOWLEDGEMENTS

We would like to thanks Drs. Jane Johnson, Michael Wegner and Sam Pfaff for helpful comments on the manuscript. We would also like to thank Dr. Michael Wegner for the generous gift of Sox10 antisera. Supported in part by Department of Veterans Affairs MERIT funding, and NIH awards HD33442 and MH065496 (E.E.T). E.E.T. is a NARSAD Investigator.

The text of Chapter Three is a reprint of the material as it appears in Developmental Biology, 2007, Lanier J, Quina LA, Eng SR, Cox E, Turner EE. I was the primary researcher, and the co-authors contributed to the research that forms the basis of this chapter. My contributions to the work included the bioinformatic analysis, chromatin immunoprecipitation assays, real-time PCR analysis, and electrophoretic mobility shift assays.

IV

POU-domain Factor Brn3a Regulates Both Distinct and Common Programs of Gene Expression in the Spinal and Trigeminal Sensory Ganglia

ABSTRACT

Background: General somatic sensation is conveyed to the central nervous system at cranial levels by the trigeminal ganglion (TG), and at spinal levels by the dorsal root ganglia (DRG). Although these ganglia have similar functions, they have distinct embryological origins, in that both contain neurons originating from the neural crest, while only the TG includes cells derived from the placodal ectoderm.

Results: Here we use microarray analysis of E13.5 embryos to demonstrate that the developing DRG and TG have very similar overall patterns of gene expression. In mice lacking the POU domain transcription factor Brn3a, the DRG and TG exhibit many common changes in gene expression, but a subset of Brn3a target genes show increased expression only in the TG. In the wild-type TG these Brn3a-repressed genes are silent, yet their promoter regions exhibit histone H3-acetylation levels similar to constitutively transcribed gene loci. This increased H3-acetylation is not observed in the DRG, suggesting that chromatin modifications play a role in cell-specific target gene regulation by Brn3a.

Conclusion: These results demonstrate that one developmental role of Brn3a is to repress potential differences in gene expression between sensory neurons generated at different axial levels, and to regulate a convergent program of developmental gene expression, in which functionally similar populations of neurons are generated from different embryological substrates.

BACKGROUND

The generation of cellular diversity in the developing vertebrate nervous system is one of the most complex problems in biology, and a large number of transcription factors have been identified that orchestrate neurodevelopment and regulate the molecular identity of neurons. Perhaps the best-studied systems for the establishment of neuronal phenotypes in vertebrates are the primary input (sensory) and output (motor) pathways of the peripheral sensory ganglia, spinal cord, and brainstem. In these areas, several key transcriptional regulators have been identified, many of which are members of the basic-helix-loop-helix (bHLH) and homeodomain transcription factor classes (Anderson 1999; Shirasaki and Pfaff 2002).

The peripheral sensory nervous system is organized anatomically according to the nature of the external information reported to the central nervous system (CNS). The sensory modalities of pain, touch, temperature and proprioception are transduced by sensory neurons innervating the skin and musculoskeletal structures, and are referred to as general somatic sensation. Peripheral sensory neurons also convey the senses of taste and hearing, and visceral sensation, which reports the state of the internal organs. At spinal levels, general somatic sensation is conveyed by the dorsal root or spinal ganglia (DRG), while in the anterior head and face, these sensory modalities are mediated by the trigeminal ganglion (TG).

Surprisingly, in spite of the similar functions of the DRG and TG, these ganglia have rather different embryological origins. The DRG are formed entirely from spinal neural crest. In contrast, the TG is formed in part from the ophthalmic and

maxillo-mandibular placodes originating in the surface ectoderm, as well as cranial neural crest cells derived from rhombomere 2, which migrate and condense to form the ganglion (Le Douarin and Smith 1988; Baker and Bronner-Fraser 2001). The trigeminal system also includes a population of sensory neurons that reside outside the anatomical ganglion, the mesencephalic trigeminal (mesV), the exact origin of which is still somewhat controversial (Hunter, Begbie et al. 2001; Lazarov 2002). The developmental mechanisms that lead to the differentiation of functionally similar populations of neurons from these different embryological sources are not well understood.

Transcriptional regulators of sensory development may be broadly divided into early factors that are essential for neurogenesis, pan-sensory factors that begin to be expressed around the time of cell-cycle exit, and late factors that characterize specific sensory subtypes. In mice, the proneural bHLH factors Ngn1 and Ngn2 are expressed transiently from embryonic day 8.5 in cranial sensory precursors and have been shown to have a crucial role in neurogenesis (Fode, Gradwohl et al. 1998; Ma, Chen et al. 1998; Ma, Fode et al. 1999). Around the time of ganglion condensation and cell cycle exit, beginning at E9.5–10.5, nearly all sensory neurons at both spinal and cranial levels co-express the homeodomain transcription factors Brn3a and Islet1 (Anderson 1999; Fedtsova, Perris et al. 2003). Later in development, factors associated with the development of specific sensory subtypes include the runt family factors Runx1 and Runx3, the variant homeodomain protein Prrxl1/DRG11, and the Ets family member

Etv1/Er81 (Anderson 1999; Arber, Ladle et al. 2000; Chen, Rebelo et al. 2001; Levanon, Bettoun et al. 2002; Chen, Broom et al. 2006; Kramer, Sigrist et al. 2006).

Studies of Brn3a knockout mice have shown that this factor is required for correct axon growth, target innervation, and survival of TG and DRG neurons (McEvilly, Erkman et al. 1996; Xiang, Gan et al. 1996; Huang, Zang et al. 1999; Eng, Gratwick et al. 2001). Microarray studies of the developing TG of Brn3a null mice have shown that Brn3a is required for a complex program of sensory gene expression (Eng, Lanier et al. 2004). In the present study, we examine the role of Brn3a in sensory neurogenesis at truncal and cranial levels. In normal mice, the developing DRG and TG have very similar patterns of global gene expression. The loss of Brn3a expression in the developing DRG leads to marked changes in the expression of specific neurotransmitters, receptors, developmental regulators, mediators of signal transduction, and transcription factors. Many of these changes are conserved between the TG and DRG of Brn3a knockout mice, but certain transcripts are markedly increased only in the cranial ganglia. The promoter regions of these normally silent but differentially regulated genes are hyperacetylated only in the TG, which may indicate a latent state of 'expressability' that can differ between spinal and cranial levels. Thus, a key developmental role of Brn3a may be to repress potential differences in gene expression between developing DRG and TG neurons, and thus promote the generation of functionally similar populations of neurons from different embryological sources.

RESULTS

Global gene expression is highly conserved in sensory neurons from different axial levels. To begin to compare the molecular program of sensory neuron development at spinal and cranial levels, we performed global analysis of gene expression in the developing DRG and TG. E13.5 was chosen for this comparison because, at this stage, most of the neurons of the DRG and TG have exited the cell cycle and have begun to express definitive markers of neurogenesis. Because sensory neurons have conserved functions at spinal and cranial levels, we hypothesized that the global pattern of gene expression in the DRG would be much more similar to the TG than to other neural tissues. Figure 4.1 illustrates a comparison of gene expression in the DRG to a replicate analysis from the same tissue, to the TG, and to the embryonic neocortex. These results show a high degree of overall similarity in gene expression between the sensory ganglia from different axial levels, and confirm that there is much greater divergence between the patterns of gene expression in the DRG and the developing cerebral cortex.

Overall, only a very small fraction of the approximately 44,000 transcripts assayed by the array showed profound differences in expression between the DRG and TG (Table 4.1). Significantly more transcripts were uniquely expressed in the DRG, and seven out of ten of the transcripts with the highest relative expression in the DRG encoded Hox transcription factors, an expected finding given the axial restriction of Hox expression. Several of the other differentially expressed genes revealed in the microarray analysis could be validated by *in situ* hybridization (online Figure S1),

although some differences appear to represent anomalous findings that may be related to factors such as high expression in peripheral glia (Ednrb, Sostdc1).

Aside from the Hox factors, the transcript with the greatest relative expression in the DRG was Etv1, an Ets-family transcription factor expressed in 1a muscle spindle afferents (Arber, Ladle et al. 2000; Patel, Kramer et al. 2003). Immunofluorescence for Etv1 expression in the E13.5 DRG confirmed expression in sensory neurons of large and intermediate size, whilst expression was entirely absent in the TG at this stage (Figure 4.2a, b). In contrast, expression of Runx3, an earlier and more general marker of proprioceptive neurons (Arber, Ladle et al. 2000; Levanon, Bettoun et al. 2002), was noted in both the DRG and TG at E13.5 (Figure 4.2c, d).

By E16.5, Etv1 expression could be detected in some neurons of the TG, as well as the DRG. In the DRG, one population of Etv1-expressing neurons displayed large soma and nuclei consistent with 1a proprioceptors. However, in the TG nearly all of the Etv1-expressing neurons were of intermediate size, and large Etv1-positive neurons consistent with 1a proprioceptors were rare. In both the DRG and the TG, this intermediate-sized and later-developing population of Etv1-expressing sensory neurons could be further distinguished from the 1a proprioceptors by the co-expression of Islet2 (Figure 4.2e, f).

One distinguishing feature of the TG is that a subset of neurons that are functionally part of the trigeminal system, the mesV, is located within the CNS. Examination of the mesV in late gestation revealed large sensory neurons positive for Etv1. These neurons co-expressed a *tauLacZ* transgene integrated into the Brn3a

locus, and Brn3a is a known marker of the mesencephalic trigeminal (Hunter, Begbie et al. 2001). The position of these neurons in the caudal midbrain is consistent with the role of some of these neurons as muscle spindle afferents for the muscles of mastication (Raappana and Arvidsson 1993). Thus, the exclusive expression of Etv1 in the DRG but not the TG at E13.5 appears to result from a population of $Etv1^{+}$, $Islet2^{-}$ proprioceptive neurons that are present in the DRG but are largely restricted to the mesV at the cranial level.

Brn3a regulates multiple downstream targets in the DRG. To understand the developmental programs regulated by Brn3a at spinal versus cranial levels, we next analyzed global gene expression in E13.5 DRG from Brn3a mutant embryos and wild-type controls. Tables 4.2 and 4.3 summarize the transcripts most increased and decreased at E13.5 in the DRG of Brn3a knockout mice, and a more complete list appears in online Supplemental Tables. *In situ* hybridization and immunofluorescence were used to verify altered expression of several of the target genes (Figures 4.3 and 4.4), with an emphasis on newly identified Brn3a targets and genes for which expression has not been previously described in the sensory ganglia.

