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Review

Mechanisms and perspectives on differentiation of autonomic neurons

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Abstract

Neurons share many features in common but are distinguished by expression of phenotypic characteristics that define their specific function, location, or connectivity. One aspect of neuronal fate determination that has been extensively studied is that of neurotransmitter choice. The generation of diversity of neuronal subtypes within the developing nervous system involves integration of extrinsic and intrinsic instructive cues resulting in the expression of a core set of regulatory molecules. This review focuses on mechanisms of growth and transcription factor regulation in the generation of peripheral neural crest-derived neurons. Although the specification and differentiation of noradrenergic neurons are the focus, I have tried to integrate these into a larger picture providing a general roadmap for development of autonomic neurons. There is a core of DNA binding proteins required for the development of sympathetic, parasympathetic, and enteric neurons, including Phox2 and MASH1, whose specificity is regulated by the recruitment of additional transcriptional regulators in a subtype-specific manner. For noradrenergic neurons, the basic helix-loop-helix DNA binding protein HAND2 (dHAND) appears to serve this function. The studies reviewed here support the notion that neurotransmitter identity is closely linked to other aspects of neurogenesis and reveal a molecular mechanism to coordinate expression of pan-neuronal genes with cell type-specific genes.
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Keywords: Neurons; Mechanisms; Perspectives; HAND2; Neural crest

Introduction

A challenge in developmental neurobiology is to delineate the molecular mechanisms governing diversification of neuronal phenotypes developing from multipotent progenitor cells. In the vertebrate nervous system, two broad classes of neuronal progenitor cells have been identified; multipotent cells resident within the ventricular zone and neural crest-derived cells. Neuronal elements (neurons, astrocytes, oligodendrocytes) in the central nervous system are derived from the cells in the ventricular zone while peripheral neurons and schwann/glial cells are derived from the neural crest. Although progenitor cells giving rise to central and peripheral neurons themselves have a different developmental history, differentiation of these cells into neurons appears to follow a general pattern. Proneural genes, members of the basic helix-loop-helix (bHLH) family of transcription factors, initially select progenitor cells previously specified to a neuronal fate. In a series of subsequent steps, the activity of proneural genes, either alone or in concert with additional "neuronal differentiation" genes, activates the NOTCH signaling pathway, cell cycle withdrawal, and pan-neuronal and cell type-specific gene expression (for review, see Bertrand et al., 2002; Briscoe and Ericson, 2001; Jessell, 2000). How the expression of pan-neuronal characteristics is coordinated with cell type-specific gene expression remains a central question for both central and peripheral neurons. Identification and characterization of choice points in the process of specification and differentiation of neurons are exemplified in neural crest-derived noradrenergic sympathetic ganglion neurons. Sympathetic ganglion neurons have provided an excellent system in which to assess the contribution of both genetic and epigenetic interactions required for the differentiation of this cell type. This review will focus on growth and

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transcription factor interactions in the generation of peripheral neurons, emphasizing the differentiation of sympathetic noradrenergic neurons.

Extrinsic signals

To understand fully how neuronal diversity is generated begs the question of how neural progenitors become fate restricted in the appropriate location; this is a particularly compelling issue for neural crest-derived neurons as the majority differentiate at a great distance from their site of origin on the dorsal neural tube. Several extrinsic signals providing instructive cues for fate determination of neural crest-derived neural progenitors have been identified (Hagedorn et al., 2000; Hari et al., 2002; Lee et al., 2004; Morrison et al., 2000; Paratore et al., 2002; Schneider et al., 1999; Shah et al., 1996). The most prominent of these factors are bone morphogenetic proteins (BMPs) (Schneider et al., 1999) and Wnt (Hari et al., 2002; Lee et al., 2004). Defining the exact timing when neural crest-derived cells become fate restricted to a particular neuronal lineage is controversial, but it is clear that multipotent progenitor cells are resident in many neural crest-derived structures (Hagedorn et al., 1999; Morrison et al., 1999; Sommer, 2001), suggesting an interplay between cell extrinsic and intrinsic factors as a mechanism regulating cell fate decisions. Sensory neuron progenitors are apparently fate restricted as they emigrate from the neural tube (Ma et al., 1999; Zirlinger et al., 2002) or very soon thereafter. This early fate restriction is the result of Wnt-activated β-catenin signaling (Hari et al., 2002; Lee et al., 2004). Forced expression of β-catenin in neural crest cells results in aberrant rostral migration, aggregation, and differentiation into ectopic neurons expressing the sensory neuron markers NeuroD, Brn-3a, and neurogenin 2 (Ngn) (Lee et al., 2004). In an elegant series of studies, Lee et al. (2004) present compelling evidence demonstrating that neural crest cells exposed to Wnt1 or cells constitutively expressing β-catenin differentiate into sensory neurons at the expense of all other neuronal phenotypes. These studies show signaling via the canonical Wnt signaling pathway serves as an instructive signal specifying neural crest cells as sensory neurons. A very interesting question raised by this and other studies is how are signals segregated so that all neural crest cells exposed to Wnt1 and Wnt3a in the dorsal neural tube are prevented from differentiating as sensory neurons. Another way to look at this issue is to ask how intrinsic heterogeneity is generated. For neural crest cells, heterogeneity could be determined stochastically or rather as a consequence of local gradients of signaling molecules. Either could provide a mechanism for coherent cohorts of cells to differentially respond to local environmentally derived instructive signals. This is a particularly interesting issue because at the time that neural crest cells remain associated on the dorsal neural tube they are exposed to Wnt1, Wnt3, BMPs, and TGF-βs; which (BMP and TGF-β) family member depends upon the species. Each of these signaling molecules provides instructive cues for fate restriction or specification to a particular phenotype (Fig. 1). Responsiveness to any particular signaling molecule present in the local environment might have spatial and temporal constraints because all neural crest cells exposed to Wnt1 in situ, for example, do not differentiate as sensory neurons. The temporal effects on cellular responsiveness are particularly evident for neural crest cells giving rise to noradrenergic sympathetic ganglion neurons.

Early studies implicated the neural tube and notochord as sources of instructive factors required for the differentiation of neural crest-derived cells into catecholaminergic neurons in the trunk of avian embryos (Cohen, 1972; Groves et al., 1995; Howard and Bronner-Fraser, 1985, 1986; Norr, 1973; Stern et al., 1991; Teillet and Le Douarin, 1983). In embryos where the neural tube or notochord was either removed or rotated through 90°, results suggested two sources of required factors for normal localization and differentiation of catecholaminergic cells (Groves et al., 1995; Stern et al., 1991; Teillet and Le Douarin, 1983). These studies suggested that neural tube and notochord provide some soluble signal(s) required for both appropriate migration and differentiation as well as implicating the dorsal aorta as the source of a signal required for the differentiation of sympathetic ganglion neurons. The neural tube-derived factor was tentatively identified as a member of the TGF-β family of factors (Howard and Gershon, 1993) while the dorsal aorta is the source of BMPs (Reissmann et al., 1996; Shah et al., 1996). Studies in vitro and in vivo establish BMP as a proximal signal supporting expression of a transcription factor cascade required for the differentiation of sympathetic ganglion neurons as well as the acquisition of a noradrenergic phenotype (Fig. 1).

