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The Basic Helix-Loop-Helix Factor Hand2 Regulates Autonomic Nervous System Development

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Abstract

Mammalian autonomic nervous system (ANS) development requires the combinatorial action of a number of transcription factors, which include Mash1, Phox2b and GATA3. Here we show that the bHLH transcription factor, *Hand2* (dHAND), is expressed concurrently with *Mash1* during sympathetic nervous system (SNS) development and that the expression of *Hand2* is not dependent on *Mash1*. This suggests that these two bHLH factors work in parallel during SNS development. We also show that ectopic expression of *Hand2* activates the neuronal program and promotes the acquisition of a phenotype corresponding to peripheral neurons including neurons of the SNS lineage in P19 embryonic carcinoma cells. We propose that *Hand2* works in parallel with other members of the transcriptional network to regulate ANS developmental but can ectopically activate the program by a cross-regulatory mechanism that includes the activation of *Mash1*. We show that this function is dependent on its interaction with the histone acetyltransferase p300/CBP, indicating that *Hand2* functions to promote ANS development as part of larger transcriptional complex.

Keywords

autonomic; sympathetic; Hand2; development; p300

INTRODUCTION

The development of the autonomic nervous system (ANS), and in particular the sympathetic division of the ANS (SNS), has long provided a model to investigate the cellular, molecular and genetic events leading to the differentiation of neurons (Reviewed in (Bertrand et al., 2002; Goridis and Rohrer, 2002)). From these studies numerous signaling pathways and transcription factors have been identified as critical components of SNS development, yet many gaps remain in our knowledge. During SNS development, neural crest (NC) cells migrate from the dorsal region of the neural folds, traversing the ventral-medial sclerotome of the somites until they reach the dorsal aorta where they are induced to differentiate in response to BMP (Reissmann et al., 1996; Varley and Maxwell, 1996; Varley et al., 1998). BMP signaling activates a number of genes encoding transcription factors essential for SNS development including the paired homeodomain genes *Phox2a* and *Phox2b* (Lo et al., 1998; Schneider et al., 1999) and the basic helix-loop-helix factors (bHLH) *Mash1* (Shah et al., 1996; Lo et al., 1998) and *Hand2* (Howard et al., 2000).

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The *Phox2* genes are closely related factors expressed in all catecholaminergic neurons and are required for the specification of catecholaminergic neurons and regulation of catecholaminergic biosynthesis in differentiated neurons (Tiveron et al., 1996; Pattyn et al., 1997). Loss of either *Phox2* gene leads to loss of the ANS by mid-gestation (Morin et al., 1997; Pattyn et al., 1999). When ectopically expressed, both *Phox2a* and *Phox2b* activate noradrenergic neuronal development in NC stem cells *in vitro* (Lo et al., 1999) and NC cells located along the peripheral nerves (Stanke et al., 1999). *Phox2* target genes involved in regulating noradrenergic specification during early development remain unknown, but in differentiated neurons, the *Phox2* genes bind directly to and regulate expression of the noradrenalin biosynthetic enzymes dopamine- β -hydroxylase (DBH) and tyrosine hydroxylase (TH) genes, suggesting that the *Phox2* genes are required for maintenance of the differentiated phenotype (Kim et al., 1998; Yang et al., 1998). Although the *Phox2* genes are required for acquisition of the catecholaminergic phenotype, their expression in non-catecholaminergic lineages during development suggests they do not specify specific lineages of the ANS.

The bHLH transcription factors are key genes in the specification and differentiation of neurons and are essential during central (CNS), peripheral nervous system (PNS) and ANS development (Reviewed in (Jan and Jan, 1994; Anderson and Jan, 1998). The bHLH factor *Mash1* is expressed in a number of neuronal lineages during development including the CNS and ANS (Guillemot and Joyner, 1993). In the ANS, loss of *Mash1* does not prevent development of neuroblasts but a majority of the neuroblasts fail to differentiate and are subsequently lost. This suggests that *Mash1* plays a role in the differentiation of ANS neurons (Guillemot et al., 1993; Sommer et al., 1995). However, *Mash1* is expressed in a number of neuronal lineages outside the ANS including those of the eye, olfactory epithelium and CNS where it does not induce ANS differentiation (Guillemot and Joyner, 1993). The broad expression of *Mash1* argues against a role for *Mash1* in the specification of ANS neurons. Further support of this, comes from ectopic expression studies, which demonstrate that *Mash1* promotes generic neuronal development and not the ANS program (Farah et al., 2000; Tomita et al., 2000).

Unlike other transcription factors found in the ANS, the bHLH transcription factor *Hand2* is restricted to ANS during neural development (Srivastava et al., 1995; Dai et al., 2004). *Hand2* is a member of the twist family of bHLH factors and can activate transcription through binding to a subset of E-boxes (Dai and Cserjesi, 2002). However, it can also function to regulate limb development without a DNA binding domain (McFadden et al., 2002). This suggests that *Hand2* can activate genes by direct binding to their regulatory regions and also as part of a transcriptional complex independent of DNA binding.

The role of *Hand2* in neuronal development has been investigated *in vitro* and *in vivo* but its function in neuronal development remains unclear. Ectopic expression of *Hand2* in quail NC cultures enhances catecholaminergic differentiation, while down regulation of *Hand2* leads to suppression of development (Howard et al., 1999). *In vivo*, ectopic expression of *Hand2* in NC cells along peripheral nerves also induces the catecholaminergic differentiation program (Howard et al., 2000). Since NC derived catecholaminergic neurons are predominantly sympathetic, these results suggest that *Hand2* specifies the sympathetic lineage. However, *Hand2* is expressed in all three lineages of the ANS suggesting that *Hand2* plays a role in development of the ANS, not just the sympathetic lineage.

Here, we address where in the transcriptional hierarchy regulating ANS development *Hand2* fits. its developmental role, and the molecular mechanism through which it regulates neuronal development. We show that *Hand2* and *Mash1* are co-expressed during SNS development and that expression of the *Hand2* gene is not dependent on *Mash1*, suggesting that these two bHLH factors function in parallel during ANS development. We also show that *Hand2* can activate

the ANS neuronal development in P19 embryonal carcinoma cells suggesting that it possesses the ability to activate the neuronal program and promote lineage-specific selection. *Hand2* also activates *Mash1* through recruitment of the histone acetyltransferase p300/CBP, suggesting that *Hand2* functions as part of a transcriptional complex during neuronal development. Based on these results, we propose that *Hand2* activates the ANS developmental program through the cross-activation of a network of transcription factors that act in parallel during development.