Most of the Brn3a-regulated genes of known function have specific roles in neurotransmission, axonogenesis/synaptogenesis, signal transduction, regulation of developmental pathways, or transcription. Among mediators of neurotransmission, multiple glutamate receptors (Gria3, Gria4, Grik1), a GABA transporter (Slc6a1), a serotonin receptor (Htr3a) and the neuropeptide somatostatin are increased, while a GABA receptor (Gabra2) and neuropeptides with specific sensory roles (Galanin,

Adcyap1/PACAP) are decreased. Also reduced is latexin, a secreted carboxypepidase inhibitor with a role in nociception (Jin, Ishida et al. 2006).

Profound changes were also observed in genes that encode known or potential mediators of sensory neurogenesis, and axon growth or guidance. Increased transcripts in this class included the cell adhesion molecule Chl1, which has a known role in the growth of cortical dendrites (Demyanenko, Schachner et al. 2004), and Nel-like 2, an epidermal growth factor (EGF)-repeat containing factor with a previously demonstrated role in sensory development (Nelson, Claes et al. 2004). Increased expression was also noted for several molecules with known or likely roles in axon guidance, including Disabled1, Sema3c, Sema3d, f-spondin, Cadherin22, Protocadherin 8, Protocadherin 17, Dcc, and NetrinG1. Decreased expression was noted for advillin, an actin binding protein that is a known mediator of sensory neurite outgrowth (Shibata, Ishii et al. 2004), as well as Ncam2 and Eph receptor A7. These changes suggest that the profound defects observed in the sensory axons of Brn3a null mice (Eng, Gratwick et al. 2001) result from the derangement of a coordinated program of expression of mediators of axon growth and guidance, rather than from a single molecular lesion.

Loss of Brn3a also resulted in significant changes in the expression of other transcription factors. Notably, deletion of Brn3a effectively resulted in a triple-knockout of the Pou4 homeodomain class, because Pou4f2 (Brn3b) expression is nearly eliminated, and Pou4f3 (Brn3c) expression is greatly reduced in Brn3a knockout ganglia. Marked decreases were also observed in specific markers of sensory

neuron subclasses, including Prrxl1/DRG11, Runx1 and Runx3. In contrast, specific bHLH genes, including those encoding NeuroD1, NeuroD6, Msc, and Nhlh2, were increased, and, perhaps consistent with this, the inhibitory bHLH factor Id1, which can act to oppose the action of neurogenic bHLH genes, was decreased. The significance of some of these changes in the hierarchy of gene regulation in developing sensory neurons is discussed below.

Loss of Brn3a expression resulted in increased expression of essentially all of the anterior *HoxA*, *HoxB* and *HoxC* genes, and the changes reached statistical significance ($p < 0.005$ or $p > 0.995$, Materials and Methods) for seven genes in this group (Figure 4.5). The posterior *Hox* genes were expressed at much lower levels than the anterior factors, as expected for brachial-level DRG, and exhibited either unchanged or decreased expression in the knockout. Members of the HoxD cluster were not significantly expressed in the DRG (data not shown), with the exception of HoxD1, which, unlike the other anterior *Hox* gene products, exhibited decreased expression in Brn3a null DRG, and was also the only *Hox* gene product expressed at significant levels in the TG (see online Supplemental Tables) (Eng, Lanier et al. 2004).

In prior studies, we have shown that Brn3a regulates its own expression via direct negative feedback, and that, in heterozygous Brn3a knockout mice, partial relief of this negative autoregulation results in increased expression of the intact allele, resulting in nearly complete gene dosage compensation. To determine whether gene dosage compensation is also present at spinal levels, we examined the levels of all

increased and decreased transcripts in Brn3a$^{+/+}$, Brn3a$^{+/-}$ and Brn3a$^{-/-}$ ganglia (see online Figure S2 and Supplemental Tables). In each case, the mean effect of the loss of one *Brn3a* allele on target gene expression was 16% to 19% of the effect of the loss of both alleles, compared to the 50% change that would be expected without any compensatory mechanism, indicating significant but incomplete compensation for gene dosage.

A subset of Brn3a targets are unique to the cranial sensory ganglia.

Comparison of the regulatory targets of Brn3a in the developing DRG with our prior analysis of the TG (Eng, Lanier et al. 2004) suggests that there are many downstream genes in common between these ganglia. However, results from the more advanced microarray used in the present study (Affymetrix 430) cannot be compared directly to the prior TG data derived from an older array (Affymetrix U74v2). To make such a comparison, we performed an additional analysis of these E13.5 DRG samples with the U74v2 array. We also updated the interpretation of the results for the TG U74v2 dataset using annotations from Build 34 of the mouse genome. This analysis confirmed that the DRG and TG of E13.5 Brn3a knockout mice have many conserved changes in gene expression (see online Supplemental Tables). Transcripts increased in both the DRG and TG of Brn3a knockout mice include the bHLH transcription factors musculin and NeuroD1, the neurotransmitters/receptors somatostatin, CCK-receptor A, 5HT-receptor 3A, and GABA-transporter 1, and the growth factor FIGF/VEGF-D. Conserved decreases include the transcription factors Hmx1, Runx1 and basonuclin, the peptides galanin and PACAP, as well as advillin and latexin.

Although the majority of the downstream targets of Brn3a were conserved in the DRG and TG, a subset of genes was uniquely regulated at each axial level. The most profound differences were observed for a set of genes markedly increased in the TG of Brn3a knockout embryos, and unchanged in the DRG (Figure 4.6). These differentially regulated genes include those encoding the transcription factors Tcfap2b (Ap2β), NeuroD4 (Math3) and Gata3, the calcium binding protein Calb2 (calretinin) and the small GTP-binding protein Rab3b. These results led us to consider possible mechanisms for cell-specific target gene regulation by Brn3a in these two closely related neural tissues.

The cell-type specific effects of transcription factors are generally attributed to distinct complements of interacting partners, or to the expression of related factors that provide redundancy in some cell types but not in others. However, with the exception of the Hox factors, the very similar gene expression profiles of the DRG and TG do not suggest many candidates for distinct Brn3a partners in these neurons. In addition, the loss of Brn3a effectively eliminates expression of all Pou4 class factors in both the DRG and TG, making selective redundancy unlikely.

One approach to understanding the cell-specific effects of the loss of Brn3a expression is suggested by recent work in which we have demonstrated a correlation between Brn3a binding to its target sites *in vivo* and H3-acetylation in the vicinity of the potential binding sites (Lanier, Quina et al. 2007). Given these results, one potential mechanism for the differential regulation of target genes by Brn3a might be distinct modifications of chromatin at the target gene loci in the DRG and TG, which

could modulate the effects of Brn3a or downstream regulatory factors. To examine this question for the differentially regulated loci Tcfap2b, NeuroD4 and Gata3, we used chromatin immunoprecipitation (ChIP) to profile histone H3 acetylation in wild-type E13.5 DRG and TG (Figure 4.7). We also examined the Msc locus as an example of a gene that shows similarly increased expression in the DRG and TG of Brn3a knockout mice. ChIP assays of the promoter regions of three constitutively expressed genes, *Gapdh*, *Mapt* (tau), and *Eno2* (neuron specific enolase), were used as positive controls, and the results were normalized to negative control assays from the promoter region of the *Alb1* (albumin) gene, which is not expressed in the nervous system. Because the genomic sequences that regulate these genes in the sensory ganglia have not been defined, we surveyed histone acetylation across these loci using multiple oligonucleotide pairs spaced at 500 to 1,000 base-pair (bp) intervals from approximately -10 kb to +15 kb relative to the start of transcription, using quantitative real-time PCR ('locus-ChIP') ["locus-ChIP", (Lanier, Quina et al. 2007).

ChIP profiling of the Msc locus revealed similar levels of H3-acetylation in the DRG and TG. However, ChIP assays of the *Tcfap2b* and *NeuroD4* loci revealed significantly greater H3-acetylation in the TG relative to the DRG ($p = 0.0003$ and $p = 0.0005$, respectively), while the Gata3 locus exhibited a trend ($p = 0.09$) toward greater acetylation in the TG. As expected, the positive control promoters were highly acetylated, and the negative control, *Alb1*, was deacetylated in both the DRG and TG. Thus, the state of H3-acetylation in wild-type ganglia appears to be correlated both

with basal expression and also with a state of latent potential expression that can be induced in Brn3a null ganglia.

DISCUSSION

Numerous transcription factors have been shown to play key roles in the differentiation of brainstem, spinal, and spinal sensory neurons, yet, for the most part, the programs of gene expression regulated by these factors remain unknown. A layer of complexity is added to the analysis of the downstream targets of these factors by the fact that their expression patterns are often very complex, and may include diverse classes of neurons and non-neuronal cell types with no obvious common characteristics. Brn3a, for example, is expressed in a majority of differentiating peripheral sensory neurons, and also in specific neurons of the spinal cord, olivo-cerebellar system, midbrain, diencephalon and retina. The LIM-domain transcription factor Islet1 is co-expressed with Brn3a in the sensory system, but in the CNS it is expressed primarily in motor neurons (Pfaff, Mendelsohn et al. 1996; Thaler, Koo et al. 2004), and also has important roles in the development of the heart (Cai, Liang et al. 2003) and pancreatic islet cells (Ahlgren, Pfaff et al. 1997). A fundamental unanswered question is whether these factors, and many others with expression patterns of similar complexity, regulate the same downstream targets in different neuronal types, and in neurons versus non-neuronal cells.

To better understand the molecular pathways of sensory development, we initiated the present study by examining global gene expression in the brachial-level DRG and TG at E13.5. At this stage, when essentially all sensory neurons have exited the cell cycle and markers of sensory subtypes are beginning to be expressed, the gene expression patterns of the DRG and TG are quite similar. Some transcripts exhibit

quantitative differences between the ganglia, which may represent relative differences in cellular composition or the timetable of development, but very few transcripts are uniquely expressed in either the DRG or TG. Most prominent among the few transcripts restricted to a particular axial level are those encoded by the *Hox* genes and Etv1, which at this stage are expressed only in the DRG. The restriction of the Hox transcripts (with the exception of HoxD1) to spinal levels is expected based on the well characterized axial expression pattern of these genes. Etv1 expression is restricted to the DRG at E13.5 because the first neurons to express this marker, the large proprioceptive neurons innervating muscle spindles, develop within the DRG at spinal levels but at cranial levels are largely sequestered in the mesV. Etv1-expressing neurons that develop in the TG by E16.5 are distinguished by smaller size and the co-expression of Islet2, and appear to represent a distinct subset of sensory neurons.