BMPs are essential for sympathetic neuron development

BMPs were shown to be essential determinants of neuronal differentiation by demonstrating an increase in the number of neural crest-derived cells expressing tyrosine hydroxylase (TH) or norepinephrine in cultures supplemented with BMP2, 4, or 7 (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996; Varley et al., 1995). The capacity of BMP4 to support neurogenesis and selective generation of autonomic and sensory progenitors has also been demonstrated in embryonic stem cells (Mizuseki et al., 2003). An in vivo requirement for BMP4 was nicely demonstrated using gain-of-function approaches in avian embryos by ectopically expressing BMP4 using retrovirus-mediated gene transfer (Reissmann et al., 1996). In cells ectopically expressing BMP4, the effectiveness of BMP4 in

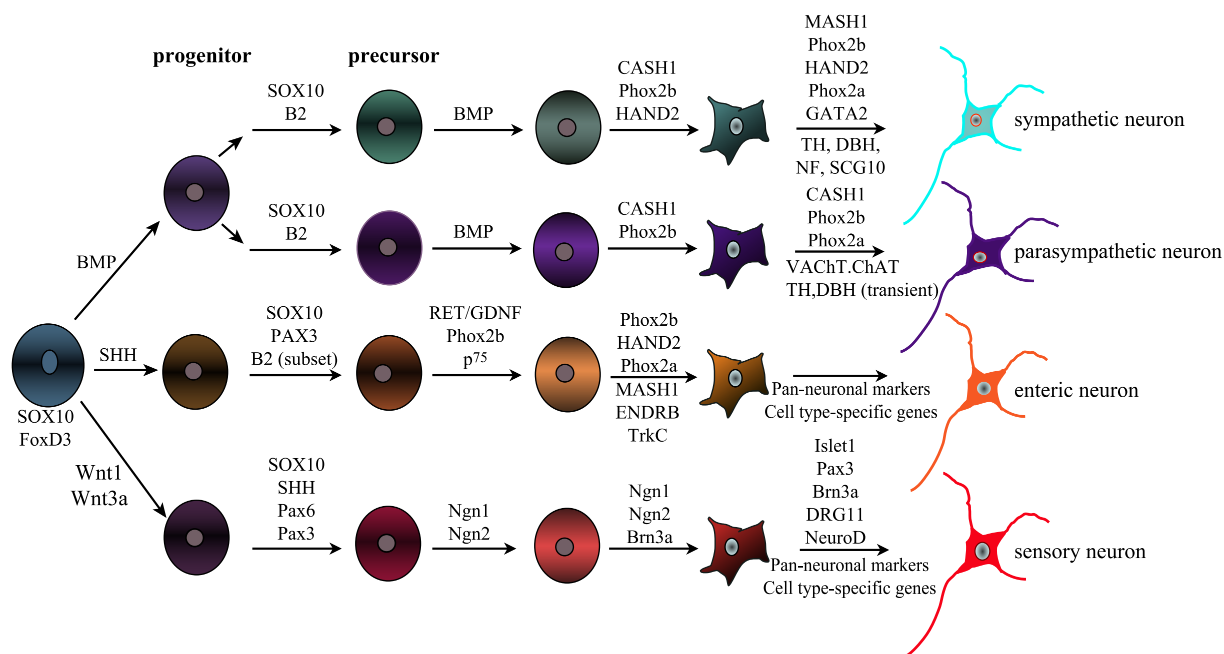

Fig. 1. Analyses in vitro and in vivo have identified a series of growth factors and transcriptional regulators (markers) affecting different stages of neurogenesis of neural crest-derived progenitor cells. This schematic diagram summarizes work from many laboratories and includes some information not explicitly described in the body of the text; the complied data provides a roadmap for following important hallmark events in the development of autonomic, enteric, and sensory neurons. Neural crest cells segregated from the neuroepithelium can be identified by the expression of FoxD3 and SOX10 (among other markers); cells expressing FoxD3 give rise to neurons and not melanocytes (Kos et al., 2001). SOX10 maintains multipotency in neural crest-derived cells as well as neurogenic potential (Kim et al., 2003). In the enteric nervous system, SOX10 and Pax3 together regulate RET (Lang and Epstein, 2003), which is required for normal development of these neurons. Progenitor cells differentiate into sympathetic, parasympathetic, enteric, or sensory neurons in part dependent upon instructive signals encountered early at or near the time of egress from the neural tube. Additionally, extrinsic cues encountered during migration or at sites where neural crest-derived cells differentiate influence patterns of gene expression. In autonomic ganglia, expression of HAND2 appears to select cells as noradrenergic sympathetic ganglion neurons as well as functioning in cell type-specific gene expression. In the sensory neuron lineage, the POU domain transcription factor Brn3a, expressed downstream of Ngn, regulates a large array of genes influencing cell death, neurotransmitter expression, and axon guidance (Eng et al., 2004). The signaling molecule sonic hedgehog (SHH) is necessary for the expression of neurogenin (Ungos et al., 2003). In the enteric nervous system, HAND2 is expressed downstream of Phox2b (Christos Goridis, personal communication) in all segments of the developing gut (Wu and Howard, 2002); the function of HAND2 in development of enteric neurons is unknown. The neurotrophin receptor TrkC is expressed early by neural crest-derived cells whose potential is restricted to neuronal or glial lineages (Luo et al., 2003) as well as a subset of enteric neurons (Chalazonitis et al., 2001).

supporting differentiation into sympathetic-like neurons was evidenced by the expression of the noradrenergic marker gene TH. The expression of BMP2, 4, and 7 in cells of the dorsal aorta in mammals (Shah et al., 1996) and avians (Reissmann et al., 1996) suggested an in vivo role of BMP in the differentiation of sympathetic ganglion neurons. Since null mutation of BMP4 is embryonic lethal prior to the generation of peripheral neurons (Winnier et al., 1995; Zhang and Bradley, 1996), a different strategy was needed to assess the effects of loss of BMP function on generation of sympathetic ganglion neurons, in situ. Loss-of-function was achieved using the BMP4/7 antagonist Noggin (Smith and Harland, 1992; Zimmerman et al., 1996) by the implantation of Noggin-releasing beads at the dorsal aorta (Schneider et al., 1999). Inhibition of BMPs at the site where neural crest-derived cells differentiate into sympathetic ganglion neurons resulted in the loss of noradrenergic marker genes TH and dopamine-β-hydroxylase (DBH) as well as the pan-neuronal markers neurofilament 160 and SCG10. Similar studies demonstrate an essential role for BMPs in the differentiation of parasympathetic ciliary ganglion neurons, with BMP5 and BMP7 as candidate signals (Muller and Rohrer, 2002). In vitro, neural crest-derived cells differentiate into noradrenergic neurons in response to BMP2, 4, and 7 (Howard et al., 2000; Reissmann et al., 1996; Varley and Maxwell, 1996; Varley et al., 1995; Wu and Howard, 2001). This response is mimicked by constitutive expression in neural crest-derived cells of BMP type IA receptors (McPherson et al., 2000; Varley et al., 1998) and most likely involves translocation of SMAD1 to the nucleus (Wu and Howard, 2001). Taken together, these studies implicate BMP2, 4, 5, and 7 as in vivo instructive cues inducing differentiation of sympathetic and parasympathetic ganglion neurons (Muller and Rohrer, 2002; Reissmann et al., 1996; Schneider et al., 1999). However, which BMP family member is the essential factor and whether BMPs provide the only instructive cue remains unclear.