MATERIALS AND METHODS

Plasmids

pRC-RSV-HAND2 was generated by subcloning the EcoRI-BamHI fragment containing the *Hand2* coding region from pRSET-HAND2 (Dai and Cserjesi, 2002) into pcDNA3-HisB (Invitrogen). The HindIII-XbaI fragment containing the epitope tag and *Hand2* cDNA was subcloned into pRC-RSV (Invitrogen). To make pEGFPJH-HAND2, pEGFP2JH was constructed by modifying pHOOK2 (Invitrogen) with a replacement of the HOOK gene sequence with EGFP by inserting the 0.74kb SpeI (blunted)-SalI fragment from pBI-EGFP (Clontech) into the EcoRV-XhoI site of pHOOK2. To generate pEGFPJH-HAND2, the BglII-XbaI *Hand2* fragment from pcDNA3-HisB-H2 was inserted into the BglII-XbaI sites of pEGFP2JH. The *Mash1* expression vector, CS2+MT MASH1, was provided by D. Turner (University of Michigan, Ann Arbor) (Farah et al., 2000). CMV β p300 wt was provided by D. Livingston, (University of Southern California, Los Angeles, CA) (Eckner et al., 1996) and CMV-12SE1A expressing E1A wild type protein by S. Chellappan (University of South Florida, Tampa, FL).

Cell Culture and generation of stable *Hand2* expressing P19 cell lines

P19 embryonal carcinoma (P19-EC) cells were cultured in D-MEM with 7.5% calf serum and 2.5% fetal bovine serum (Gibco). P19-EC cell lines stably expressing *Hand2* (P19-H2) were generated by transfection with pRC-RSV-HAND2 and selection of neomycin resistant clones using 100 μ g/ml Geneticin (Gibco). Expression of *Hand2* protein in cell lines was examined by Western blot analysis monitored by immunocytochemistry with *Hand2* specific antibody (Dai and Cserjesi, 2002).

Growth and differentiation of P19-EC and P19-H2 cells were described previously (Bain et al., 1994) with the following modifications. After 4 days of aggregation, aggregates were collected, dispersed, and cultured for 2 days. The media was then replaced with media supplemented with 5 μ g/ml cytosine arabinoside (Sigma) to select for non-dividing neurons.

Neuro-2a cells were cultured and transfected as described previously (Dai et al., 2004).

Immunocytochemistry

For immunostaining, cells were fixed in 3% paraformaldehyde for 10 min and washed with PBS. The cells were incubated with blocking solution (1% goat serum in PBS) for 10 min then incubated with primary antibodies in blocking solution for 1 hr. After washing, cells were incubated with fluorescein conjugated anti-mouse and rhodamine-conjugated anti-rabbit secondary antibodies (Chemicon). Antibodies were obtained from: peripherin, R. Liem (Columbia University, New York); TuJ1 (Sigma); TH (Chemicon); *Mash1*, J. Johnson (University of Texas, Southwestern, Dallas); SSEA1, Developmental Studies Hybridoma Bank; *Hand2* as described previously (Dai and Cserjesi, 2002).

RNA analysis

Total RNA was isolated from P19 cells and adult sympathetic ganglia using Trizole (Gibco) following manufacturers instructions. For Northern analysis, 15 μ g of total RNA was separated

in a formaldehyde-agarose gel and transferred to a Duralon membrane (Stratagene). Blots were hybridized with ^{32}P -dCTP-labeled probes generated by random primer labeling (Ambion) in Quick-Hybrid Buffer (Stratagene). To detect endogenous *Hand2* transcripts, the 3' untranslated region of the *Hand2* cDNA was used as a probe.

RT-PCR analysis was performed on 0.5 μg of total RNA as described previously (Morikawa and Cserjesi, 2004). Primer sequences are available upon request.

***In situ* hybridization**

In situ hybridization of whole mount mouse embryos and sections were performed with digoxigenin-labeled probes as described previously (Dai et al., 2004). Plasmids used to generate *in situ* probes were pBSK-HAND2 (Dai et al., 2004), *Mash1* (Guillemot and Joyner, 1993), cRet (cRet9-pBKSII), provided by F. Costantini (Columbia University, New York), and *Phox2b* (pcDNA3-*Phox2b*) provided by J. -F. Brunet (CNRS, Ecole Normale Supérieure, Paris).

RESULTS

Expression of *Hand2* and *Mash1* during ANS development

As a first step in understanding the potential role of *Hand2* during mouse ANS development, we examined *Hand2* expression in the developing ANS by *in situ* hybridization (Fig. 1). *Hand2* is expressed in all lineages of the ANS, the sympathoadrenal, parasympathetic, and enteric (Fig. 1A). *Hand2* expression was not detected in the CNS or in the sensory lineage of the PNS. In the SNS, *Hand2* expression was first detected in the rostral region of the sympathetic trunk at 10 dpc in the NC cells coalescing by the dorsal aorta. *Hand2* expression was not detected in migrating NC, the precursors of the ANS. The expression of *Hand2* in post-migratory NC cells suggests it is first expressed after NC cells are specified to form the SNS, overlapping the expression of *Mash1*.

To determine the exact temporal relationship between *Hand2* and *Mash1*, we focused our studies on the SNS. Since SNS development proceeds from rostral to caudal, we sectioned through the length of a 10.5 dpc mouse embryo obtaining sections containing SNS ganglia at all stages of differentiation. Alternate serial sections were analyzed for *Hand2* and *Mash1* expression by *in situ* hybridization (Fig. 1C–F). The cervical region of the embryo contains a large population of sympathetic cells expressing both *Hand2* and *Mash1* transcripts (Fig. 1C, D). Sections through more caudal regions contain progressively fewer *Hand2* and *Mash1* expressing cells (Fig. 1E, F). The analysis showed that the initiation of *Hand2* and *Mash1* expression in the SNS occurs concurrently suggesting they are activated in parallel.