Global analysis of gene expression in Brn3a knockout and wild-type DRG demonstrates that Brn3a regulates, directly or indirectly, an extensive program of gene expression. Advances in microarray technology and in the annotation of the mouse genome have allowed us to significantly expand the number of identified Brn3a targets from a prior study of the TG (Eng, Lanier et al. 2004). A large number of the downstream targets of Brn3a in the DRG have known roles in neurogenesis or neural function, and include components of axons and synapses, neurotransmitters and their receptors, mediators of intracellular signaling, and transcription factors. Because the regulatory sequences of many of these genes have not been described, in many cases it is not possible to distinguish direct regulatory targets from secondary or compensatory

effects. However, in recent studies we have used locus-ChIP to demonstrate that Brn3a is a direct repressor of NeuroD1 and NeuroD4 in the embryonic TG (Lanier, Quina et al. 2007), as well as a negative modulator of its own expression (Trieu, Rhee et al. 1999; Trieu, Ma et al. 2003).

Most Brn3a target genes are conserved between sensory neurons at cranial and spinal levels. However, a subset of genes with the most increased expression in the TG do not change expression in the Brn3a knockout DRG, and are generally undetectable in the E13.5 DRG of animals of any genotype. These differentially regulated transcripts include the transcription factors Tcfap2b, Gata3 and NeuroD4, the calcium binding protein calbindin2 (calretinin), and the small GTP-binding protein Rab3b, implicated in synaptic vesicle release (Schluter, Schmitz et al. 2004). Because there are normally few transcripts that distinguish the TG from the DRG at this stage of development, the gene expression patterns of the DRG and TG are significantly more different in Brn3a knockout ganglia than in the wild type. This suggests that one important role for Brn3a is to suppress potential differences in gene expression between the spinal and cranial ganglia, mediating a process of 'convergent development' in which functionally similar populations of neurons are generated from different embryological sources.

The state of histone H3 acetylation of Brn3a target gene loci offers some insight into the underlying mechanism of the differential regulation of these targets at spinal and cranial levels. Normally, H3 acetylation is associated with the regulatory sequences in the promoters of actively transcribed genes. Consistent with this, we

have previously shown that the promoter region of *Pou4f1* itself is highly acetylated in the TG, and the promoters of genes that are silent in the sensory ganglia regardless of Brn3a genotype are deacetylated (Lanier, Quina et al. 2007). In wild-type DRG and TG, the differentially regulated genes *NeuroD4* and *Tcfap2b* are nearly silent, yet these gene loci can be distinguished in trigeminal neurons by increased H3 acetylation over a region encompassing the transcription start site. The *Msc* gene is also normally silent, but it exhibits increased expression at both cranial and spinal levels in Brn3a null mice, and is accordingly H3 acetylated in both the DRG and TG. Thus, in the case of these genes with increased expression in the Brn3a knockout, H3 acetylation appears to reveal a latent state of potential expression, which is normally repressed by Brn3a or its downstream effectors, and de-repressed in Brn3a knockout ganglia.

The failure of DRG neurons to increase *NeuroD4* and *Tcfap2b* expression in Brn3a knockout ganglia suggests that a redundant mechanism of repression exists for these genes at spinal but not cranial levels. The action of such a repressor may be reflected in the low levels of histone acetylation at these loci in the DRG. Because the most prominent distinguishing feature of sensory gene expression at spinal levels is the expression of multiple *Hox* genes, these factors are candidate mediators of this selective repression of *NeuroD4* and *Tcfap2b* in the DRG. Transcriptional repressor functions have been described for the *Hox* genes (Svingen and Tonissen 2006), and there is evidence that Hox proteins can directly block the activity of the widely expressed histone acetyltransferase CBP-p300 (Shen, Krishnan et al. 2001). *Hox* gene repression of a subset of Brn3a target gene loci could be an active process at E13.5, or

Hox gene expression at earlier developmental stages could produce persisting modifications of chromatin, reflected in the deacetylation of these loci observed here, resulting in reduced transcription even when direct repression by *Hox* genes is no longer active.

MATERIALS AND METHODS

Matings, embryos, and RNA isolation for array analysis. To generate tissue for microarray analysis, timed matings of Brn3a heterozygote animals were performed, and the embryos were harvested at E13.5. Only embryos corresponding to E13.5 ± 0.5 days based on the staging system of Theiler (Theiler 1972) were used for microarray analysis. TG were removed by blunt dissection and carefully freed of adherent non-neural tissue with fine forceps. DRG were isolated by stripping of the spinal cord with its adherent ganglia, followed by dissection of the ganglia with fine forceps. DRG were harvested from brachial region only, including the C5-T1 levels. Dissected ganglia were placed in RNAse inhibitor solution (RNAlater, Ambion, Austin TX), and RNA was prepared using the RNeasy system (Qiagen, Valencia, CA). Embryos were genotyped for Brn3a alleles as previously described (Eng, Gratwick et al. 2001) from a sample of tail or hind-limb tissue harvested at the time of ganglion dissection. Genotyped TG or DRG from five embryos were sufficient to provide approximately 5 μg of total RNA for a single microarray analysis. 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, Santa Clara, CA).

Analysis of expression array data. The principal microarray datasets presented here for the mouse DRG and TG were generated using the Affymetrix 430A and 430B microarrays. In addition, the same DRG samples were analyzed using Affymetrix U74A and U74B arrays for direct comparison with prior data sets for the TG (Eng, Lanier et al. 2004). 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 samples ('change *p*') was performed with Microarray Suite 5.0 (Affymetrix). Default Microarray Suite 5.0 parameters were used for increase (I) and decrease (D) calls. For the 430 array set these cutoff values were $p < 0.005$ and $p > 0.995$ for I and D, respectively, and for the U74 arrays, the values were $p < 0.003$ and $p > 0.997$. All array values were initially scaled to a mean value of 500 using global scaling. To permit more meaningful comparison of the expression levels of transcripts assayed by the 430A and 430B arrays, the initial expression values for the 430B array were rescaled based on the expression levels of 100 probe sets in common between the 430A and 430B arrays. Microarray probe sets were related to the corresponding mouse transcripts using the NetAffx database (Affymetrix), based on the NCBI Build 34 annotation of the mouse genome. The array data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE5658.

Comparisons of gene expression between wild-type DRG and TG (Table 4.1) and between the DRG from Brn3a wild-type, heterozygous, and knockout mice (Tables 4.2 and 4.3) were performed in duplicate, using separate pools of isolated ganglia. To be included in the tables of changed transcripts for a given tissue or genotype, the following criteria were met: both replicates were called as 'present' in the condition of greater expression; both replicates exhibited an increased or 'I' call for

the condition of higher expression; and the probe set identified an annotated and named transcript in NCBI Build 34 of the mouse genome.

Duplicate probe sets identifying the same transcript were eliminated, and the tables are annotated with the number of concordant probe sets for each transcript. Concordance was identified by the agreement of the present and increased calls for the duplicate probe set, and a minimum fold change of 2.0. In the case of multiple concordant probe sets, the probe set with the greatest fold change is listed in the tables. Results for the probe sets meeting the inclusion criteria were ranked by fold change, and the cutoff value for inclusion appears in the tables.

In situ *hybridization and immunofluorescence.* Non-isotopic *in situ* hybridization was performed as previously described (Birren, Lo et al. 1993). A list of probes used and their sources appears in online Supplemental Tables. Immunofluorescence for Brn3a was performed with rabbit polyclonal antisera as previously as previously described (Fedtsova and Turner 1995). Other antisera used included rabbit antisera against Etv1/Er81 and Runx3 obtained from Dr Sylvia Arber (Arber, Ladle et al. 2000; Kramer, Sigrist et al. 2006), and guinea pig antisera against Islet2, obtained from Dr Sam Pfaff (Thaler, Koo et al. 2004). Immunofluorescence for other antigens was performed with commercially available antibodies, including rabbit anti-calretinin (Swant, Bellinzona, Switzerland), rabbit anti-galanin (Bachem, King of Prussia, PA), rabbit anti-somatostatin-14 (Peninsula Laboratories), and goat anti-β-galactosidase (Biogenesis (MorphoSys), Kingston, NH).

Locus-wide chromatin immunoprecipitation. Locus-ChIP assays were performed as previously described (Lanier, Quina et al. 2007). In brief, embryos for ChIP assays were generated from timed matings of ICR mice. DRG and TG were dissected from E13.5 embryos and fixed in 4% paraformaldehyde for 30 minutes, then quenched with 150 mM glycine. The fixed tissue was washed with phosphate-buffered saline and stored at -80°C until analysis.

Selection of chromatin complexes from embryonic sensory ganglia was performed by a modification of a widely used procedure (Luo, Postigo et al. 1998). For each analysis, fixed ganglia from 30 embryos (60 TG or approximately 300 DRG) were pooled and suspended in lysis buffer containing 50 mM Tris-HCl, pH 8.1, with 10 mM EDTA and 1% SDS, 1 mM 4-(2-Aminoethyl)benzenesulfonylfluoride, HCl (AEBSF) and a proprietary protease inhibitor mix (1 × Complete Mini, Roche, Indianapolis, IN; used according to instructions). Chromatin was then fragmented to an average size of 500 bp by sonication, and insoluble cellular debris was removed by centrifugation. The supernatant containing fragmented chromatin was diluted in 15 mM Tris-HCl, pH 8.1, with 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.01% SDS, and protease inhibitor mix. An unselected ('input') sample of 10% of the total homogenate was removed prior to antibody selection.

ChIP was performed using rabbit anti-acetylated histone H3 antibody (Upstate Biotechnology, Inc., Bellerica, MA, catalog no. 06–599), which recognizes histone H3 acetylated at lys9 and lys14. For each selection, 50 μg of anti-histone antibody was coupled to 250 μL of anti-rabbit IgG magnetic beads (Dynal M-280), in lysis buffer

containing 50 mM Tris-HCl, pH 8.1, with 10 mM EDTA and 1% SDS. To reduce non-specific background, the chromatin sample was pre-cleared using the magnetic beads with the secondary antibody alone. The sample was then incubated overnight with secondary antibody-coupled beads to select the acetyl H3-containing chromatin complexes. The beads were then washed for 5 minutes at room temperature with each of the following solutions: 20 mM Tris-HCl, pH 8.1, with 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, and 1% Triton-X-100; 20 mM Tris-HCl, pH 8.1, with 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton-X-100; 10 mM Tris-HCl, pH 8.1, with 1 mM EDTA, 0.25 M LiCl, 1% NP-40, and 1% deoxycholate. Salt and detergent were removed by washing twice with 10 mM Tris-HCl, 1 mM EDTA, pH 8. DNA was extracted from the antibody-chromatin complexes and the input sample by heating in 0.1 M $NaHCO_3$ with 200 mM NaCl and 1% SDS at 65°C for 4 hours with constant shaking. The input and selected samples were then digested with proteinase K, extracted with phenol/chloroform, and precipitated with ethanol.