Once neural crest-derived cells have aggregated around the dorsal aorta, the first marker of their specified "autonomic" fate is expression of CASH1 in avians (Ernsberger et al., 1995; Groves et al., 1995) and MASH1

in rodents (Guillemot and Joyner, 1993; Lo et al., 1991), the vertebrate homologue of the *Drosophila* proneural genes acheate-scute (Guillemot and Joyner, 1993; Jasoni et al., 1994; Johnson et al., 1990). Transcripts encoding CASH1 are first detected in neural crest-derived cells in the region of the dorsal aorta of chick embryos at Hamburger and Hamilton's (1951) stage 15 (Ernsberger et al., 1995; Groves et al., 1995). Thus, CASHI is expressed prior to the time that BMP can be detected in the dorsal aorta (Ernsberger et al., 1995; Groves et al., 1995; McPherson et al., 2000) and prior to the time that BMP Type I receptors are expressed in precursors of sympathetic ganglion neurons (McPherson et al., 2000). Additionally, in avian embryos when Noggin-releasing beads are implanted around the dorsal aorta at a time when migrating neural crest-derived cells localize there, sympathetic ganglion neurons fail to differentiate and CASH1 expression is only reduced but not extinguished (Schneider et al., 1999). In rat neural crest-derived cells, BMP2 supports the maintained expression of MASH1 (Lo et al., 1997), suggesting that the role of BMP is to maintain the expression of CASH1 (MASH1) rather than to induce its expression (Lo et al., 1997; Schneider et al., 1999). Alternatively, it is possible that in the primordia of the sympathetic ganglia, BMP growth factors synthesized and secreted by cells resident within the developing ganglion function as a paracrine signal following initial induction by aorta-derived BMP (McPherson et al., 2000). Regardless of how this conundrum is resolved, these studies establish BMP as an essential determinant of the noradrenergic neuron phenotype.

Intrinsic factors involved in the generation of peripheral neurons

The requirement for BMP growth factors in the differentiation of autonomic neurons is supported by both in vitro and in vivo studies (Howard and Gershon, 1993; Muller and Rohrer, 2002; Reissmann et al., 1996; Schneider et al., 1999; Shah and Anderson, 1997; Shah et al., 1996; Varley et al., 1995). The mechanism underlying BMP-induced differentiation of autonomic neurons, although not completely understood, is based on the coordinate expression of transcription factors in neural progenitors once they localize at the sites where they will ganglionate; for the primary sympathetic chain, this is the dorsal aspect of the dorsal aorta and a site along the oculomotor nerve in the retro-orbital mesenchyme for parasympathetic ciliary ganglion neurons (Fig. 1). Common to both sympathetic and parasympathetic neurons, this group of transcription factors includes the homeodomain (HD) DNA binding proteins Phox2b and Phox2a (Lo et al., 1999; Morin et al., 1997; Pattyn et al., 1997, 1999; Stanke et al., 1999) and the bHLH DNA binding protein CASH1 (MASH1) (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998). Additional transcription factors induced in sympathetic ganglion precursors are the bHLH DNA binding protein HAND2 (dHAND) (Howard et al., 1999, 2000) as well as the zinc finger proteins GATA2 expressed in avian embryos (Groves et al., 1995) and GATA3 expressed in mouse embryos (Lim et al., 2000; Pandolfi et al., 1995). Examination of the temporal expression profile of these DNA binding proteins in neural crest-derived cells destined to differentiate as noradrenergic neurons suggested a model of two parallel but interacting pathways of gene regulation (Fig. 2). Further, the compiled data suggested that specificity in the function of these genes is based, in part, on their ability to cross-regulate expression as well as act as co-transcriptional regulators (see below).

In vertebrates, bHLH DNA binding proteins have several functions including specification of neuronal progenitors, neuronal fate, and differentiation (for review, see Bertrand et al., 2002; Massari and Murre, 2000). However, as our understanding of the function of this family of genes has increased, it has become evident that for some of these factors their function is determined by the cellular context in which they are expressed. As a rule, homeodomain DNA binding proteins function in specification of cell type-specific identity. Interestingly, it has become a common theme to find that bHLH and HD factors together function in transcription factor cascades to regulate the steps required for cell determination and differentiation (Bertrand et al., 2002; Jan and Jan, 1993; Kintner, 2002; Lee, 1997; Lee and Pfaff, 2003; Weintraub, 1993; Xu et al., 2003). In addition, it has become increasingly evident that homeodomain and zinc-finger DNA binding proteins function in concert to modify or regulate the activity of bHLH factors. In the context of developing sympathetic noradrenergic neurons, results of gain-of-function or loss-of-function studies did not always provide predicted outcomes, suggesting novel functions/interactions for some of the transcriptional regulators important for generation of this phenotype.

Our understanding of how neural precursors are selected for the generation of neural crest-derived peripheral neurons remains limited. For some lineages, notably sensory neurons, proneural genes have been identified and their function well studied (Bertrand et al., 2002; Ma et al., 1999; Nakada et al., 2004; Quan et al., 2004; Scardigli et al., 2003; Schuurmans et al., 2004). Identification of a proneural gene that accounts completely for the selection of precursors of autonomic neurons remains obscure. In the central nervous system, MASH1 functions as a proneural gene (for review, see Bertrand et al., 2002; Ross et al., 2003) as well as in neuron subtype specification (Parras et al., 2001), but this does not seem to be the case in peripheral neural crest-derived neurons (Parras et al., 2001; Sommer et al., 1995); in peripheral ganglia, MASH1 is required for expression of pan-neuronal marker genes. In mice null for expression of MASH1, all noradrenergic neurons, both central and peripheral, are affected (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998). MASH1 expression is extinguished prior to or just after neural differentiation (Guille-

