HAND transcription factors are required for neonatal sympathetic neuron survival

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Expression of the basic helix–loop–helix transcription factor HAND2 begins early in sympathetic neuron development and is essential for the differentiation of noradrenergic neurons. Here, we show that the expression of HAND2 and related HAND1 are maintained in sympathetic neurons throughout fetal and post-natal development when these neurons depend on target-derived nerve growth factor (NGF) for survival. Short interfering RNA knockdown of endogenous HAND2 and, to a lesser extent, HAND1 in neonatal sympathetic neurons cultured with NGF, reduced the expression of the NGF receptor tyrosine kinase TrkA (tropomyosin-related kinase A), as well as neuronal survival. Chromatin immunoprecipitation analysis showed that NGF promotes HAND2 binding to the TrkA minimal enhancer and that transfection of sympathetic neurons with a TrkA expression plasmid rescued the neurons from HAND knockdown. These findings show that HAND transcription factors have a crucial function in sustaining the survival of neonatal sympathetic neurons with NGF by a feed-forward loop that maintains the expression of TrkA.

Keywords: HAND2; survival; TrkA; sympathetic neuron

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INTRODUCTION

Paravertebral sympathetic neurons and their neural crest cell-derived precursors are an extensively studied model for investigating the molecular basis of neuronal differentiation, survival and death (Glebova & Ginty, 2005). Precursor cell specification and the early appearance of noradrenaline biosynthetic enzymes,

tyrosine hydroxylase and dopamine β -hydroxylase (D β H), are controlled by a network of cross-regulatory transcription factors initiated by bone morphogenetic proteins (Rohrer, 2003). An important member of this transcriptional cascade is the basic helix–loop–helix transcription factor HAND2 (also known as dHAND). The expression of HAND2 begins in the sympathetic ganglion primordia after the neural crest precursor cells have been specified to become sympathetic neurons (Howard *et al*, 2000). Gain-of-function and loss-of-function studies have shown that HAND2 is essential for the differentiation of noradrenergic neurons. Overexpression of *Hand2* in neural crest precursor cells induces the differentiation of noradrenergic neuron *in vitro* and *in vivo* (Howard *et al*, 1999, 2000; Xu *et al*, 2003), and ectopic expression of HAND2 in embryonic parasympathetic ganglia maintains the expression of tyrosine hydroxylase and D β H that is otherwise lost during development (Muller & Rohrer, 2002). *Hand2*-null zebrafish and mice with conditional inactivation of *Hand2* in the neural crest cell lineage have greatly reduced expression of tyrosine hydroxylase and D β H in early sympathetic ganglia (Lucas *et al*, 2006; Morikawa *et al*, 2007). Owing to the lethality of these latter HAND2 conditional mutants shortly after sympathetic ganglion formation (Morikawa *et al*, 2007), nothing is known of the potential roles of HAND2 at later stages of sympathetic neuron development.

In addition to HAND2, the related Hand1 (also known as eHAND) is also expressed in the early sympathetic chain (Cserjesi *et al*, 1995; Morikawa & Cserjesi, 2004). Its expression follows the appearance of tyrosine hydroxylase (Howard *et al*, 2000) and is regulated by HAND2 (Rychlik *et al*, 2005). However, the early embryonic lethality of HAND1-null embryos (Firulli *et al*, 1998; Morikawa & Cserjesi, 2004) has hampered the investigation of the role of HAND1 in sympathetic neuron development.

Here, we find that the HAND1 and HAND2 messenger RNAs are maintained at a constantly high level of expression in the sympathetic neurons of the mouse superior cervical ganglion (SCG) throughout fetal and early postnatal development when these neurons are dependent on target-derived nerve growth factor (NGF) for survival. Manipulating the expression of the HAND

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Fig 1 | HAND1 and HAND2 messenger RNAs are expressed in developing sympathetic neurons. The levels of HAND1 and HAND2 mRNAs relative to GAPDH mRNA in freshly purified E14, E16, E18, P0, P3 and P10 SCG neurons (normalized to a value of 1.0 at P0). Mean and s.e.m. of four measurements for each data point. E, embryonic day; GAPDH, glyceraldehyde phosphate dehydrogenase; P, postnatal day.

transcription factors in cultured neonatal mouse SCG neurons has shown a new and important role for these transcription factors in regulating cell survival.