Real-time locus-wide PCR analysis. Chromatin fragments recovered from the immunoprecipitated and input samples were then assayed by real-time PCR using an ABI 7300 thermocycler and SYBR Green fluorimetric detection. To screen an entire gene locus, oligonucleotide pairs were designed at 500 to 1,000 bp intervals throughout the region, and selected and unselected samples were run in parallel in a 96-well plate format. The primer pairs used for locus-ChIP assays of the *NeuroD4*, *Msc*, *Tcfap2b* and *Gata3* loci appear in online Supplemental Tables.

The enrichment of immunoprecipitated chromatin fragments was assayed by the cycle-threshold difference method (Livak and Schmittgen 2001). For this method, real-time PCR signals are measured using the 'cycle threshold', or Ct parameter, which is the number of cycles required for the amplification product to reach an arbitrary level of fluorescence intensity (threshold), and is logarithmic to the initial abundance of the target sequence in the sample. For each PCR amplicon, a ΔCt value comparing the unselected (input) and antibody-selected DNA samples was then calculated by subtracting the $Ct_{selected}$ from the Ct_{input} signal:

$$\Delta Ct = Ct_{input} - Ct_{selected}$$

Fold enrichment values for target sequences bound by the selecting antibodies, corresponding to the y-axis of the locus-ChIP plots, were calculated using the following equation:

$$E = 2^{(\Delta Ct - \Delta Ctcontrol)}$$

Because product formation approximately doubles with each cycle in the linear range of amplification, a ΔCt of one cycle represents a two-fold difference in starting template. A significant advantage of this method is that, for each primer pair, a selected sample is compared directly to its unselected control, which differs only by the antibody selection process. Potentially confounding factors such as small differences in the PCR amplification efficiency of different primer pairs are eliminated in this comparison.

The ΔCt assays for each pool of selected material were normalized to an arbitrary baseline (one-fold enrichment) determined using two primer pairs in the

promoter region of the *Alb1* (albumin locus), which was chosen as a negative control because it is not transcribed in the nervous system. Fold enrichment values for the *Alb1* locus were very similar to values for the unselected, intergenic regions of the target gene loci. Primer pairs in the promoter regions of *Mapt* (microtubule-associated protein tau) and *Eno2* (neuron specific enolase), which exhibit tissue specific expression in the nervous system, and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), which is expressed ubiquitously, were used as positive controls. Comparisons of histone H3 acetylation at a given locus in the DRG and TG were made using three- to six-fold enrichment values using oligonucleotide pairs in a contiguous genomic region near the transcription start site of the analyzed gene loci. The statistical significance (*p* values) of the difference in H3 acetylation was determined using two-sample, unequal variance *t*-tests.

FIGURES

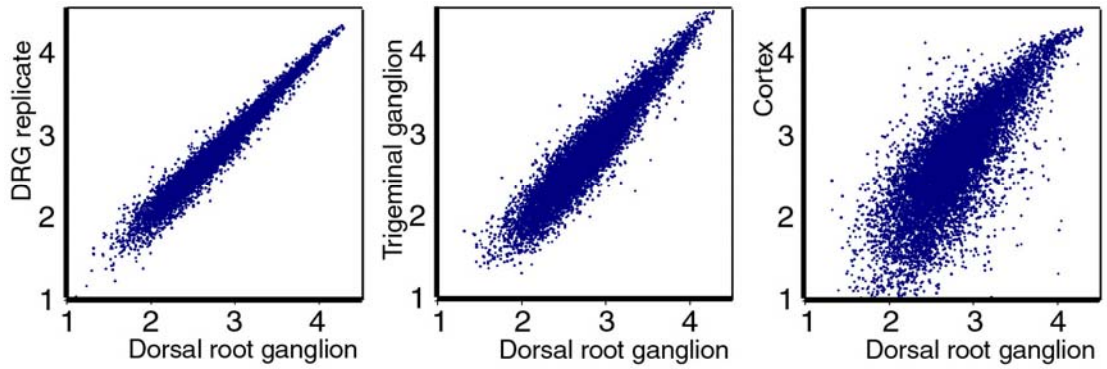
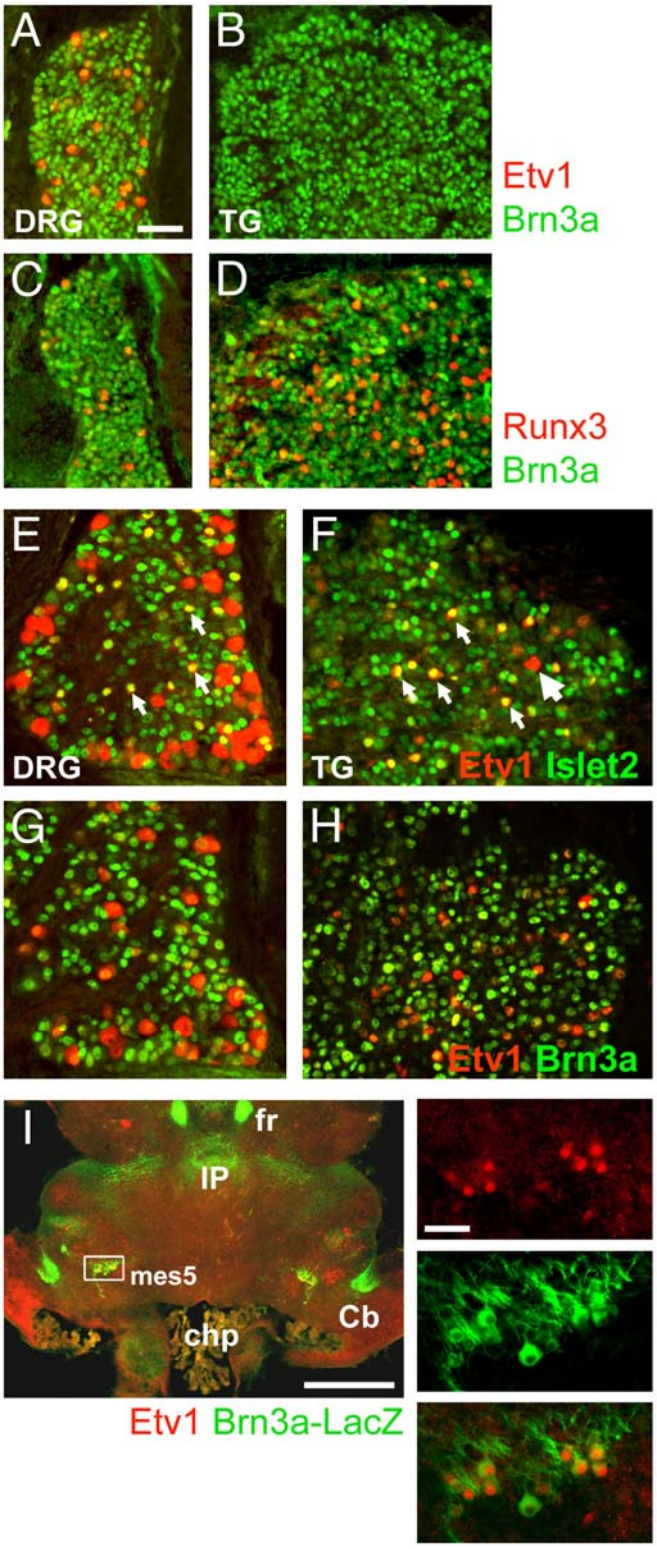


Figure 4.1. Analysis of global gene expression in embryonic neural tissue. Analysis of global gene expression was performed using E13.5 DRG, E13.5 TG and E16.5 cerebral cortex samples. Only probe sets on the Affymetrix 430A array are shown, and transcripts with "absent" calls in all three samples were excluded from the analysis. (A) Plot of DRG expression versus a replicate DRG assay closely approximates a diagonal line indicating equal expression in the two samples. (B) Plot of DRG expression versus the TG indicates very similar global gene expression in the two samples, with few points lying far from the diagonal. (C) Plot of DRG expression versus the embryonic cerebral cortex indicates a large number of transcripts which are differentially expressed between the two samples. Axis is graduated in log10 of scaled signal..

Figure 4.2. Selective expression of proprioceptor markers in the DRG and trigeminal system. The sensory ganglia of wildtype embryos were examined at E13.5 and E16.5 for the expression of transcription the factors Brn3a, Etv1, Runx3 and Islet2. (A-D) At E13.5, Etv1 expression is restricted to the DRG, while Runx3 is expressed in both the DRG and TG. (E-H) At E16.5, Etv1 is expressed in both the DRG and the TG, but in the TG Etv1 positive neurons with large nuclei consistent with 1a proprioceptors are rare (large arrow). Instead, the majority of Etv1-positive neurons in the TG have nuclei of intermediate size and co-express Islet2; similar cells are also found in the DRG (small arrows). The 1a proprioceptors of the DRG co-express Brn3a, but at relatively low levels. (I) Etv1 expression in the mesV of an E18.5 embryo expressing a tauLacZ transgene integrated into the Brn3a locus (Quina, Pak et al. 2005), which is thus heterozygous for Brn3a, but phenotypically normal. Numerous neurons are noted which co-express Etv1 and the Brn3a-LacZ marker. The caudal location and large size of these neurons are consistent with proprioceptors innervating the muscles of mastication. Cb, cerebellum; chp, choroid plexus; fr, fasciculus retroflexus; IP, interpeduncular nucleus; mes5, mesencephalic trigeminal. Scale (A-H) 50μM, (I) 400μM, (I) inset 50μM.