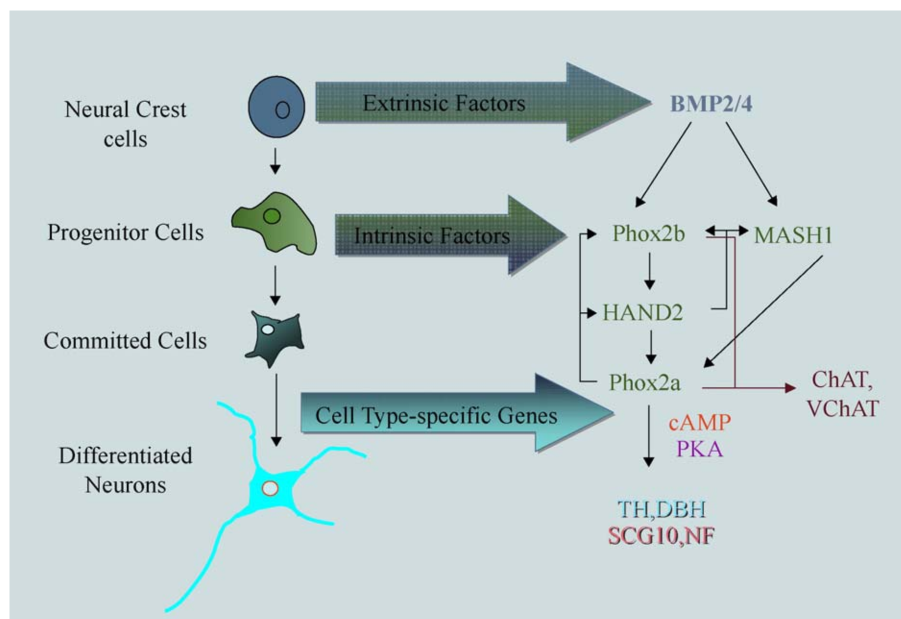

Fig. 2. Specification and differentiation of peripheral autonomic neurons are dependent upon the interplay between cell extrinsic and cell intrinsic factors. Initial instructive cues from the neural tube influence neural crest cells that then respond to BMPs derived from the dorsa aorta or retro-orbital mesenchyme. Induction of Phox2b and MASH1 is followed by the induction of HAND2 and Phox2a resulting in expression of pan-neuronal (SCG10, NF) and cell type-specific (TH, DBH, ChAT, VAChT) genes. Since the recognized transcription factors required for the differentiation of noradrenergic sympathetic and cholinergic parasympathetic neurons are the same, with the notable exception of HAND2. It is compelling to suggest that this diversity could be the result of exclusive expression of HAND2 in precursors of noradrenergic sympathetic ganglion neurons. Cross-regulation of transcription factor expression suggests patterns of regulation based on generation of local gradients.

mot et al., 1993), suggesting that additional regulators are required for specification of neurotransmitter and other aspects of a neuronal phenotype.

In vertebrates, the list of proneural genes is confined to MASH1, Ngn, MATH1, and MATH5. In mice null for expression of Ngn1 or Ngn2, as well as in zebrafish where expression of Ngn1 is blocked, cranial sensory ganglia are affected (Andermann et al., 2002; Fode et al., 1998; Ma et al., 1998, 1999; Scardigli et al., 2001). However, in animals carrying a double mutation for Ngn1 and Ngn2, in addition to lacking cranial sensory ganglia, spinal sensory ganglia and a subgroup of ventral spinal cord neurons are affected as well (Fode et al., 1998; Gowan et al., 2001; Ma et al., 1998, 1999; Scardigli et al., 2001). Deletion of MASH1 results in deficits in the generation of neural progenitors in the olfactory sensory epithelium and in the ventral telencephalon (Casarosa et al., 1999; Cau et al., 2002; Guillemot et al., 1993; Horton et al., 1999; Ross et al., 2003), but not in the sympathetic chain ganglia (Sommer et al., 1995). This suggests that additional gene products with proneural activity in the autonomic branch of the peripheral nervous system remain to be identified (Hassan and Bellen, 2000).

The activity of HAND2 has some aspects of proneural function. In vitro, ectopic expression of HAND2 increases withdrawal of neural crest-derived cells from the cell cycle and their subsequent differentiation into NE neurons (Howard et al., 1999). Ectopic expression of HAND2, both in vitro and in vivo, is sufficient for the expression of pan-neuronal and NE-specific marker genes (Howard et al., 1999, 2000). HAND2 therefore couples neurogenesis with cell-type-specific gene expression but is not expressed early enough to classify it as a proneural gene. Additionally, HAND2 has not been shown competent to induce differentiation of NE neurons at the expense of other phenotypes. Although a proneural gene for autonomic derivatives has not yet been identified, the function of MASH1 is closely linked to Phox2a and Phox2b, homeodomain DNA binding proteins that regulate expression of the NE marker genes TH and DBH (Brunet and Pattyn, 2002; Goridis and Brunet, 1999; Hirsch et al., 1998; Stanke et al., 2004; Swanson et al., 1997, 1998; Zellmer et al., 1995; Yang et al., 1998); the encoded proteins are the rate limiting and final (respectively) biosynthetic enzymes required for NE synthesis (Fig. 2). Since the proper development of neural circuits requires that neurons differentiate at the appropriate time, in the correct place, and that they express characteristics specific to their function and location, the function in the periphery of MASH1 as a neuronal differentiation gene is quite important and is likely required for maintaining the expression of Phox2a (see below).

Co-regulation of pan-neuronal and cell type-specific genes

One aspect of specification of neuronal identity is the coordinated expression of genes that regulate expression of pan-neuronal (generic) properties with those that specify

specific aspects of cellular identity. Several lines of experimental evidence suggest that MASH1 might coordinate expression of pan-neuronal and cell type-specific genes via its influence on the expression of Phox2a (Hirsch et al., 1998; Lo et al., 1998). This conclusion is based on both gain-of-function and loss-of-function phenotypes where both neurogenesis and expression of Phox2a are affected. In rodents, BMP2 induces expression of MASH1 and Phox2a, but neither TH nor DBH: this suggests that additional factors are required for the expression of NE marker genes as well as implicating a non-overlapping pathway in which HAND2 is expressed (Fig. 2). While expression of Phox2a alone is not sufficient to support expression of noradrenergic characteristics (Lo et al., 1998), expression of Phox2a with co-activation of PKA supports expression of TH but not neuronal differentiation in rodent neural crest-derived cells (Lo et al., 1999). Under these same conditions (expression of Phox2a and activated PKA), the addition of BMP2 supports expression of TH and DBH as well as differentiation into neurons (Lo et al., 1999). Several lines of evidence suggest that HAND2 might link the function of MASH1 and Phox2a with BMP, supporting both neurogenesis and cell type-specific gene expression (Howard et al., 2000; Xu et al., 2003). Ectopic expression of HAND2, both in vitro (Howard et al., 1999) and in vivo (Howard et al., 2000) is sufficient for expression of pan-neuronal and NE cell type-specific genes as well as generation of NE neurons. The most parsimonious model suggests that MASH1 is required for the sustained expression and regulation of Phox2a; the majority of data describing the epistatic relationship of transcriptional regulators thus far identified as required for specification/differentiation of NE neurons combined with functional consequences of gain-of-function or loss-of-function assays indicates a complex of factors that can cross-regulate each other's expression (Fig. 2).

At the proximal level of the transcription network required for noradrenergic differentiation are Phox2b and MASH1. The Phox2 proteins are widely expressed in the nervous system and are necessary for the specification/differentiation of both central and peripheral neurons (for review, see Brunet and Pattyn, 2002; Young et al., 2003). In the periphery, loss-of-function studies demonstrate that both enteric and autonomic neuron precursors depend upon Phox2b for neuronal determination (for review, see Brunet and Pattyn, 2002; Goridis and Rohrer, 2002; Pattyn et al., 1997, 1999). In addition, Phox2b is an essential regulator of the NE phenotype and possibly the cholinergic phenotype (Morin et al., 1997; Muller and Rohrer, 2002; Pattyn et al., 1999). Based on loss-of-function studies, autonomic neurons do not develop in mice lacking Phox2b and only marginally in animals null for MASH1 (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999). However, the effect of loss-of-function of MASH1 or Phox2b on subsequent expression of pan-neuronal and cell type-specific genes suggests that two interacting but parallel pathways of transcriptional regulation account for differentiation of sympathetic noradrenergic neurons.