RESULTS AND DISCUSSION

HAND mRNAs are expressed in postnatal SCG neurons

To investigate whether HAND transcription factors might have a function in sympathetic neuron development after acquisition of the noradrenergic phenotype, we quantified the levels of HAND1 and HAND2 mRNAs in SCG neurons purified (>95% neurons) from freshly dissociated SCG of fetal and postnatal mice. To compare the relative levels of HAND mRNAs at various ages, we also quantified the levels of mRNA for the ubiquitous house-keeping protein glyceraldehyde phosphate dehydrogenase (GAPDH). Fig 1 shows that the levels of HAND1 mRNA and HAND2 mRNA were maintained at almost constant levels relative to GAPDH mRNA at stages from embryonic day 14 (E14) to postnatal day 10 (P10). Immunocytochemistry confirmed the expression of HAND1 and HAND2 in cultured P0 SCG neurons (Fig 2). Although HAND1 showed predominantly nuclear staining, HAND2 was predominantly cytoplasmic or perinuclear, similar to the perinuclear staining observed for HAND1 in a proportion of trophoblast cells (Martindill *et al*, 2007). All neurons expressed

Fig 2 | Localization of HAND1, HAND2 and TrkA in cultured SCG neurons. Representative confocal images of P0 SCG neurons stained with antibodies to HAND1, HAND2 amino terminus (HAND-NT), HAND2 carboxyl terminus (HAND-CT) and TrkA (left-hand panels, rhodamine-labelled or Alexa Fluor 546-labelled secondary antibodies), and co-stained with an antibody to the neuron-specific protein β III-Tubulin (second panels, FITC-labelled secondary antibody) and with the DNA stain DAPI (third panels). Merged images are shown in the fourth panel. DAPI, 4',6-diamidino-2-phenylindole; E, embryonic day; FITC, fluorescein isothiocyanate; P, postnatal day; SCG, superior cervical ganglion; TrkA, tropomyosin-related kinase A.

HAND1, HAND2 and TrkA, although the intensity of labelling varied markedly among neurons.

HAND2 siRNA reduces D β H expression in P0 SCG neurons

We investigated the roles of HAND1 and HAND2 in newborn SCG neurons by nucleofecting these neurons with short interfering RNAs (siRNAs) that knock down HAND mRNAs. The effectiveness of stealth siRNAs was assessed by measuring the levels of HAND1 and HAND2 mRNAs in P0 SCG neurons cultured for 48 h after nucleofection with NGF and the irreversible caspase inhibitor Q-VD-OPH to prevent neuronal death and to ensure similar numbers of neurons under all experimental conditions. HAND1 siRNA, which is 100% complementary to a unique sequence in mouse HAND1 mRNA, caused about 70% reduction in HAND1 mRNA relative to scrambled (control) RNA and had no significant effect on HAND2 mRNA (Fig 3; supplementary Fig S1 online). HAND2 siRNA, which is 100% complementary to a unique sequence of mouse HAND2 mRNA, caused around 60% reduction in HAND2 mRNA relative to scrambled RNA and had no significant effect on HAND1 mRNA (Fig 3; supplementary Fig S1 online). HAND1/HAND2 siRNA, which is 96% complementary to HAND1 mRNA (single base pair difference in the centre of siRNA) and 100% complementary to HAND2 mRNA,