A
DRG
B
TG
Etv1
Brn3a
C
D
Runx3
Brn3a
E
DRG
F
TG
Etv1 Islet2
G
H
Etv1 Brn3a
I
fr
IP
mes5
chp
Cb
Etv1 Brn3a-LacZ

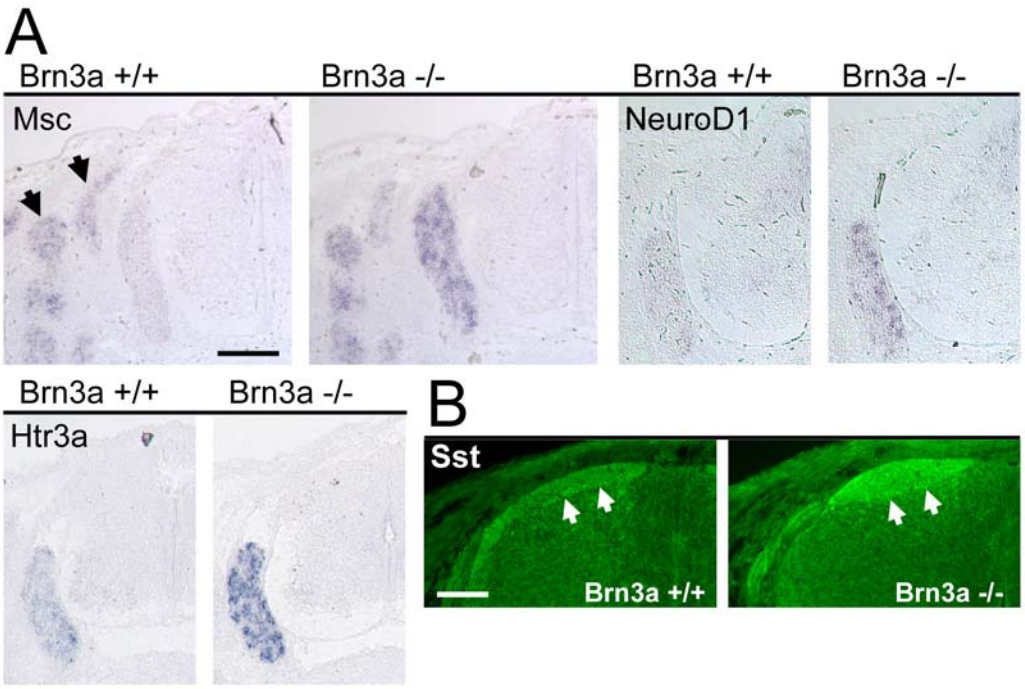
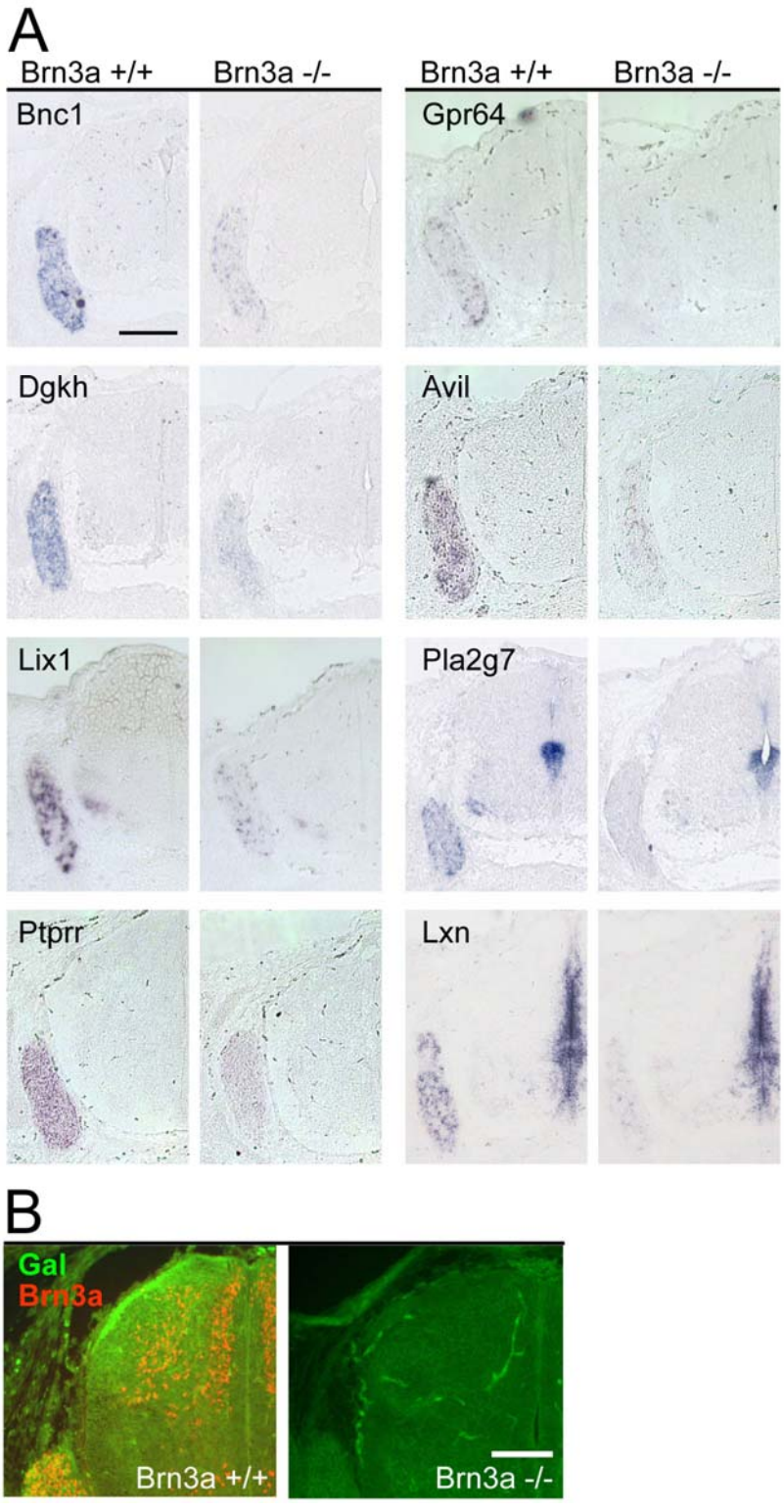


Figure 4.3. Target genes with increased expression in the DRG of Brn3a null mice. (A) In situ hybridization confirms increased expression of Msc (musculin), NeuroD1, and Htr3a (5HT receptor 3A) transcripts in the dorsal root ganglia of Brn3a wild-type and knockout embryos. Note that the expression of Msc in the surrounding musculature (arrows) is unchanged. (B) Immunofluorescence for the somatostatin-14 peptide shows increased expression concentrated in the dorsal root entry zone (arrows). The distribution of somatostatin peptide also reveals an abnormal accumulation of axons in the superficial dorsal horn in the Brn3a null mutant. All views show lower cervical (brachial) level cross sections of E13.5 embryos. Scale (A) 200μM, (B) 50μM

Figure 4.4. Target genes with decreased expression in the DRG of Brn3a null mice. (A) In situ hybridization confirms decreased expression of multiple Brn3a downstream targets. (B) Immunofluorescence for the galanin neuropeptide shows marked reduction in the dorsal horn and dorsal root entry zone of Brn3a null DRG. All views show lower cervical (brachial) level cross sections of E13.5 embryos. Scale (A) 200μM, (B) 100μM.


A
Brn3a +/+
Brn3a -/-
Brn3a +/+
Brn3a -/-
Bnc1
Gpr64
Dgkh
Avil
Lix1
Pla2g7
Ptprr
Lxn
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Gal
Brn3a
Brn3a +/+
Brn3a -/-

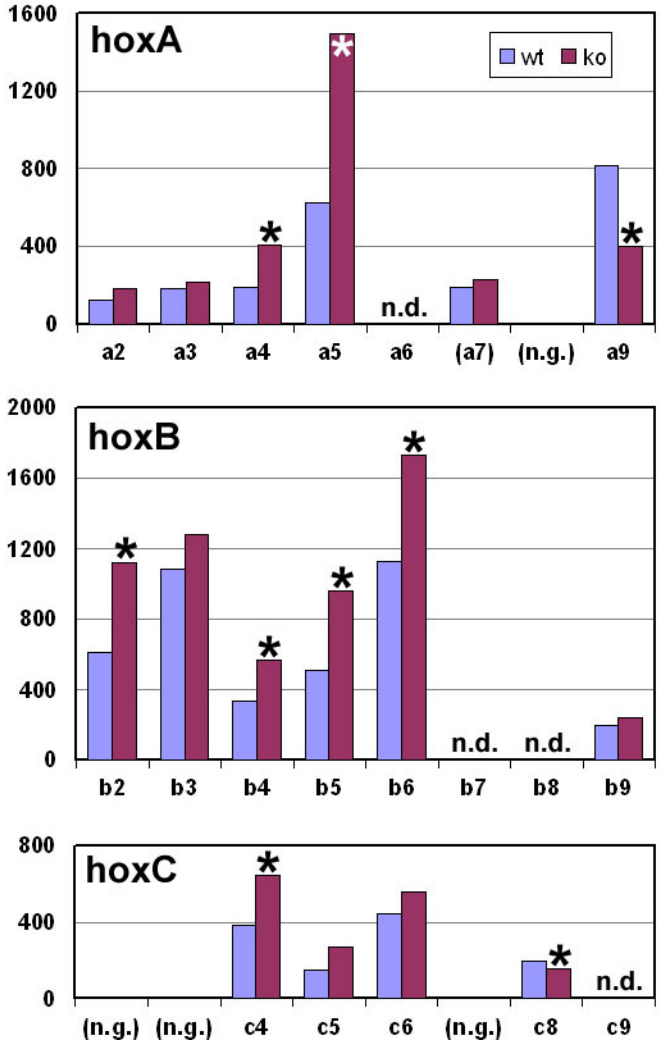


Figure 4.5. Brn3a regulation of Hox gene expression in the DRG. Hox gene expression data were derived from 430A+B array data for E13.5 Brn3a wildtype and knockout DRG. Statistically significant increases ($p < 0.005$) in the knockout were observed for 7 anterior hox transcripts, and significant decreases were observed ($p>0.995$) for two posterior hox transcripts (asterisks). Similar changes reached statistical significance in 5 anterior hox genes and one posterior hox gene in a replicate assay. HoxA7 (parentheses) did not reach statistical significance for detection, indicating high background signal in this probe set. Legend: n.g., no gene at the corresponding position in the cluster; n.d., not determined.