Expression of Phox2b and MASH1 initially occurs independently, supporting the notion of two interdependent cascades of transcriptional regulators required for the differentiation of noradrenergic neurons (Fig. 2). It is noteworthy that expressions of both Phox2b and MASH1 depend upon the HMG-box factor SOX10 (Kim et al., 2003). Inasmuch as one function of SOX10 is to inhibit overt neuronal differentiation, it is tempting to speculate that neural crest cells that will differentiate into noradrenergic neurons are maintained as progenitors until exposed to BMP4 at the dorsal aorta. This would provide a mechanistic basis for the findings that while ectopic expression of MASH1 is not sufficient to induce differentiation of noradrenergic neurons, ectopic expression of HAND2 induces Phox2a, TH, DBH, NF, and SCG10, and synthesis of norepinephrine (Howard et al., 1999, 2000). While the expression of Phox2a may be dependent upon both Phox2b and MASH1, the expression of HAND2 appears independent of MASH1 (Howard, unpublished results) but dependent upon Phox2b (Howard et al., 2000; Goridis, personal communication). However, there must be an interaction between gene products expressed downstream of Phox2b and MASH1 because although noradrenergic neuron differentiation is essentially lacking in MASH1 null mice, Phox2b is expressed in precursor cells (Hirsch et al., 1998; Lo et al., 1998). Support for the notion that there are additional factors that link the function of Phox2b and MASH1 is based on the finding that HAND2 is not expressed in parasympathetic ciliary ganglion neurons (Muller and Rohrer, 2002), although MASH1 and Phox2b are essential for the differentiation of these parasympathetic neurons. Based on the current available data, we favor a model suggesting that HAND2 and Phox2a integrate neurogenesis and cell type-specific gene expression in the precursors of sympathetic ganglion neurons (Figs. 1 and 2). This model would predict that in cholinergic and non-autonomic noradrenergic neurons there are additional factors that either inhibit the expression of HAND2 or induce expression of additional transcriptional co-activators. The idea that HAND2 might be inhibited in inappropriate neurons or their precursors is supported by the finding that ectopic expression of HAND2 in young (ED6) or older (ED18) chick-dissociated dorsal root ganglia does not induce expression of NE marker genes in either neurons or support cells (Howard, unpublished observation). However, ectopic expression of HAND2 in precursors of parasympathetic cholinergic ciliary ganglion neurons supports sustained expression of NE marker genes (Muller and Rohrer, 2002). Interestingly, ectopic expression of BMP in the precursors of ciliary ganglion neurons induces expression of Phox2b but not HAND2, and the resulting neurons are cholinergic (Muller and Rohrer, 2002). One conclusion that can be drawn from these data is that expression of

HAND2 identifies autonomic neurons that will remain noradrenergic. With the exception of HAND2, all of the transcriptional regulatory genes identified as important for the generation of NE neurons are also required for the development of cranial parasympathetic ganglia (Fig. 1). Both MASH1 and Phox2 are necessary determinants of cranial parasympathetic ganglia (Hirsch et al., 1998; Morin et al., 1997; Pattyn et al., 1999). The fact that in the environment of the ciliary ganglion, BMP does not induce HAND2 or the differentiation of NE neurons implies early segregation of sympathetic and parasympathetic neuronal precursors (White and Anderson, 2001). Although not completely understood at this time, these data suggest not only the presence of additional transcriptional regulators controlling NE differentiation but also functions for HAND2 independent of specification or differentiation of NE neurons.

Additional transcriptional regulators shown to have a functional role in specification and differentiation of autonomic and serotonergic neurons are GATA-2 and GATA-3 (Groves et al., 1995; Patient and McGhee, 2002). The GATA family of zinc-finger DNA binding proteins has six members, some of which function in cell fate specification or cell type-specific gene expression (Karumaratne et al., 2002; Patient and McGhee, 2002; Zhou et al., 2000). GATA-4 and GATA-6 are highly expressed in neural crest, embryonic ectoderm, and neural tube (Nemer and Nemer, 2003), and are both upstream regulators of BMP4 (Nemer and Nemer, 2003). In very compelling studies employing avian embryos, the expression of GATA-2 has been demonstrated to be dependent upon BMP4 and induced by both Phox2b and HAND2 (Tsarovina et al., 2004; H. Rohrer, personal communication). Using ectopic expression of a dominant-negative form of GATA-2 in chick, a significant reduction in expression of TH as well as reduced neurogenesis implicates GATA-2 as an important transcriptional regulator in the differentiation of noradrenergic sympathetic ganglion neurons. In mouse embryos, GATA-2 functions in the specification of subgroups of serotonergic neurons but is also expressed downstream of homeodomain DNA binding proteins, implying an unusual epistatic relationship between GATA-2 and bHLH DNA binding proteins and HD proteins required for specification and differentiation of noradrenergic and serotonergic neurons. Because GATA-2 specifies cell fate in a subclass of rostral serotonergic neurons but downstream of required homeodomain DNA binding proteins (Craven et al., 2003), this raises the question of the function of GATA-2 in the specification or differentiation of noradrenergic sympathetic ganglion neurons. GATA-2 is expressed in precursors to sympathetic ganglion neurons (Groves et al., 1995) and has a functional role in both neurogenesis (Tsarovina et al., 2004) and expression of norepinephrine (Groves et al., 1995). The epistatic relationship of GATA-2 to other transcriptional regulators required for expression of TH and DBH in precursors of sympathetic NE neurons suggests the possible involvement of GATA-2 in the regulation of neurotransmitter biosynthesis. This idea is supported by the phenotype of animals carrying a null mutation of GATA-3. The phenotypes and defects described in animals carrying targeted mutation of GATA-3 suggest that HAND2 is one affected transcriptional regulator. In these animals, although Phox2 genes are expressed, expression of neither TH nor DBH is detectable. These animals appear to die due to lack of norepinephrine in sympathetic neurons (Lim et al., 2000). Our data implicate HAND2 as the critical component regulating expression and function of DBH in noradrenergic autonomic neurons (Howard et al., 2000; Xu et al., 2003). The link between GATA factors and HAND2 is functional. GATA family members are co-transcriptional regulators of cell-type-specific genes with HAND2 in the heart (Dai et al., 2002; McFadden et al., 2000) as well as in regulating transcription of DBH transiently expressed in P19 cells (Howard, unpublished observation). These results suggest GATA-2, in avian embryos, as a co-transcriptional activator with HAND2 and Phox2a of DBH.