Fig 3 | siRNA HAND knockdown reduces the levels of DβH messenger RNA in newborn SCG neurons. The levels of HAND1, HAND2 and DβH mRNAs relative to GAPDH mRNA in purified P0 SCG neurons that were transfected with HAND1, HAND2, HAND1/HAND2 or control scrambled sequence siRNAs and were cultured for 48 h with 10 ng ml⁻¹ NGF (normalized to a value of 1.0 for scramble-transfected neurons). The means and s.e.m. of the results of 6–12 separate experiments are presented. ****P* < 0.001, statistical comparison with scramble-transfected neurons (*t*-test). DβH, dopamine β-hydroxylase; GAPDH, glyceraldehyde phosphate dehydrogenase; NGF, nerve growth factor; P, postnatal day; SCG, superior cervical ganglion; siRNA, short interfering RNA.

reduced the expression of HAND1 and HAND2 mRNAs by around 80 and 70%, respectively. There were no significant differences in the levels of HAND mRNAs between scrambled RNA-transfected and non-transfected neurons (data not shown).

As HAND2 is known to induce the differentiation of noradrenergic neurons in sympathetic neuroblasts (Howard *et al*, 1999, 2000; Xu *et al*, 2003), we examined the effects of HAND siRNAs on the expression of DβH mRNA in cultured newborn SCG neurons to investigate whether HAND transcription factors have a function in regulating the expression of this crucial noradrenergic marker at this late stage in development. Although HAND1 siRNA had no significant effect on the levels of DβH mRNA compared with scrambled RNA, HAND2 siRNA and HAND1/HAND2 caused around 60% reduction in the level of DβH mRNA (Fig 3; supplementary Fig S1 online). This suggests that HAND2 has an ongoing function in maintaining the noradrenergic phenotype of differentiated sympathetic neurons.

HAND siRNA inhibits NGF-promoted survival

Naturally occurring neuronal death is a prominent feature of sympathetic neuron development during late fetal and early

postnatal stages when competition for target-derived NGF matches the number of neurons to the size and requirements of their targets (Glebova & Ginty, 2005). To investigate whether HAND transcription factors have a function in regulating the survival of sympathetic neurons, we studied the effect of siRNA knockdown on the *in vitro* survival of newborn SCG neurons. Neuronal survival was assessed at intervals in P0 SCG neurons nucleofected with siRNAs, and cultured for up to 96 h with NGF and no caspase inhibitor. In the absence of NGF, all neurons died within 24 h of plating (data not shown). About 70% of neurons nucleofected with scrambled (control) RNA survived for 96 h with NGF (Fig 4), which was similar to the level of survival of non-nucleofected neurons grown with NGF (data not shown). The survival of neurons nucleofected with HAND1 siRNA was consistently lower than scrambled siRNA-nucleofected neurons at all time points, but the reduction was only statistically significant at 48 h (*P* < 0.05). HAND2 siRNA caused highly significant reductions in survival at all time points (*P* < 0.001), ranging from a 25% decrease relative to scrambled RNA after 24 h to a 32% decrease relative to scrambled RNA after 96 h. HAND1/HAND2 caused an even greater reduction in survival than HAND2 siRNA, most noticeably after 48 and 96 h (43 and 54% decrease, respectively, relative to scrambled RNA). These findings suggest that HAND2 and, to a lesser extent, HAND1 are important components of the molecular machinery that allows postnatal neurons to survive with NGF. The proportionately greater decrease with HAND1/HAND2 might represent some degree of cooperation between HAND1 and HAND2 in NGF-promoted survival, or it might reflect the marginally more effective knockdown of HAND2 mRNA by HAND1/HAND2 siRNA than by HAND2 siRNA (Fig 3). However, by comparison with the effects of siRNAs on the expression of DβH mRNA, in which HAND1 siRNA has no effect and HAND1/HAND2 is no more effective in reducing the levels of DβH mRNA than HAND2 siRNA, it seems most likely that some degree of cooperation between HAND1 and HAND2 occurs in NGF-promoted survival.