To determine if *Hand2* expression is dependent on continued *Mash1* expression, we examined the expression of *Hand2* in later stage embryos. *Mash1* is expressed at high levels until 13 dpc followed by a rapid down regulation at 14.5 dpc (Guillemot and Joyner, 1993). At 14.5 dpc of development, *Hand2* expression remains high (Fig. 1G) while *Mash1* transcripts were undetectable (Fig. 1H). To determine if *Hand2* expression is maintained in the adult SNS and if *Mash1* expression is reactivated, we examined the levels of *Hand2* and *Mash1* transcripts by semi-quantitative RT-PCR. *Hand2* is detected in the adult SNS while *Mash1* expression was not detected (data not shown). The continued expression of *Hand2* after cessation of *Mash1* suggests that *Hand2* expression is not dependent of *Mash1*.

Hand2* expression is not regulated by *Mash1

Although our expression results show that continued expression of *Hand2* is independent of *Mash1*, it is possible that initiation of *Hand2* expression is *Mash1* dependent. To test this, we

examined *Hand2* expression in embryos lacking the *Mash1* gene (Fig. 2). *Mash1*^{+/+} and *Mash1*^{-/-} embryos were analyzed by *in situ* hybridization using *Hand2* probes to examine *Hand2* expression and *Phox2b* probes to identify SNS neuroblasts. Sagittal (Fig. 2A, B) analysis showed that at 12.5 dpc, *Hand2* was expressed in the sympathetic trunk in *Mash1*^{+/+} embryos although fewer *Hand2* expressing cells were observed. At 10.5 dpc, a half day after the initiation of *Mash1* expression, *Hand2* expression was observed in the sympathetic trunk of *Mash1*^{-/-} embryos (data not shown). These data suggest that activation and continued expression of the *Hand2* gene are independent on *Mash1*.

The smaller number of *Hand2* expressing cells in *Mash1*^{-/-} embryos could be due either to a dependence on *Mash1* expression in a subset of cells or to the loss of cells during development (Guillemot and Joyner, 1993; Sommer et al., 1995). To determine if the decrease in *Hand2* expressing cells in the sympathetic trunk was due to *Mash1* dependence or to the presence of fewer cells (neuroblasts), alternate thin sections from *Mash1*^{+/+} and *Mash1*^{-/-} embryos were hybridized with *Hand2* and a marker for SNS neuroblasts and neurons, *Phox2b*. More cells in the sympathetic trunk express *Phox2b* and *Hand2* in *Mash1*^{+/+} (Fig. 2C, E) than in *Mash1*^{-/-} (Fig. 2D, F) embryos. A comparison of the number of *Hand2* and *Phox2b* expressing SNS cells in *Mash1*^{-/-} embryos was determined in a series of cross sections along the embryo. An equivalent number of cells express *Hand2* and *Phox2b*. This suggests that the reduced *Hand2* expression in *Mash1*^{-/-} embryos is due to the loss of developing sympathetic neurons. These results further demonstrate that *Hand2* expression is not dependent on *Mash1*.

Hand2 activates the ANS developmental program

Previous studies have shown that Ectopic expression of *Hand2* in primary NC stem cell cultures enhances the differentiation of catecholaminergic neurons (Howard and Cserjesi, 1996). However, in the culture conditions used, the NC cells spontaneously differentiated into catecholaminergic neurons suggesting that *Hand2* may not be activating the SNS developmental program but may instead promote their terminal differentiation. To investigate if *Hand2* can activate the ANS developmental program, we ectopically expressed *Hand2* in P19 embryonic carcinoma (P19-EC) cells and examined the ability of *Hand2* to activate the neuronal program and more specifically, the SNS developmental program.

P19-EC cells are pluripotent cells that can be induced to differentiate into CNS neurons when aggregated into embryoid bodies in the presence of retinoic acid (RA) (Bain et al., 1994). As reported previously, transient *Hand2* expression or RA addition to monolayer cultures was not sufficient to induce neuronal differentiation (Farah et al., 2000). To determine if *Hand2* can activate neuronal development in P19-EC cells after embryoid body formation, we generated cell lines stably expressing *Hand2* (P19-H2). When cultured as a monolayer, P19-H2 cell lines are morphologically indistinguishable from control cells and express the inner cell mass antigen SSEA1 indicating they remain pluripotent (data not shown).

To determine if *Hand2* has the ability to activate the neuronal program after embryoid body formation, cells were aggregated, replated, and examined for morphological changes and the expression of neural markers (Fig. 3). P19-EC cells that were aggregated for 4 days and replated did not develop neuronal projections, express the panneuronal marker TuJ1 or the PNS marker peripherin (Fig. 3A, B). We next examined if the neuronal inductive agent RA could activate the PNS developmental program in P19-EC cells. Embryoid bodies were formed and treated with 0.3 μ m RA for 4 days, replated, and examined for expression of TuJ1 or peripherin. P19-EC cells express TuJ1 but not peripherin (Fig. 3C, D). When P19-H2 cells were aggregated and replated in the absence of RA, the cultures developed extensive neuronal projections, and cells expressed TuJ1 and peripherin (Fig. 3E, F). The expression of peripherin in the P19-H2 cultures suggests that *Hand2* can activate formation of PNS neurons.

In the developing nervous system, *Hand2* expression is restricted to the peripheral autonomic lineage suggesting a role in the development of peripheral neurons. To determine if the neurons that form in the P19-H2 cultures are all peripheral neurons or a mixture of CNS and PNS neurons, we examined if all TuJ1 expressing cells also co-express the peripheral neuronal marker peripherin (Fig. 3G, H). We found that all neurons that express the pan-neuronal marker TuJ1 co-express peripherin. Because *Hand2* has been shown to enhance SNS development in cultured neural crest cells (Howard et al., 1999), we asked if *Hand2* also activates the SNS differentiation program in P19-EC cells. We found that a subset of *Hand2* induced neurons, which express the pan-neuronal marker TuJ1, also co-express TH (Fig. 3I, J). To confirm that these catecholaminergic cells are indeed SNS neurons, we examined the co-localization of peripherin and TH (Fig. 3K, L). All *Hand2* induced TH expressing neurons also expressed peripherin showing that expression of *Hand2* is able to activate the SNS developmental program in P19-EC cells.