How specification of cell-type-specific characteristics occurs in relation to the control of neuronal differentiation remains unclear. A necessary step in generating specific neuronal phenotypes from previously specified progenitors requires expression of genes encoding cell fate determinants. In the neural tube, organizing centers in the dorsal and ventral domain are well established. It is possible that neural crest cells receive some signals prior to their egress from the neural tube or that soluble factors provide signals to cells as they migrate. In whatever manner this signaling occurs, it is evident that bHLH transcription factors regulating general aspects of neuronal specification also regulate cell type-specific gene expression. We have presented evidence linking the function(s) of HAND2 with Phox2 proteins and BMP4 in the development of sympathetic (Howard et al., 2000; Xu et al., 2003) and enteric neurons (Howard, unpublished data; C. Goridis, personal communication; Wu and Howard, 2002). One aspect of the differentiated neuronal phenotype that is accessible to experimental manipulation and assessment is the coordinated expression of pan-neuronal and cell type-specific phenotypic characteristics. Although the mechanisms governing these interactions have not been well established for the majority of neurons, inroads have been made in our understanding of cell type-specific gene expression in noradrenergic sympathetic ganglion neurons. We suggest that HAND2 is a pivotal player in the differentiation of neural crest-derived noradrenergic sympathetic ganglion neurons linking expression of pan-neuronal genes (SCG10, NF) with the noradrenergic marker genes TH and DBH. In the nervous system, the expression of transcripts encoding HAND2 is restricted to sympathetic noradrenergic and enteric neurons (Howard et al., 1999; Wu and Howard, 2002). This pattern of expression sets HAND2 apart from other transcription factors required for the differentiation of noradrenergic neurons, such as Phox2a, Phox2b, and MASH1, which are also expressed in a diverse array of

non-noradrenergic neurons. To date, none of the transcriptional regulators identified as important in the determination of neurotransmitter phenotype appear to function exclusively in this role; specifications of other aspects of neural identity are linked to neurotransmitter determination (reviewed in Bertrand et al., 2002; Brunet and Ghysen, 1999; Goridis and Brunet, 1999). A review of our current knowledge suggests that proposing a simple hierarchical model of transcription factor function will not account for all available data on NE neuron development. In parasympathetic neurons, such as found in the ciliary ganglion, MASH1, Phox2b, and Phox2a do not induce the expression of HAND2 and noradrenergic phenotypic characteristics are only transiently expressed (Muller and Rohrer, 2002), suggesting that one function of HAND2 is to maintain the expression of noradrenergic characteristics in the appropriate neurons. Clearly, the different transcriptional regulators controlling specification and differentiation of sympathetic noradrenergic neurons have distinct functions within the various neuronal populations in which they are expressed.

Transcriptional regulation of neurotransmitter biosynthesis

Although only a few *cis*-acting regulatory elements that control expression of neuron subtype-specific genes have been identified, it is becoming evident that interactions between homeodomain and bHLH DNA binding proteins are key mechanisms coordinating expression and function of target genes (Dubreill et al., 2002; Lee and Pfaff, 2003; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001). We have expanded our understanding of one regulatory circuit involved in the determination of neuronal identity by demonstrating an interaction between HAND2 and Phox2a in transcriptional control of DBH (Xu et al., 2003). Using transient transfection in P19 and NT-2 cells, we have shown that HAND2 synergistically enhances Phox2a-driven transcriptional activity at the DBH promoter (Fig. 3). Transactivation of DBH by either Phox2a or Phox2a plus HAND2 is enhanced by elevating cAMP (Kim et al., 1994, 1998; Swanson et al., 1997, 1998, 2000; Xu et al., 2003). This was an intriguing result because of potential interaction with signaling cascades downstream of cAMP, BMP, and retinoic acid (RA), and involving MAPK pathways required early for the differentiation of noradrenergic neurons (Bilodeau et al., 2000; Dupin and Le Douarin, 1995; Holzschuh et al., 2003; Howard et al., 2000; Maxwell and Forbes, 1990; Rockwood and Maxwell, 1996; Wu and Howard, 2001).

That the BMP-induced generation of noradrenergic sympathetic ganglion neurons also involves signaling downstream of cAMP was inferred from studies showing that Phox2a induces the expression of TH in collaboration with cAMP downstream of BMP2 (Lo et al., 1999; Swanson et al., 1997). Additional sites of action of cAMP in the generation of NE neurons can be inferred from results demonstrating that elevated cAMP results in a loss of noradrenergic cells from neural crest-derived precursors in vitro (Bilodeau et al., 2000; Maxwell and Forbes, 1990). This result suggested that cAMP-responsive elements might regulate some of the genes required for expression of noradrenergic phenotypic characteristics. This is true for TH where at least three transcription factors, CREB, ATF1/2, and CREM, have been shown to regulate basal (CRE/AP-1) and induced (CRE/AP-2) transcriptional activity in a variety of cells (Ghee et al., 1998; Kim et al., 1993; Lazaroff et al., 1995; Lewis-Tuffin et al., 2004; Nagamoto-Combs et al., 1997; Suzuki et al., 2002); CREB appears to be one site of convergence for extrinsic factor signaling (reviewed in Lewis-Tuffin et al., 2004; Lonze and Ginty, 2002) regardless of the proximal effector (Fig. 3). In zebrafish, if Ap2a expression is knocked down, the number of cells expressing TH near the dorsal aorta is significantly reduced, suggesting an essential function in the regulation of noradrenergic marker genes (O'Biren et al., 2004). Recent evidence suggests that in a manner similar to what occurs at the DBH promoter (see below), additional cell or tissue-specific co-activators influence TH transcriptional regulation (Lewis-Tuffin, 2004; Quinn, 2002). Detailed investigation of TH regulation in neural crest-derived autonomic neurons at early stages of development will likely identify these cell type-specific co-activators.

Agents that increase cAMP are present in 10-day chick embryo extract (CEE) and are required to support the expression of noradrenergic phenotypic characteristics in vitro. Under these conditions, transcripts encoding HAND2 and Phox2a are expressed, and norepinephrine is synthesized and stored. Elevating PKA by the addition of the membrane permeable analogue of cAMP, 8-Bromo-cAMP, results in a significant decrease in transcripts encoding HAND2 and Phox2a as well as a loss in NE neurons. This result suggested that PKA and cAMP may be important in intracellular signaling in the context of NE differentiation but that PKA might not be signaling downstream of cAMP (Liu and Howard, unpublished observation). PKA has been implicated in growth factor signaling in a variety of tissues including the kidney (Gupta et al., 1999), the central nervous system (Epstein et al., 1996; Hammerschmidt et al., 1996), and the limb (Lee and Chuong, 1997). BMP can activate PKA, independent of cAMP, and phosphorylate CREB independent of SMAD proteins (Gupta et al., 1999; Lee and Chuong, 1997). The possibility that cAMP and PKA may have differential effects on the noradrenergic differentiation of neural crest-derived cells is supported by earlier findings showing that elevation of PKA is inhibitory for expression of norepinephrine (Bilodeau et al., 2000; Maxwell and Forbes, 1990) but that increased cAMP resulted in enhanced expression of TH and differentiation of noradrenergic neurons (Dupin et al., 1993). The apparent conflicts in these results most likely reflect differences in the treatment and growth conditions used in the various studies