HAND siRNA reduces TrkA in NGF-treated neurons

The survival response of developing sympathetic neurons to NGF is mediated by the TrkA receptor tyrosine kinase (Reichardt, 2006). The expression of this receptor in developing sensory and sympathetic neurons is controlled by a minimal enhancer that contains consensus binding sites for 15 different transcription factors, including a putative site for HAND1/HAND2 (Ma *et al*, 2000). Mutation of this latter site eliminated the expression of *lacZ* in the sympathetic chain of E13 transgenic embryos having a TrkA minimal enhancer/*lacZ* reporter construct, suggesting that this site is required for the expression of TrkA in early sympathetic neurons (Ma *et al*, 2000). For this reason, we investigated whether HAND1 and HAND2 are required for the expression of TrkA transcripts in newborn SCG neurons. P0 SCG neurons were nucleofected with scrambled and HAND siRNAs, and the levels of TrkA mRNA were quantified after 48 h incubation in medium containing NGF and caspase inhibitor. Highly significant reductions in the levels of TrkA mRNA were observed in neurons nucleofected with HAND1, HAND2 and HAND1/HAND2 siRNAs as compared with scrambled siRNA-nucleofected neurons (Fig 5A). The greatest reduction was observed in HAND1/HAND2 siRNA-nucleofected neurons (33% reduction),

Fig 4 | siRNA HAND knockdown inhibits NGF-promoted survival of newborn SCG neurons. P0 SCG neurons were transfected with HAND1, HAND2, HAND1/HAND2 or scrambled (control) sequence siRNAs and then cultured with 10 ng ml⁻¹ NGF. Percentage survival was assessed after 24, 48 and 96 h (means and s.e.m. of 6–12 separate experiments; 200–300 neurons per data point per experiment). **P* < 0.05, ****P* < 0.001, statistical comparison with scramble-transfected neurons at each time point (*t*-test). NGF, nerve growth factor; P, postnatal day; SCG, superior cervical ganglion; siRNA, short interfering RNA.

suggesting that both HAND1 and HAND2 have a function in sustaining the levels of TrkA (tropomyosin-related kinase A) mRNA in postnatal sympathetic neurons.

When the above experiments were repeated in the absence of NGF, but with caspase inhibitors in the medium to prevent the death of SCG neurons, the level of TrkA mRNA was lower than in NGF-containing cultures, and HAND siRNAs failed to significantly reduce the levels of TrkA mRNA further. For this reason, we carried out a detailed NGF dose–response analysis with and without siRNA HAND knockdown to determine whether NGF regulates the expression of TrkA mRNA through a HAND-dependent mechanism. P0 SCG neurons were nucleofected with scrambled or HAND1/HAND2 siRNAs, and cultured with caspase inhibitor and NGF over a range of concentrations for 48 h. A dose-dependent increase in TrkA mRNA was observed with increasing concentrations of NGF, with twofold higher levels of TrkA mRNA in control-nucleofected neurons grown with 10 ng ml⁻¹ NGF compared with neurons grown without NGF (Fig 5B). HAND1/HAND2 siRNA did not significantly reduce the expression of TrkA mRNA in the absence of NGF, but caused significant reductions in the medium containing NGF at all concentrations used (Fig 5B). These findings suggest that the elevation in the expression of TrkA mRNA brought about by NGF is, to a large extent, dependent on HAND transcription factors, but that these factors are not required for maintaining basal expression of TrkA mRNA in the absence of NGF.