The SNS developmental program involves activation of a number of transcription factors including *Mash1* and *Hand2*. To determine if the *Hand2* induced neuronal development in P19-EC cells involves activation of these bHLH factors, we examined their expression by Northern analysis. The generation of embryoid bodies from P19-EC cells is not sufficient to induce neuronal differentiation or the expression of *Mash1* while RA treatment robustly activated both (Fig. 3M). When P19-H2 cells are aggregated and replated in the absence of RA, *Hand2* was able to activate *Mash1* expression. To further investigate the gene hierarchy induced by *Hand2* expression in P19-EC cells, we examined the ability of *Hand2* to activate expression of the endogenous *Hand2* gene. Aggregation of P19-EC cultures and activation of the neuronal program with RA is insufficient to activate the endogenous *Hand2* gene (Fig. 3N). However, aggregation of P19-H2 cultures is sufficient to activate endogenous *Hand2* expression (Fig. 3N). These data suggests that *Hand2* regulates the ANS developmental program by auto and cross regulation of the ANS transcriptional network.

Hand2 activates expression of *Mash1* by a p300-dependent mechanism

Hand2 has been shown to act through recruitment of the histone acetyltransferase p300 during cardiac gene regulation (Dai et al., 2002). To determine if *Hand2*'s function in the activation of neuronal development also depends on p300 recruitment, we examined *Hand2*'s dependence on p300 in the activation of *Mash1* (Fig. 4). *Hand2* was expressed in the mouse neuroblastoma cell line, neuro-2a, a cell line that does not normally express *Mash1*. Neuro2a cells were transfected with the dual promoter construct pEGFPJH-H2 that expresses *Hand2* and EGFP from independent promoters. Transfection was monitored by EGFP expression (Fig. 4A) and *Mash1* expression was determined by immunostaining with Mash1 monoclonal antibody (Fig. 4B). In *Hand2* expressing cells, over 80% of cells co-express Mash1. Although *Hand2* activated Mash1 in neuro-2a cells, it was unable to promote terminal differentiation, which may reflect the transformed nature of this cell line.

To determine if p300 or the functionally redundant factor CBP, was an essential co-factor for *Hand2* dependent Mash1 activation, *Hand2* was co-expressed with the p300/CBP inhibitory protein E1A (Fig. 4D). The activation of Mash1 by *Hand2* was repressed by E1A in a dose dependent manner. To determine if the effect of E1A was due to an inhibition of p300 or by non-specific repression, p300 was co-expressed with *Hand2* and E1A to determine if p300 could rescue E1A repression of *Mash1*. E1A inhibition of Mash1 expression was rescued by p300 (Fig. 4D) suggests that *Hand2* activation of *Mash1* requires the recruitment of p300/CBP.

To analyze the mechanism by which p300 regulates *Hand2* function, we examined its ability to regulate the transcriptional activity of *Hand2* using a mammalian one-hybrid assay (Fig. 4E). We fused *Hand2* with the yeast Gal4 minimal DNA binding domain to create a chimeric protein dependent on *Hand2* for transcriptional activity but not DNA binding. The Gal4-*Hand2*

fusion plasmid and a CAT reporter plasmid containing five Gal4 DNA binding sites upstream of the E1b basal promoter was transfected into neuro-2a cells. Hand2 activated transcription by 23 fold. Co-transfection of E1A with Gal4-Hand2 reduced transcriptional activity in a dose-dependent manner. E1A repression of Hand2 transcription is rescued by co-transfection with p300 showing that E1A repression is acting through p300/CBP. Taken together, our results suggest that Hand2 promotes neuronal development by functioning in a transcriptional complex that includes p300/CBP.

DISCUSSION

Our understanding of the transcriptional network regulating ANS development remains incomplete. Only two transcription factors, Phox2b and Mash1, have been assigned essential early roles in ANS development. *Phox2b* is expressed soon after NC cells have reached the dorsal aorta and have become specified to form the SNS suggesting it may function as an early determination factor. However, genetic analysis has shown it is not required for the initial formation of the SNS, based upon the transient expression of *Mash1* and β -galactosidase knock into the *Phox2b* gene (Pattyn et al., 1999). Phox2b is required for the continued expression of *Mash1*, a bHLH transcription factor that is required for differentiation and survival of the SNS during terminal differentiation but not its initial formation (Guillemot et al., 1993; Sommer et al., 1995; Pattyn et al., 1999). Genetic analysis in mice suggests that these two transcription factors are not essential for commitment of NC cells to an ANS lineage but are essential for their survival. In addition, both factors are expressed in a number of neuronal lineages other than the ANS including the CNS, further suggesting that these factors, whether acting alone or in concert, are insufficient to specifically activate the ANS developmental program. These data suggest that other transcription factors must be involved in the specification and differentiation of the ANS.

The work presented here demonstrates that Hand2 is a member of the gene regulatory network that controls ANS development. Unlike other neuronal transcription factors, *Hand2* is expressed exclusively in the ANS lineage, indicating that it plays a unique role in its development. We have shown that the expression of *Hand2* and *Mash1* begins concurrently, or within a short time of each other. The close timing of expression suggests that *Hand2* is regulated in parallel with *Mash1* and expression of these two bHLH transcription factors may initially be co-regulated by an upstream factor. However, it has been reported that *Hand2* expression is dependent on *Mash1* (Anderson and Jan, 1998; Goridis and Rohrer, 2002) suggesting a linear relationship where *Hand2* is downstream and regulated by *Mash1*. Our analysis of *Mash1* null mice shows that *Hand2* expression is independent of *Mash1* during SNS differentiation. Although there is reduced *Hand2* expression in the SNS of *Mash1* null embryos, this is due to a reduced cell number, not a dependence on *Mash1*. After *Mash1* is no longer expressed, *Hand2* continues to be expressed during development and in the adult SNS, indicating that *Mash1* does not play a role in regulating *Hand2*. However, our finding that Hand2 is expressed in the adult SNS does support a role in maintaining the noradrenergic phenotype by regulating expression of the noradrenalin biosynthetic enzymes dopamine- β -hydroxylase (DBH) gene in combination with Phox2 (Kim et al., 1998; Yang et al., 1998; Rychlik et al., 2003; Xu et al., 2003).