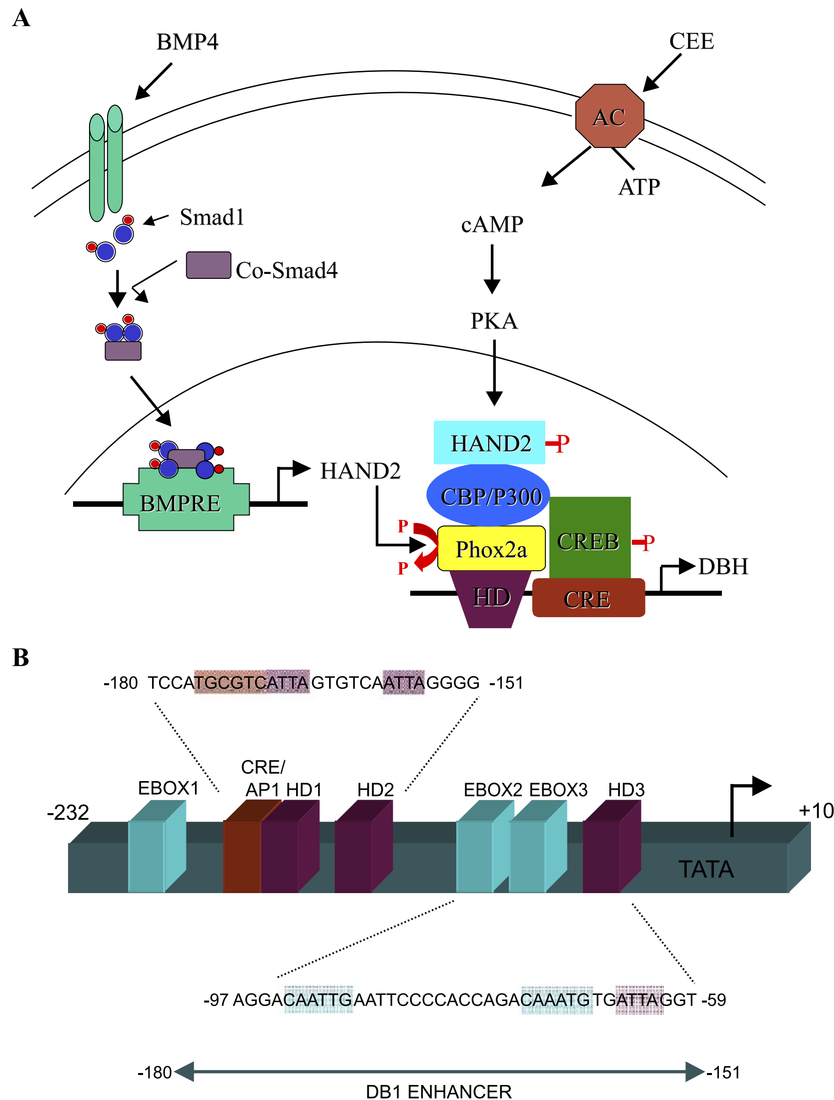

Fig. 3. (A) HAND2 regulates cell type-specific expression of NE in concert with Phox2a by a novel mechanism for bHLH DNA binding proteins. Domain mapping studies have demonstrated that HAND2 interacts in a scaffold of proteins and does not appear to directly bind DNA. Signaling downstream of both BMP4 and cAMP/PKA influences expression and function of HAND2 and Phox2a. (B) The functional activity of HAND2 at the DBH promoter resides in the bHLH domain (Fig. 4) and requires the CRE/AP1 site in the DB1 enhancer. Phox2a binds at three homeodomain (HD) binding sites for maximal activity. There are three putative HAND2 binding sites (E-box) in the proximal DBH promoter. HAND2 serves a dual role, influencing both neurogenesis, downstream of BMP4, and NE biosynthesis in a cAMP-dependent manner. The schematic of the Phox2a proximal promoter is adopted from Swanson et al. (2000) and based in part on sequence data (Hong et al., 2001).

as well as unappreciated complexities in intracellular signaling downstream of cAMP and PKA. The biphasic response to cAMP, depending upon the presence in the growth medium of factors that activate cAMP, suggested more than one site of action of cAMP. This divergence in signaling via cAMP or PKA independent of cAMP is supported by our finding that agents that block PKA but not cAMP inhibit the increased differentiation of noradrenergic cells by BMP4 (Liu and Howard, in preparation). Converging lines of evidence thus suggest complicated interactions in growth factor-mediated signaling in not only the generation of neural crest-derived noradrenergic neurons but also in the biosynthesis of neurotransmitter as well (Howard and Gershon, 1993; Howard et al., 2000; Reissmann et al., 1996; Schneider et al., 1999; Wu and Howard, 2001; Xu et al., 2003).

Attention was focused on possible interaction with transcriptional co-activators downstream of cAMP and PKA because of functional consequences on transcriptional regulation of DBH by Phox2a in response to activation of these signaling pathways. In intriguing studies, it was shown that while expression of TH by neural crest-derived cells is supported by Phox2a in the presence of cAMP, DBH is not expressed (Lo et al., 1999). This differential expression pattern of TH and DBH might be explained by results demonstrating that at the TH promoter, Phox2a and PKA act

independently to stimulate transcription, whereas Phox2 and PKA act synergistically to transactivate DBH. The differential effects of cAMP and PKA are likely based on the fact that, other than basal activity, transactivation of DBH by Phox2a requires that Phox2a be dephosphorylated (Adachi and Lewis, 2002). This dephosphorylation occurs in response to PKA-mediated events and enhances the DNA-binding ability of Phox2a. Interestingly, HAND2 is phosphorylated in helix1 (dimerization domain) at threonine 112 and serine 114 in a PKA-dependent manner (Fig. 4), and this phosphorylation is required for proper dimerization and function in both limb morphogenesis (Firulli et al., 2003) as well as at the DBH promoter (Liu and Howard, in preparation). Detailed analysis of the interactions of HAND2 and Phox2a at the DBH promoter (Fig. 3B) demonstrated that while mutation of the Phox2a homeodomain binding sites, HD1, HD2, and HD3, results in the loss of HAND2/Phox2a transactivation of DBH, it is the interaction of HAND2 at the CRE/AP1-HD1/2 domains in the DBH enhancer that is required for synergistic activation by HAND2 (Rychlik et al., 2003; Shaskus et al., 1992; Xu et al., 2003). HAND2 functions as a transcriptional activator without directly binding to E-box sequences in the DB1 enhancer of the DBH promoter, suggesting that HAND2-mediated DBH transcriptional activity occurs by protein–protein interactions with other transcriptional regulators (Fig. 3A). Although we were unable to demonstrate physical contact between HAND2 and Phox2a in IP/Western blot assays, such interaction has been reported (Rychlik et al., 2003); differences in experimental approach most likely account for this discrepancy, but additional studies will be needed to resolve this issue. HAND2 synergistic activation of DBH is blocked by E1A (CBP/P300 inhibitory protein; Arany et al., 1995), suggesting that HAND2 interacts with cAMP response element binding protein (CBP) in this transcriptional complex. Both HAND2 and Phox2a have been shown to physically interact with CBP/P300 (Fig. 4); Phox2a and HAND2 interact with CBP at the CH3 domain (Dai et al., 2002; Swanson et al., 2000). In HAND2, interaction with the CH3 domain occurs via its bHLH domain (Dai et al., 2002). Our data suggest that HAND2 interacts in a scaffold of proteins as one of several transcriptional regulators of DBH and that part of this functionality resides in the bHLH domain. In addition to a functional role in regulating activity of DBH in a DNA binding-independent manner, we have evidence suggesting that posttranslational modification in helix 1 of HAND proteins is a novel mechanism regulating choice of dimerization partner (Firulli et al., 2003).