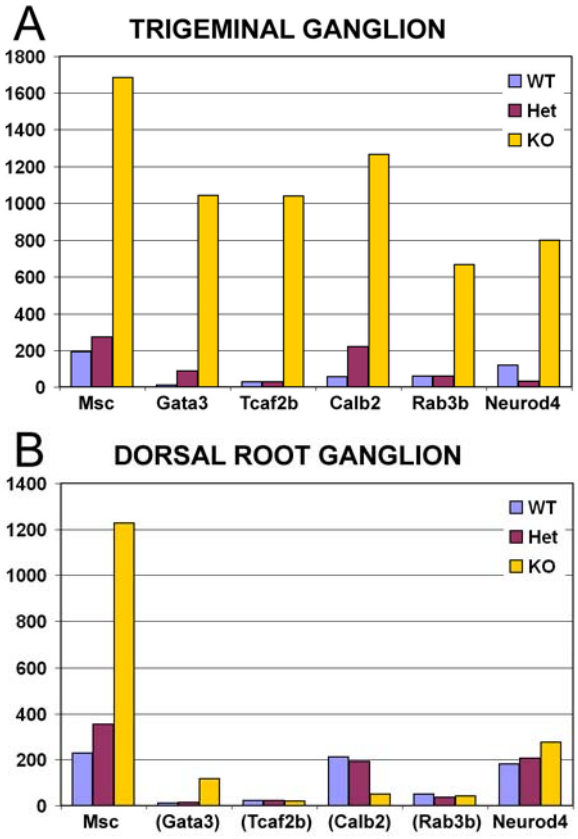
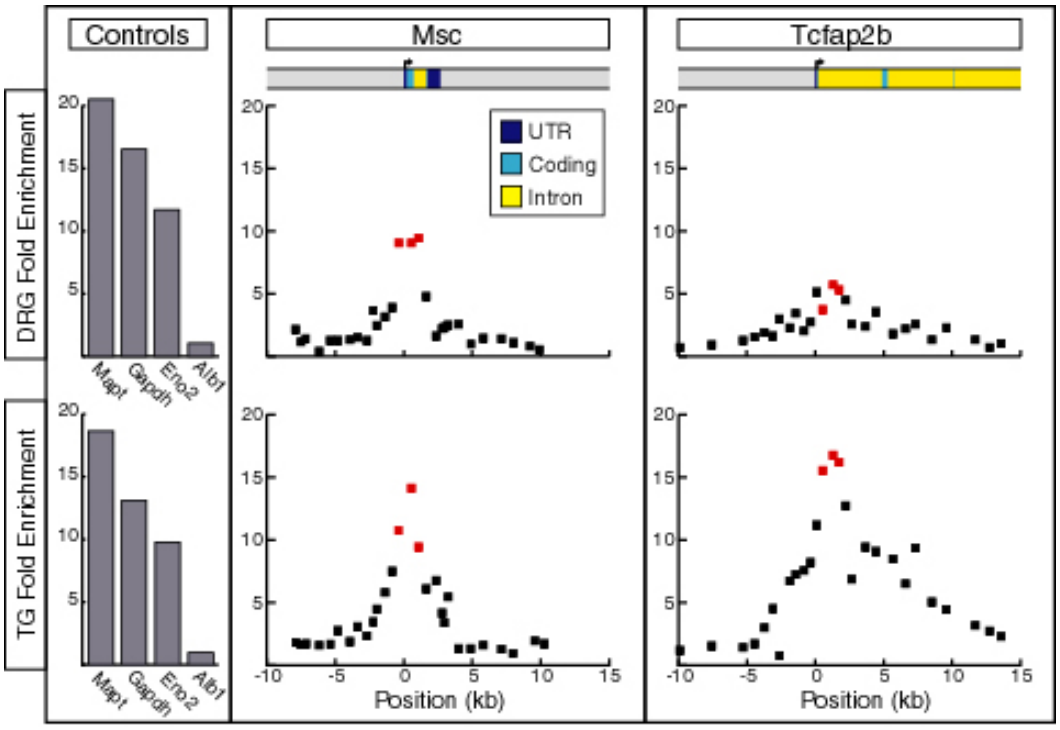
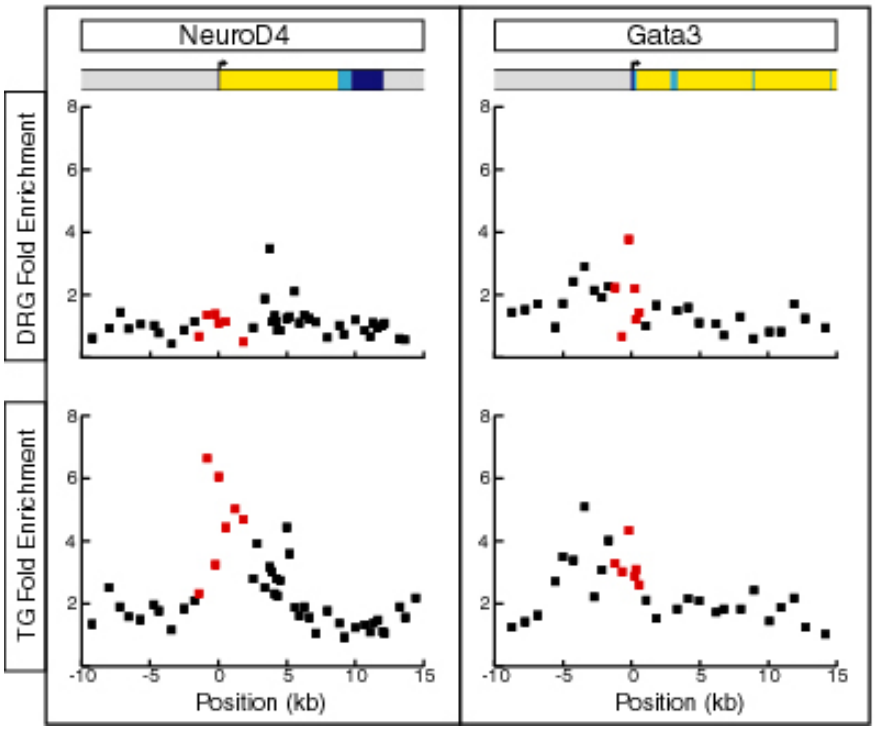


Figure 4.6. Trigeminal-specific targets of Brn3a regulation. Brn3a wildtype, heterozygote and knockout E13.5 DRG were analyzed for global gene expression using U74Av2+U74v2B arrays to allow direct comparison with a prior analysis of the embryonic TG (online Table S3-S6). Trigeminal data are from a previously reported data set, and changes in Gata3, Tcfap2b, Calb2 and NeuroD4 have been previously verified in the TG of Brn3a knockout mice by in situ hybridization or immunofluorescence (Eng, Lanier et al. 2004). Rab3b is newly identified as a Brn3a target in the trigeminal due to progress in the annotation of the mouse genome. Gene abbreviations in parentheses indicate expression below the statistically significant threshold of detection (absent call) in all three genotypes.

Figure 4.7. Acetyl-histone H3 profiling of Brn3a target gene loci. Histone H3 acetylation was assayed using ChIP and tiled PCR primer pairs from -10kb to 15kb of the *Msc*, *Tcfap2b*, *NeuroD4* and *Gata3* gene loci. Positive controls for these assays included primer pairs located in the promoter regions of the *Mapt* (tau), *Gapdh*, and *Eno2* loci, which are highly expressed in the DRG and TG and are unchanged in Brn3a null mice. The silent *alb1* locus was used as a negative control and to set the baseline of 1-fold enrichment (yellow line). H3 acetylation of these loci in chromatin samples from the E13.5 DRG and TG were compared by t-tests using fold enrichment values for primer pairs flanking the transcription start site of each locus (points shown in red). H3 acetylation was not significantly different at the msc locus (p=0.25), while *Tcfap2b* (p=0.0003) and *NeuroD4* (p=0.0005) showed significantly greater acetylation in the TG, and *Gata3* (p=0.09) showed a trend toward greater acetylation in the TG


Controls
Msc
Tcfap2b
DRG Fold Enrichment
TG Fold Enrichment
Mapt
Gapdh
Eno2
Alb1
UTR
Coding
Intron
Position (kb)


NeuroD4
Gata3
DRG Fold Enrichment
TG Fold Enrichment
Position (kb)

TABLES

Table 4.1. Transcripts differentially expressed in the DRG and TG.
The principal analysis was conducted with the murine 430 array, with selected results identified using the U74A+B array set added. All listed transcripts showed increased expression (change $p<0.005$) in two independent replicates. Fold values represent the ratio of the means of two determinations for each tissue. Fold changes in parentheses indicate an absent call, i.e. below the statistically reliable limit of detection, in the lower expressing sample. Transcripts with expression levels less than 40% of the scaled mean are excluded. Superscript numerals represent the number of probe sets concordant for changed expression for a given gene. AX, axonogenesis; Dev, development; NT, neurotransmission; ST, signal transduction; Syn, synaptogenesis; TX, transcription; unk, unknown.

A. Greater expression in the DRG. Confirmation of selected results by ISH and immunofluorescence appears in Figures 4.2 and 4.3. Differential expression of Ramp2 could not be confirmed by ISH. Different levels of Zic1 and follistatin appear to be due to high expression in the spinal cord adjacent to the DRG, rather than the DRG itself. Sostdc1 and EDNRB showed increased DRG expression by ISH but in a pattern more characteristic of glial than neuronal expression, with signal concentrated in nerve roots. AX, axonogenesis; Dev, development; NT, neurotransmission; ST, signal transduction; SY, synaptogenesis; TX, transcription; unk, unknown.

B. Greater expression in the TG. In situ hybridization confirmed some changes. However, Mfap4 was widely expressed in embryonic tissues. Socs3 ISH exhibited relatively greater expression in the TG, but overall expression levels were low.

A. Greater expression in the dorsal root ganglion

Gene	Symbol	Class	Fold
Homeo box B3	Hoxb3[2]	TX	(90.4)
Homeo box A9	Hoxa9	TX	(90.2)
Homeo box A5	Hoxa5[2]	TX	(44.2)
Etv1; ER81	Etv1[3]	TX	(40.2)
Homeo box C8	Hoxc8[2]	TX	(38.4)
homeo box B6	Hoxb6	TX	(27.6)
Hoxd4	Hoxd4	TX	(16.6)
Follistatin	Fst[2]	Dev	(12.9)
Leucine-rich repeat-containing G protein-coupled receptor 6	Lgr6	Other	(11.8)
Receptor (calcitonin) activity modifying protein 2	Ramp2[3]	ST	10.9
Ca^+ channel, L type, alpha 1C subunit	Cacna1c	NT	9.2

Table 4.1. Transcripts differentially expressed in the DRG and TG (Continued).

Homeo box B8	Hoxb8	TX	(9.1)
BTB and CNC homology 2	Bach2	TX	(8.8)
Sclerostin domain containing 1	Sostdc1	Dev	8.5
Laminin, alpha 2	Lama2	Other	(7.3)
Selected:			
Purkinje cell protein 4	Pcp4	Unk	(5.7)
LIM domain binding 2	Ldb2[2]	TX	5.6
Zinc finger protein of the cerebellum 1	Zic1[2]	TX	5.3
Gap junction membrane channel protein alpha 1	Gja1[2]	NT	5.1
Protein tyrosine phosphatase, receptor type, E	Ptpre[3]	ST	5.0
Early B-cell factor 1; Olf1	Ebf1	TX	5.0
U74 array:			
Regulator of G-protein signaling 4	RGS4	NT	6.1
Endothelin receptor type B	EDNRB	Other	3.0
Anthrax toxin receptor 2	Antxr2	Unk	3.0

B. Greater expression in the trigeminal ganglion

Gene	**Symbol**	**Class**	**Fold**
Microfibrillar-associated protein 4	Mfap4	Unk	(20.6)
Lectin, galactose binding, soluble 7	Lgals7	AX	(11.3)
MyoD family inhibitor	Mdfi	TX	(9.6)
Suppressor of cytokine signaling 3	Socs3[2]	ST	(6.4)
RNA imprinted and accumulated in nucleus	Rian	Unk	6.1
Musashi homolog 2	Msi2h	Other	6.0
Selected:			
T-cell leukemia, homeobox 1	Tlx1	TX	(4.4)
SNAP-associated protein	Snapap	SY	4.0
SRY-box containing gene 11	Sox11	TX	3.2
Ring-box 1	Rbx1	Other	3.1
T-box 3	Tbx3	TX	2.9
U74 array:			
Neuroipeptide Y receptor 1	NpyR1	NT	8.6

Table 4.2. Increased transcripts in the DRG of Brn3a knockout mice. Data for one of two independent experiments is shown. All listed transcripts showed increased expression (change $p<0.005$) in two independent replicates. Complete data are shown for all replicated changes above 3-fold, and selected data appear for transcripts exhibiting 2-3 fold change. A full data set appears in online Figure S1. AX, neurogenesis/axon growth and guidance; TX, transcription, NT, neurotransmission; ST, signal transduction; SY, synapse formation and function.