When ectopically expressed in NC derived cells, Hand2 has been shown to activate the SNS developmental program (Howard et al., 1999; Howard et al., 2000). Whether this activity is permissive or instructive is unclear. We have extended these results by demonstrating that Hand2 activates the ANS neuronal developmental program in P19 EC cells, a cell line not predisposed to form ANS neurons. This supports an instructive role for Hand2 in neuronal specification. The ability of *Hand2* to activate the ANS program and the expression of *Mash1* also suggests it acts upstream of *Mash1* and functions as a proneural gene. However,

in the embryo, *Hand2* expression begins after *Phox2b*, a marker for NC cells committed to ANS lineage (Howard et al., 2000). Since expression of *Hand2* begins after ANS specification *in vivo*, *Hand2* does not initiate the ANS program. The proneural activity of *Hand2 in vitro* most likely reflects an ability to activate neuronal development through a cross-regulatory mechanism where *Hand2* activates other members of the transcriptional network that act concurrently during ANS development. This supports a parallel transcriptional regulatory model for ANS development in which *Hand2*, *Mash1* and *Phox2* genes act in parallel to promote differentiation (Fig. 5). The genes act in concert during neuroblast to neuron transition with *Hand2* and *Phox2* continuing to function in the maintenance of the catecholaminergic phenotype by directly regulating expression of the DBH gene (Rychlik et al., 2003; Xu et al., 2003). However, the ability of *Hand2* to activate the ANS-specific developmental program in non-NC cells appears to be a property unique to *Hand2*.

The ability of *Hand2* to activate other members of the transcriptional network regulating ANS development is through recruitment of p300. An interaction between p300 and *Hand2* has also been shown to occur during regulation of the ANF gene (Dai et al., 2002). During ANF regulation, the interaction between *Hand2* and p300 is direct and forms a complex that includes the zinc finger transcription factor GATA4 (Dai et al., 2002). The formation of a transcriptional complex between a bHLH factor and p300 is not unique to *Hand2*. Other members of the Twist family (Hamamori et al., 1999; Huang et al., 1999) and the *atonal* and *achaete-scute* families of neuronal bHLH factors (Sun et al., 2001; Vojtek et al., 2003) also require formation of a transcriptional complex with p300/CBP for their function. Further support of the requirement for *Hand2* to act through the formation of multi-protein complexes to regulate developmental events is its ability to regulate limb development in the absence of a DNA binding domain (McFadden et al., 2002). The ability of *Hand2* to function without direct DNA binding suggests it can function through protein-protein interactions *in vivo*.

Because the p300/CBP proteins bind a large number of transcription factors (Reviewed in (Goodman and Smolik, 2000), the number of factors modulating *Hand2* function during ANS development may also be large and dynamic. The requirement for *Hand2* to bind p300/CBP may also explain the apparent contradictory role of *Hand2* regulation of *Mash1*. *Hand2* activates *Mash1* yet continues to be expressed in SNS neurons after cessation of *Mash1* expression. During early SNS development, *Hand2* can recruit p300/CBP to a transcriptional complex that activates *Mash1* transcription, as development proceeds, this ability is lost. This dynamic interaction between *Hand2* and p300/CBP may occur through the extensive post-translational modifications that have been reported previously to affect the transcriptional activity of *Hand2* (Firulli et al., 2005).

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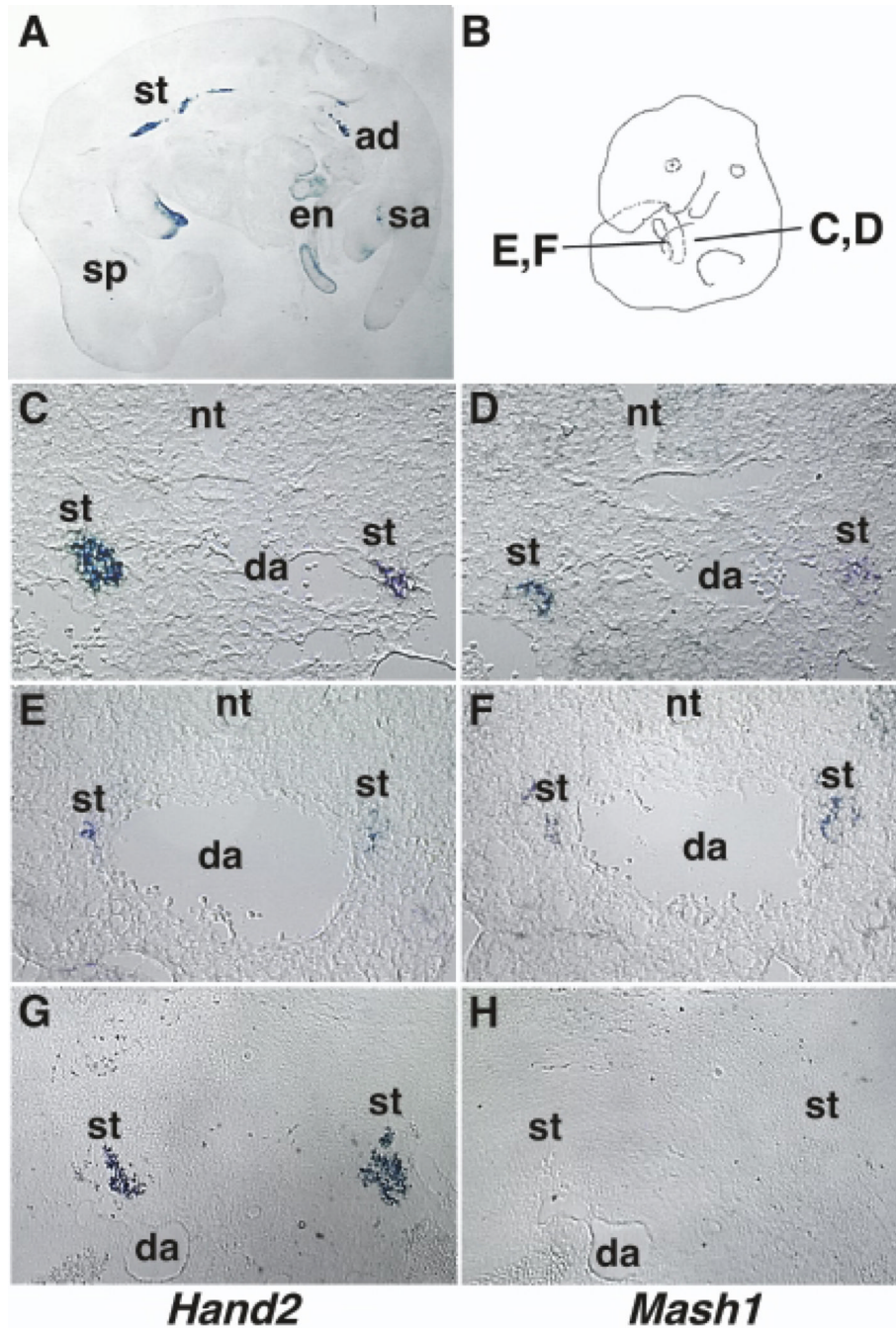