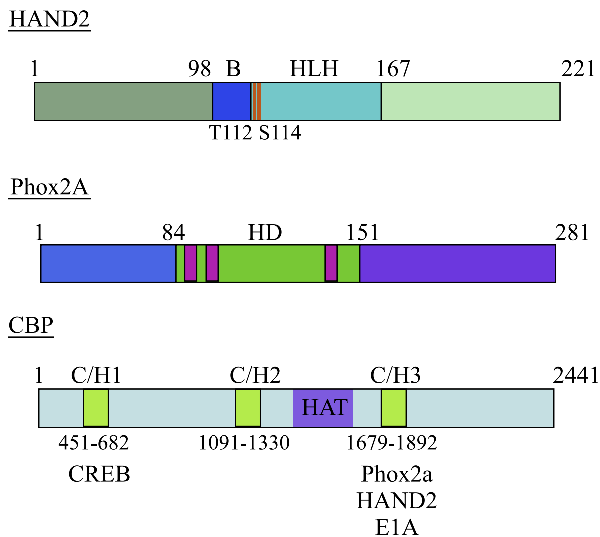

Fig. 4. Schematic drawings of the protein structure of HAND2 (Howard, unpublished data), Phox2a (Hong et al., 2001), and CBP (Harrod et al., 2000; Swenson et al., 2001) show sites identified as functionally important for the interaction of HAND2 and Phox2a at the DBH promoter. In HAND2, activity at the DBH promoter requires the HLH (cyan) dimerization domain but not the basic (royal blue) DNA binding domain. Phosphorylation by PKA of threonine (T) 112 and serine (S) 114 (orange bars) is required for dimerization as well as synergistic activation of DBH with Phox2a. Three homeodomain (HD) binding sites (red bars) must be occupied by Phox2a for maximal transactivation of DBH but are not sufficient for HAND2 synergistic activation (Rychlik et al., 2003; Xu et al., 2003). In CBP, HAND2, Phox2a, and E1A bind at the C/H3 domain (HAT, histone acetyltransferase domain).

In the presence of the putative HAND2 dimerization partner E12, synergistic activation of DBH transcription is titrated away, suggesting that HAND2 does not functionally dimerize with E12 in the DBH transcription complex. We have demonstrated that HAND2 forms homodimers and that the strength of dimerization is determined, in part, by posttranslational modification of threonine 112 or serine 114 in helix1 of HAND2 (Anthony Firulli, personal communication; Firulli et al., 2003). Analysis of HAND2 activity in the regulation of cardiac-specific genes, limb morphogenesis, and neurotransmitter biosynthesis suggests that the diversity of HAND2-mediated responses is due in part to the ability of HAND2 to function under some circumstances independent of DNA binding, to functionally dimerize to a large array of partners, and to show cooperative interactions in scaffolds of transcription factors and co-activators including CBP/P300, GATA4, and Nkx2.5 (Dai et al., 2002; Firulli et al., 2003; McFadden et al., 2002; Thattaliyath et al., 2002; Xu et al., 2003).

Expression of cholinergic marker genes

In addition to parasympathetic ganglion neurons that are primarily cholinergic, a subset of sympathetic ganglion neurons is cholinergic as well. For some of these neurons, especially those innervating the rodent footpad or periosteum, expression of the cholinergic marker genes choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT) occurs in response to target-derived factors (for review, see Asmus et al., 2001; Ernsberger and Rohrer, 1999; Francis and Landis, 1999); the inducing factor has been tentatively identified as cardiotrophin-1 (James

Coulombe, personal communication). Coincident with the upregulation of cholinergic marker genes is reduced expression of the noradrenergic marker genes TH, DBH, and Phox2a (Cervini et al., 1994; Dziennis and Habecker, 2003, 2004; Rao and Landis, 1993). Although the molecular mechanisms underlying this regulation are not well understood, some aspects of cholinergic differentiation have been extensively studied. Expression of ChAT and VAChT is indicative of cholinergic differentiation regardless of whether their expression is dependent upon target-derived factors or occurs independently earlier in development. The genes encoding these proteins comprise a cholinergic gene locus and their expression is coordinately regulated (for review, see Berrard et al., 1995; Berse and Blusztajn, 1995; Shimojo and Hersh, 2004). Expression of this pair of cholinergic marker genes is regulated in a PKA-dependent manner (Inoue et al., 1995; Lonnerberg et al., 1995; Misawa et al., 1995; Shimojo et al., 1998), although many of the growth factors (e.g., CNTF, LIF) shown to influence expression of ChAT or VAChT are not known to signal via PKA (Gould and Butcher, 1989; Hersh and Shimojo, 2003; Kamegai et al., 1990; Knusel et al., 1991). Expression of ChAT/VAChT is regulated by a complex comprised, in part, by a silencer element (NRSE) and an enhancer element (Lonnerberg et al., 1995; Maue et al., 1990; Shimojo and Hersh, 2004). Activity at the NRSE is regulated by the transcription factor RE-1 silencing transcription factor (REST) (Chong et al., 1995); REST is also known as neuron-restricted silencing factor (NRSF) (Schoenherr and Anderson, 1995). This element is a silencer and represses expression of neuronal genes in non-neuronal cells (Chong et al., 1995; Mieda et al., 1997) as well as regulating neuron-specific gene expression in neurons (to reference Kallunki et al., 1997; Palm et al., 1998; Timmusk et al., 1999). There is evidence that REST or truncated forms of REST (e.g., REST4) can also function as enhancers of some neuronal genes. This has been demonstrated for VIP, which is co-expressed in most neural crest-derived peripheral cholinergic neurons (Hamelink et al., 2004). Interestingly, putative RE-1 regulatory domains have been reported as present in genes encoding TH, DBH, ChAT, VIP, and VAChT (Afar et al., 1996; Hahm et al., 1997; Hamelink et al., 2004; Ishiguro et al., 1993, 1995; Lonnerberg et al., 1995; Schoenherr et al., 1996). Clearly, cell type-specific regulation of cholinergic (and noradrenergic) marker genes is quite complex and likely involves additional *cis*-regulatory elements yet to be identified.

Concluding remarks

The specification and differentiation of peripheral neural crest-derived neurons depend, in a large part, on the gene regulatory activities of members of the bHLH and homeodomain families of DNA binding proteins. Exactly how integration of extrinsic and intrinsic signaling is parlayed into differentiation and cell type-specific gene expression in different classes of neurons remains a focus of intense interest. The hierarchical nature of transcriptional regulation and the demonstrated effects of MASH1, HAND2, and Phox2 proteins in differentiation of subsets of peripheral neural crest-derived neurons lead one to speculate that the repertoire of functional consequences downstream of transcriptional regulatory protein activation will involve domains of functionality not yet identified. Several lines of evidence indicate that multiple functional domains in an individual DNA binding protein may serve as a mechanism to expand their capacity to regulate genes in more than one manner. We speculate that in addition to the identification of new *cis*-regulatory mechanisms and new transactivating factors, in the future, studies will focus on expanding our understanding of how modifications in chromatin structure function in determining which genes are transcribed and which genes are silenced.

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