Gene	Symbol	Class	WT	HT	KO	KO/ WT
Chordin-like 1	Chrdl1	Dev	5	2	134	27.4
Musculin (MyoR)	Msc	TX	211	675	1260	6.0
Insulinoma-associated 1	Insm1[2]	TX	153	301	827	5.4
GABA transporter 1 (Gabt1)	Slc6a1	NT	74	93	397	5.3
Microfibrillar-associated 4	Mfap4	Unk	112	370	559	5.0
Secretogranin II	Scg2	SY	206	253	966	4.7
C-fos induced growth factor (VEGF-D)	Figf	Dev	76	102	319	4.2
Guanylate cyclase 1, alpha 3	Gucy1a3	ST	50	82	205	4.1
Neurogenic differentiation 6 (Math2, Nex)	Neurod6	TX	83	200	344	4.1
Somatostatin	Sst	NT	215	382	830	3.9
Neurexin III	Nrxn3[4]	SY	367	961	1412	3.8
Bruno-like 4	Brunol4[3]	Other	154	274	588	3.8
Cell adhesion molecule with homology to L1CAM	Chl1	AX	128	127	466	3.6
Junctophilin 1	Jph1	Other	74	119	262	3.5
Glutamate receptor, ionotropic, AMPA4	Gria4[3]	NT	46	80	161	3.5
Glutamate receptor, ionotropic, AMPA3	Gria3	NT	40	66	138	3.5
Zinc finger homeobox 1b	Zfhx1b[2]	TX	81	127	270	3.3
Nel-like 2	Nell2	AX	383	331	1215	3.2
Follistatin-like 5	Fstl5[2]	Dev	281	389	885	3.2
Disabled homolog 1	Dab1[2]	AX	90	42	272	3.0
Mannan-binding lectin serine protease 1	Masp1	Other	95	101	282	3.0
Selected:						
Semaphorin 3C	Sema3c[2]	AX	589	723	1732	2.9
Cholecystokinin A receptor	Cckar	NT	95	161	269	2.8
Serotonin receptor 3A	Htr3a	NT	778	1160	2090	2.7
Homeo box A5	Hoxa5	TX	628	896	1493	2.4
Neurogenic differentiation 1	Neurod1[2]	TX	1048	1468	2440	2.3
p21-activated kinase 3	Pak3[2]	SY	246	270	568	2.3
Semaphorin 3D	Sema3d	AX	318	365	725	2.3

Table 4.2. Increased transcripts in the DRG of Brn3a knockout mice (Continued).

Protocadherin 17	Pcdh17[2]	AX	331	340	746	2.3
Spondin 1, (f-spondin)	Spon1	AX	629	778	1413	2.2
K^+ voltage-gated channel, Isk family	Kcne1l	NT	613	813	1328	2.2
Deleted in colorectal carcinoma	Dcc	AX	93	101	200	2.2
Netrin G1	Ntng1	AX	388	556	812	2.1
Neuropilin 2	Nrp2	AX	509	485	1063	2.1
Cadherin 22	Cdh22	AX	199	226	414	2.1
Synapsin II	Syn2[4]	SY	643	688	1324	2.1
Glutamate receptor, ionotropic, kainate 1	Grik1	NT	221	241	455	2.1
Protocadherin 8	Pcdh8[2]	AX	278	398	569	2.0
GDNF receptor alpha 2	Gfra2	Dev	363	436	735	2.0
Nescient helix loop helix 2 (Nscl2)	Nhlh2	TX	1222	1996	2446	2.0

Table 4.3. Transcripts decreased in the DRG of Brn3a null mice. All listed transcripts showed decreased expression (change p>0.995) in two independent replicates. Complete data appear for all replicated changes above 3-fold, and selected data appear for transcripts exhibiting change in the 2-3 fold range. A full data set appears in online Supplemental Tables. Note that Brn3a transcripts are still detected in the Brn3a null genotype, consistent with the detection of the 3'-end of residual non-coding transcripts. AX, neurogenesis, axon growth and guidance; Dev, developmental processes (differentiation, migration, survival); TX, transcription, NT, neurotransmission; ST, signal transduction; SY, synapse formation and function. Superscript numerals indicate number of concordant probes.

Gene	Symbol	Class	WT	HT	KO	WT/KO
Limb expression 1	Lix1	Unk	984	923	46	21.4
Vascular endothelial growth factor C	Vegfc	Dev	55	54	3	16.1
Neural cell adhesion molecule 2	Ncam2[2]	AX	280	212	22	12.9
Runt related 3	Runx3	TX	251	421	22	11.3
Brn3b	Pou4f2	TX	613	772	61	10.0
Brn3a	Pou4f1[2]	TX	1107	1119	115	9.6
Phospholipase A2, group VII	Pla2g7	ST	618	438	77	8.1
K+ channel, shaker-related, member 1	Kcna1[3]	NT	438	523	59	7.4
Advillin	Avil	AX	2494	1735	364	6.9
Galanin	Gal	NT	5388	4414	824	6.5
Basonuclin 1	Bnc1	TX	875	740	165	5.3
Insulin-like growth factor 1	Igf1[3]	Dev	820	609	160	5.1
G protein-coupled receptor 64	Gpr64[2]	NT	371	298	72	5.1
Adenylate cyclase activating polypeptide 1, PACAP	Adcyap1	NT	283	198	57	5.0
Regulator of G-protein signalling 10	Rgs10	NT	1793	1596	362	5.0
Parvalbumin	Pvalb	ST	403	223	95	4.3
K+ channel, shaker-related, beta member 2	Kcnab2	NT	952	684	238	4.0
Diacylglycerol kinase, eta	Dgkh[2]	ST	1348	1230	353	3.8
G protein-coupled receptor 73	Gpr73	NT	336	157	90	3.7
Serine proteinase inhibitor, clade A, member 3G; Spi2A	Serpina3g	Other	426	346	116	3.7
Copine IV	Cpne4	Unk	767	636	216	3.6
PQ loop repeat containing 1	Pqlc1	Unk	911	596	259	3.5
Reticulon 4 receptor-like 2; nogo receptor-like 3	Rtn4rl2	AX	789	694	226	3.5

Table 4.3. Transcripts decreased in the DRG of Brn3a null mice (Continued).

Spermatogenesis associated glutamate-rich protein 1	Speer1-ps1	Unk	349	307	106	3.3
Pappalysin 2	Pappa2	Other	694	794	225	3.1
Brn3c	Pou4f3	TX	394	361	130	3.0
Protein tyrosine phosphatase, receptor type, J	Ptprj	ST	853	918	284	3.0
Docking protein 4	Dok4	ST	2014	1655	679	3.0
Selected:						
Eph receptor A7	Epha7	AX	206	202	77	2.7
Latexin	Lxn	NT?	2948	2564	1105	2.7
DRG11	Prrxl1	TX	1514	1339	569	2.7
Protein tyrosine phosphatase, non-receptor type 3	Ptpn3	ST	1638	1881	623	2.6
GABA-A receptor, subunit alpha 2	Gabra2	NT	62	90	24	2.6
Na+ channel, type VII, alpha	Scn7a	NT	1199	1386	506	2.4
Protein tyrosine phosphatase, receptor type, R	Ptprr	ST	3376	3207	1467	2.3
Rap1, GTPase-activating protein 1	Rap1ga1	ST	1721	1686	749	2.3
LIM domain binding 2	Ldb2	TX	794	492	349	2.3
Anthrax toxin receptor 2	Antxr2	Unk	1355	1095	608	2.2
Runt related txn factor 1	Runx1	TX	360	343	162	2.2
Homeo box D1	Hoxd1	TX	852	751	404	2.1
Inhibitor of DNA binding 1	Id1	TX	1248	1051	624	2.0

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ACKNOWLEDGEMENTS

We would like to acknowledge Bill Wachsman and Lutfunnessa Shireen for assistance with microarray technology. Dr Sylvia Arber generously provided antisera for Runx3 and Etv1/Er81, and Islet2 antiserum was the generous gift of Dr Sam Pfaff. We would also like to thank Drs Jean-Francois Brunet, Allan Basbaum, Ryoichiro Kageyama, Nobuyuki Itoh, Eric Olson and their associates for *in situ* hybridization probes. Microarray data used in this study are deposited in the Gene Expression Omnibus, series accession number GSE5658. Supported in part by Department of Veterans Affairs MERIT funding and VISN 22 MIRECC (EET), and NIH awards HD33442 and MH065496. EET and NF are a NARSAD Investigators.

The text of Chapter Four is a reprint of the material as it appears in Neural Development, 2007, Eng SR, Dykes I, Lanier J, Fedtsova N, and Turner EE. I was a secondary author and participated in the research that forms the basis of this chapter. My contributions to the work included the chromatin immunoprecipitation assays and real-time PCR analysis.

V

Conclusions

Programs of gene regulation in the nervous system.

Brn3a is one of many tissue-specific transcription factors whose function is critical for neural development. The relatively recent development of microarray gene expression technology has created a means by which a comprehensive set of Brn3a target genes may be identified. By using microarrays to compare transcript expression levels between tissues harvested from wild-type animals and those carrying a homozygous deletion of a particular transcription factor, one may identify the transcriptional targets of the transcription factor. However, due to the technical demands of microarray analysis, very few studies of this type have been reported for neural transcription factors. Implementation of microarray analysis to identify the targets of a transcription factor requires the ability to obtain a relatively homogeneous population of cells which, under normal conditions, express the transcription factor of interest. The majority of the nervous system consists of multiple interspersed subtypes of neurons and glia. Harvesting RNA from a heterogeneous population of cells results in poor signal to noise ratio in the measurement of gene expression changes caused by loss of the transcription factor.