Fig. 1. Expression of *Hand2* and *Mash1* during PNS development

Hand2 and *Mash1* gene expression was examined by *in situ* hybridization using digoxigenin-labeled anti-sense cRNAs. A: *In situ* hybridization of a 12.5 dpc mouse embryo with *Hand2*. *Hand2* is expressed throughout the length of the sympathetic trunk, in the coalescing adrenal gland, and in the parasympathetic and enteric nervous systems. B–H: Comparison of *Hand2* (C, E, G) and *Mash1* (D, F, H) expression in the SNS of 10.5 dpc (C–F) and 14.5 dpc (G–H) mouse embryos by *in situ* hybridization using alternate 10 μ m cryosections. The regions of the embryo where sections were obtained for plates C–F are shown in plate B. *Hand2* and *Mash1* are co-expressed during early SNS development (C–F) but *Hand2* expression continues after down regulation of *Mash1* at 14.5 dpc (G–H). ad: adrenal gland; da: dorsal aorta; en:

enteric plexus; nt: neural tube; sa: sacral parasympathetic plexus; sp: sphenopalatine ganglia;
st: sympathetic trunk.

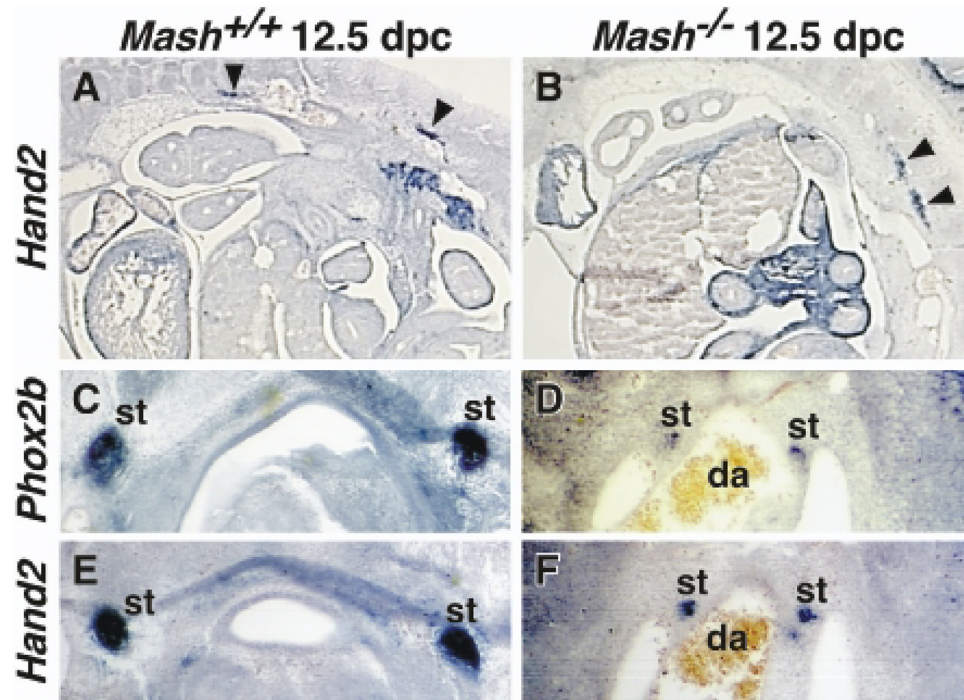


Fig. 2. *Hand2* expression is not dependent on *Mash1*

Expression of *Hand2* and *Phox2b* genes was examined in 12.5 dpc *Mash*^{+/+} (A, C, E) and *Mash1*^{-/-} (B, D, F) littermates using *in situ* hybridization. *In situ* hybridization was performed using digoxigenin -labeled anti-sense probes on 10 μ m sagittal and 25 μ m paraffin embedded cross sections. A, B: Sagittal sections stained with *Hand2* probe. *Hand2* is expressed in the developing SNS of both *Mash*^{+/+} and *Mash1*^{-/-} embryos including the trunk (arrowheads). C–F: Cross sections hybridized with *Phox2b* (C, D) and *Hand2* (E, F) on alternate sections. *Hand2* and *Phox2b* expression overlaps in the sympathetic trunk of both *Mash*^{+/+} and *Mash1*^{-/-} embryos. The decreased level of transcript in the *Mash1*^{-/-} embryos reflects a decreased cell number in the forming SNS ganglia. da: dorsal aorta; st: sympathetic trunk.

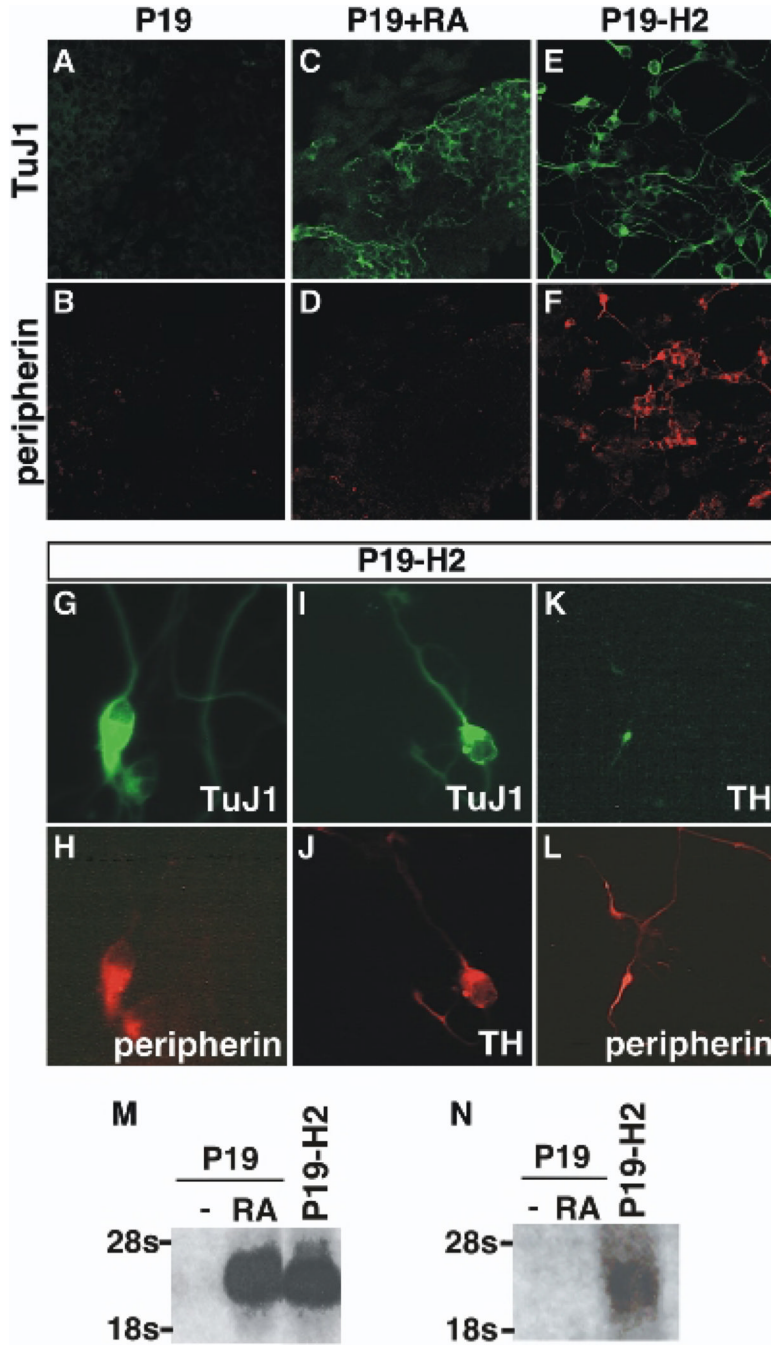


Fig. 3. Hand2 activates neuronal development in P19-EC cells

The ability of Hand2 to activate the neuronal program was examined by constitutively expressing Hand2 in P19-EC cells. P19-EC and P19-H2 cells were aggregated to form embryoid bodies, allowed to reattach as a monolayer and then examined for expression of neuronal markers. A–F: Cells were cultured for 4 days after reattachment and analyzed for expression of the pan-neuronal neuronal marker TuJ1 or the peripheral neuronal marker peripherin. P19-EC untreated (A, B), P19-EC treated with RA (C, D) and P19-H2 untreated (E, F) cells were examined for expression of TuJ1 (A, C, E) and peripherin (B, D, F). G–L: Untreated P19-H2 embryoid bodies were dissociated, re-plated and cultured for 12 days prior to analysis. P19-H2 cultures were examined for the co-expression of the pan-neural marker

TuJ1 (G) and peripherin (H), co-expression of TuJ1 (I) and TH (J), and for TH (K) and peripherin (L). M–N: Northern analysis of *Mash1* (M) and endogenous *Hand2* (N) gene expression in P19-EC and P19-H2 cultures was performed 4 days after replating embryoid bodies.

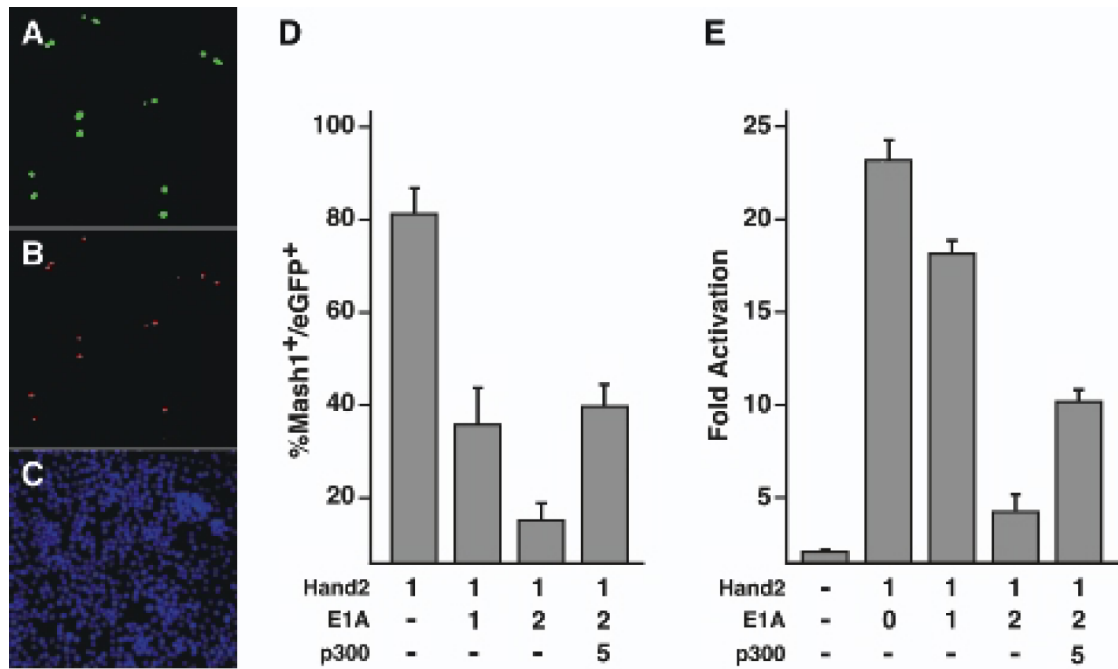


Fig. 4. Hand2 activation of Mash1 and transcriptional activity is dependent on p300/CBP