The E13.5 trigeminal ganglia and dorsal root ganglia are ideal tissues for analyzing the transcriptional targets of Brn3a because they are nearly homogeneous populations of cells (with respect to Brn3a expression), and are reproducibly

dissectible from wild-type and Brn3a knockout embryos. In addition to Brn3a expressing neurons, the E13.5 sensory ganglia also contain glial precursors which comprise a relatively small percentage of the nuclei in the ganglia. Microarray analysis of the trigeminal and dorsal root ganglia in Brn3a null embryos demonstrated highly reproducible changes in the expression of several transcripts. Nearly all of the changed transcripts were members of a few categories with clear relevance to neural development, including neurotransmitters, neurotransmitter receptors, mediators of axon growth and guidance, and neural transcription factors.

A distinct limitation of microarray analysis of transcription factor target genes is that direct and indirect transcriptional targets are indistinguishable. Because microarrays measure only relative transcript levels, it is impossible to determine whether a transcript is modulated directly by the transcription factor of interest. Many of the Brn3a target genes identified by microarray analysis are transcription factors which are likely to regulate the expression of other genes. Thus, some of the genes with changed expression in Brn3a knockout sensory ganglia are likely to be indirectly regulated by Brn3a.

Direct vs indirect regulation.

In order to understand the role of tissue-specific transcription factors, it is critical to identify their direct transcriptional targets. Because transcription factors exhibit sequence specific binding, one requirement for direct regulation is the presence of a specific DNA binding site within the regulatory regions of the target gene. Much

effort has been focused on creating sequence-based bioinformatic methods for identifying the target genes of transcription factors by searching for consensus binding sites in the genome. However, as we have shown here, the number of potential binding sites in the genome far exceeds the number of regulatory targets for most transcription factors. Although bioinformatic analysis provides a good starting point for the identification of direct transcriptional targets, current bioinformatic tools are incapable of distinguishing between functional and nonfunctional binding sites.

In order to empirically identify sites occupied by Brn3a in vivo, we employed ChIP analysis of microdissected embryonic sensory ganglia. ChIP assays have been most commonly performed using cultured cells, and very few studies have been reported using ChIP analysis of dissected neural tissue(Skowronska-Krawczyk, Ballivet et al. 2004; Zhou, Le et al. 2004). The cellular heterogeneity of the nervous system and the difficulty of obtaining sufficient amounts of neural tissue make in vivo ChIP assays of the developing nervous system difficult. However, for the study of midgestation embryonic sensory neurons, no suitable cell culture model exists. By performing the analysis using dissected sensory neurons, we were able to ensure that the assays provided information that is directly relevant to sensory neural development.

We employed an additional modification to established ChIP methods by using multiple real-time PCR primer pairs (Locus-ChIP), spaced at short intervals across target gene loci to measure Brn3a binding in vivo (Litt, Simpson et al. 2001). The use of multiple real-time PCR primer pairs allowed us to perform an unbiased search for

sites of Brn3a occupancy, without making any assumptions about the sites to which Brn3a might bind in vivo and without arbitrarily restricting our analysis to the promoter regions of the genes of interest. Furthermore, each primer pair served as an independent assay, providing multiple verifications of enrichment. Utilization of multiple primer pairs to survey entire gene loci allowed us to approximate the array-based ChIP method while using a fraction of the amount of starting material.

Prior analysis using a transgenic reporter demonstrated that Brn3a directly regulates its own expression in vivo. Locus-ChIP analysis of E13.5 trigeminal ganglia showed in vivo Brn3a occupancy of binding sites within the autoregulatory enhancer, verifying the direct nature of Brn3a autoregulation. Locus-ChIP assays also demonstrated that Brn3a directly binds to consensus sites within the NeuroD1 and NeuroD4 loci, both of which are increased in Brn3a null mice. NeuroD1 and NeuroD4 are neurogenic factors whose expression is temporarily required for the induction of neural cell fate in early development (Kageyama and Nakanishi 1997; Takebayashi, Takahashi et al. 1997; Ma, Chen et al. 1998; Bertrand, Castro et al. 2002). As Brn3a expression commences, around the time that neurons are exiting the cell cycle, a plausible role for Brn3a is to repress the expression of early neurogenic factors which are no longer required.

Context-dependent binding and activity of transcription factors.

The work presented in this dissertation provides insight into understanding the nature of target gene selection by tissue-specific transcription factors. Since it is clear

that many transcription factor binding sites are not involved in transcriptional regulation, an important question is whether Brn3a occupies all of its potential genomic binding sites at levels proportional to site affinity or whether its activity is regulated at the level of binding. Multiple studies using ChIP and serial analysis of chromatin occupancy (SACO) have provided evidence that transcription factors selectively occupy specific binding sites in the genome in a context-dependent manner (Impey, McCorkle et al. 2004; Im, Grass et al. 2005; Zhang, Odom et al. 2005; Marson, Kretschmer et al. 2007; Zheng, Josefowicz et al. 2007). In the course of performing ChIP analysis using Brn3a antibody, we observed several Brn3a consensus binding sites within the regions of analysis which were not occupied by Brn3a in vivo. In contrast, although ChIP assays clearly demonstrate that Brn3a associates with its autoregulatory enhancer in vivo, the binding sites associated with Brn3a autoregulation are somewhat diverged from the Brn3a consensus sequence. This suggested that Brn3a occupancy of its potential binding sites in vivo may not be directly proportional to in vitro site affinity.

We tested this possibility by making a comparison between in vitro affinity of Brn3a binding sites and Brn3a occupancy of the same sites in vivo. The results of this analysis clearly showed that Brn3a affinity for DNA binding sites is not always predictive of site occupancy in vivo. Additional factors must influence the ability of Brn3a to associate with potential binding sites in the genome. One potential explanation for this phenomenon is that Brn3a binding to DNA may be stabilized by an additional transcription factor bound to an adjacent site. Consistent with this

hypothesis, analysis of the genomic sequences containing occupied Brn3a binding sites revealed that nearly all of the occupied Brn3a binding sites occur within regions of extended interspecies conservation. However, careful analysis of the conserved sequences has failed to identify any DNA motif that would suggest a potential binding partner common to these sites.

An additional possibility is that the underlying chromatin may affect the ability of Brn3a to occupy potential binding sites in vivo. Clearly the state of chromatin exerts enormous influence over the physical conformation and accessibility of DNA. Active transcription of DNA requires an open, accessible conformation of chromatin characterized by the presence of specific covalent modifications of histone proteins. A growing body of evidence suggests that the binding of tissue-specific transcription factors to regulatory elements may also require specific chromatin modifications. In rat cortical progenitor cells, the presence of methylated H3K9 at the GFAP promoter prevents the binding of the STAT3 complex. FGF2 stimulation catalyzes a switch from methylated H3K9 to methylated H3K4, allowing STAT3 to occupy the GFAP promoter and activate transcription (Song and Ghosh 2004). Similarly, in developing muscle cells, binding of the bHLH transcription factor MyoD to specific binding sites at the myogenin locus may require ATP-dependent chromatin remodeling and hyperacetylation of H4 (de la Serna, Ohkawa et al. 2005). CpG methylation has also been shown to influence transcription factor binding. Genome-wide analysis of CREB binding revealed that methylation of CREs blocks CREB binding in vivo (Zhang, Odom et al. 2005).

In order to test whether chromatin conformation may contribute to the binding specificity of Brn3a, we performed ChIP analysis using antibodies recognizing specifically modified histones. We assayed the genomic loci of genes found to be directly regulated by Brn3a for acetylation of H3K9/K14 and dimethylation of H3K4. We found a striking correlation between occupancy of Brn3a binding sites and the state of chromatin. Brn3a preferentially associates with binding sites located within regions of chromatin characterized acetylated H3K9/K14 and dimethylated H3K4. For example, low affinity Brn3a binding sites within the autoregulatory enhancer, which contains acetylated H3K9/K14 and dimethylated H3K4, are occupied by Brn3a in vivo. In contrast, several optimal Brn3a binding sites located in the promoters of inactive genes and characterized by deacetylated H3 were unoccupied by Brn3a in vivo. These results demonstrate that, rather than being determined exclusively by DNA sequence, Brn3a binding in vivo is context-dependent. Brn3a preferentially occupies evolutionarily conserved sites located in genomic regions containing chromatin modifications which are representative of relaxed, accessible chromatin.

Cell-specific functions of transcription factors.

Several known tissue-specific transcription factors are required for the proper development of multiple diverse tissues. Islet1, for example, is critical for the development of the heart, sensory neurons, spinal motor neurons, and endocrine pancreas. Likewise, Brn3a expression is required for the proper development of two similar but distinct sensory structures, the trigeminal and dorsal root ganglia and

specific populations of neurons in the central nervous system. Thus a key question centers on whether these factors regulate the same or different sets of target genes in different tissues. Our microarray analyses have shown that the majority of Brn3a targets are conserved between the trigeminal and dorsal root ganglia, but have also revealed a few clear differences. Specifically, these include a subset of genes that are upregulated in Brn3a null trigeminal, but remain unchanged in the dorsal root ganglia.

Our results demonstrating the influence of chromatin modification over Brn3a binding suggested a potential mechanism by which a gene could be regulated by a particular transcription factor in one tissue but not another. Evidence for a role of chromatin conformation in cell-type specific transcriptional regulation has been provided by an examination of CREB occupancy of CRE-containing promoters in PC12 and H4IIE cells, and primary cortical neurons which showed that CREB occupies these promoters in a cell-type specific manner. The promoters of four genes with high CREB occupancy in stimulated PC12 cells also showed high H3K4 methylation even in the unstimulated state, but unoccupied promoters were H3K4 demethylated (Cha-Molstad, Keller et al. 2004). We assayed the state of chromatin of the differentially regulated genes in the trigeminal and dorsal root ganglia. We found that the gene loci that were specifically upregulated in Brn3a null trigeminal but not dorsal root ganglia were also specifically H3K9/K14 acetylated in the trigeminal ganglia even in wild type ganglia in which they were minimally expressed or silent.

This suggests a model in which Brn3a is not required for silencing these genes in the dorsal root ganglia, due to an existing repressive conformation of chromatin.

Transcriptional repression by Brn3a in these cases is redundant. Cells in the trigeminal and dorsal root ganglia have accumulated distinct modifications of chromatin at certain loci, presumably because of differences in developmental history. Chromatin modifications may constitute a developmental memory for a cell and its lineage. Thus, by inducing chromatin modifications, a transcription factor may continue to influence target gene transcription even after it is no longer expressed. Such modifications regulate the expression of many genes and also regulate the activity of tissue specific transcription factors. The work presented here has demonstrated that, in addition to the sequence of a Brn3a binding site, its genomic location and chromatin conformation may also be critical determinants of site occupancy in vivo.

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