The role of p300/CBP in Hand2 function was examined by inhibition of p300/CBP function with E1A. (A–C) Neuro2a cells were transfected with pEGFP-HAND2 and monitored for co-expression of Hand2 by GFP fluorescence (A), expression of Mash1 with a Mash1 specific monoclonal antibody (B) and cell number with DAPI nuclear staining (C). Mash1 expression was activated in over 80% of cells co-expressing Hand2 and eGFP. (D) Hand2 was co-expressed with the p300/CBP inhibitor protein E1A (μ g of plasmid transfected). Hand2 activation of Mash1 expression was inhibited in a dose dependent manner by E1A. Co-expression of p300 rescues the inhibitory effect of E1A suggesting E1A inhibition targets the Hand2-p300/CBP complex. (E) Expression of E1A repressed the transcriptional activity of Hand2 in a dose dependent manner that was overcome by co-expression of p300.

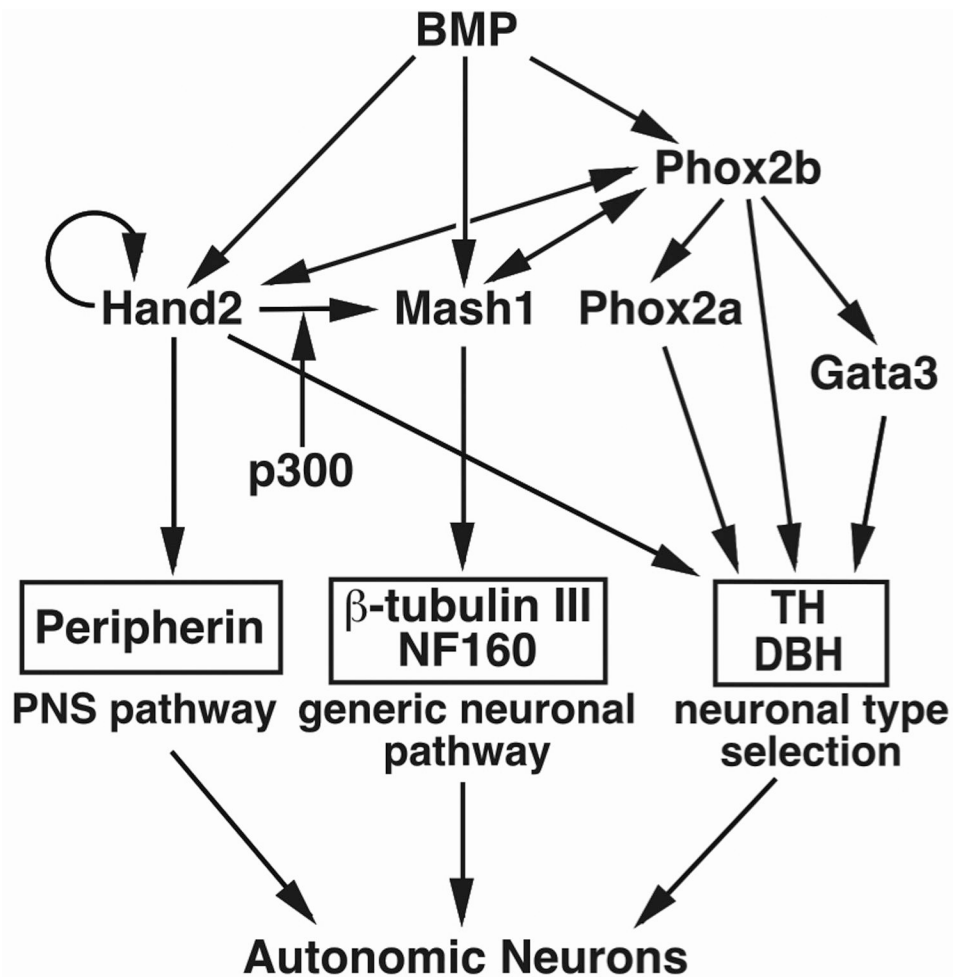


Fig. 5. Transcriptional regulatory network during development of the ANS

During ANS development, transcription factors act in a network to induce other transcription factors. BMP is an inductive cue for the SNS lineage and activates the expression of the transcription factors Phox2b (Lo et al., 1998; Schneider et al., 1999), Mash1 (Shah et al., 1996; Lo et al., 1998) and Hand2 (Howard et al., 2000). The bHLH factor Mash1, is able to cross-activate the paired homeodomain transcription factor Phox2b (Lo et al., 1998; Lo et al., 1999) which is required for maintenance of Mash1 expression (Pattyn et al., 1999) and for expression of another bHLH factor Hand2 (Goridis and Rohrer, 2002). Hand2 activates Phox2b (Howard et al., 2000), itself and Mash1 (this study). These three factors together activate the expression of the neuronal structural genes. Each factor activates different pathways that converge during autonomic neuronal development. **Generic neuronal pathway:** Expression of Mash1 is sufficient for the expression of generic neuronal structural genes such as TuJ1 and NF160 (Farah et al., 2000). Mash1 is required for the expression of the noradrenergic biosynthetic genes TH, DBH (Guillemot et al., 1993) and peripherin (Sommer et al., 1995) but transcription regulation of these genes must be indirect since *Mash1* expression stops at mid-gestation (Guillemot and Joyner, 1993). **Neuronal type selection:** Phox2b regulates expression of the zinc finger factor GATA3 (Tsarovina et al., 2004) and Phox2a (Pattyn et al., 1999). Phox2a/b and Gata3 directly regulate the expression of noradrenergic biosynthetic enzymes TH and DBH in sympathetic neurons (Kim et al., 1998; Yang et al., 1998; Lim et al., 2000) and may regulate other neuronal genes in parasympathetic and enteric neurons. **Peripheral pathway:** *Hand2* is only expressed in the ANS lineages of the nervous system suggesting a

role in its specification (Srivastava et al., 1995; Dai et al., 2004). Ectopic expression of Hand2 activates the PNS program and regulates the expression of TH in the SNS. The ability of Hand2 to activate Mash1 is p300/CBP dependent.