Binding of HAND2 to the TrkA minimal enhancer

The most parsimonious explanation for the above results is that NGF signalling promotes binding of HAND transcription factors to the TrkA minimal enhancer with a consequent increase in gene transcription. To explore this possibility, we used chromatin immunoprecipitation (ChIP) to determine whether NGF promotes binding of HAND2 to the TrkA minimal enhancer. To provide sufficient nucleoprotein for these studies, experiments were carried out on Neuro2A cells, as these cells express TrkA and

respond to NGF with decreased proliferation (Castellon & Mirkin, 2003). The Neuro2A cells were treated with and without NGF for 30 min, and sheared genomic DNA with its associated proteins was immunoprecipitated with a HAND2 antibody. PCR analysis of immunoprecipitated DNA from untreated Neuro2A cells showed the association of HAND2 with genomic sequences encompassing the putative HAND1/HAND2 site of the TrkA minimal enhancer and the DβH promoter (positive control), but not with parts of the coding sequences of GAPDH and β-actin (negative controls). NGF treatment significantly increased the association of HAND2 with both the TrkA minimal enhancer and the DβH promoter (Fig 5C; supplementary Fig S2 online). This suggests that HAND2 has a crucial function in mediating the effects of NGF on the expression of TrkA.

TrkA rescues neurons from HAND1 knockdown

The above findings suggest that HAND transcription factors have a crucial function in sustaining the survival of newborn SCG neurons with NGF by enhancing the expression of TrkA. As overexpression of TrkA in SCG neurons enhances NGF-promoted survival (Lavoie *et al*, 2005), we tested directly the importance of the regulation of the expression of TrkA by HAND transcription factors in maintaining the survival of SCG neurons with NGF. Newborn SCG neurons were co-nucleofected with HAND1/HAND2 siRNA (or scrambled siRNA) and a TrkA expression plasmid (or an empty control expression plasmid) to investigate whether overexpression of TrkA could rescue SCG neurons from HAND knockdown. In neurons nucleofected with an empty control expression plasmid, the survival of HAND1/HAND2 siRNA-nucleofected neurons was substantially lower than scrambled RNA-nucleofected neurons, as expected (Fig 5D). However, in neurons nucleofected with the TrkA plasmid, the survival of HAND1/HAND2 siRNA-nucleofected neurons was restored to the level observed in neurons co-nucleofected with scrambled RNA and control plasmid, and a further increase in survival was observed in scrambled RNA-nucleofected neurons

Fig 5 | NGF promotes binding of HAND2 to the TrkA minimal enhancer, upregulating the expression of TrkA and enhancing NGF-promoted survival. (A) Levels of TrkA messenger RNA relative to GAPDH mRNA in purified P0 SCG neurons nucleofected with scrambled, HAND1, HAND2 or HAND1/HAND2 siRNAs and cultured for 48 h with 25 mM Q-VD-OPH and 10 ng ml⁻¹ NGF (normalized to a value of 1.0 for scramble-transfected neurons). The mean and s.e.m. of eight measurements for each data point are presented. ** $P < 0.01$, *** $P < 0.001$, statistical comparison with scramble-transfected neurons (t -test). (B) Levels of TrkA mRNA relative to GAPDH mRNA in purified P0 SCG neurons cultured for 48 h with 25 mM of the broad-spectrum, irreversible caspase inhibitor Q-VD-OPH and either no NGF or NGF over a range of concentrations (normalized to a value of 1.0 for scramble-transfected neurons without NGF). The neurons were nucleofected with either scrambled siRNA or HAND1/HAND2 siRNA. The mean and s.e.m. of five measurements for each data point are presented. * $P < 0.05$, statistical comparison with scramble-transfected neurons at each concentration (t -test). (C) ChIP assay showing the association of HAND2 with the DβH promoter and TrkA minimal enhancer in Neuro2A cells treated with or without 50 ng ml⁻¹ NGF for 30 min. The relative fold difference between immunoprecipitated amplified genomic sequence was calculated relative to no antibody control. The mean and s.e.m. of 4–9 measurements for each data point are presented. ** $P < 0.01$, *** $P < 0.001$, statistical comparison with and without NGF (t -test). (D) Survival in NGF-supplemented cultures of purified P0 SCG neurons nucleofected with either scrambled or HAND1/HAND2 siRNAs together with either an empty pcDNA3.1 plasmid (control transfection) or a pcDNA3.1-TrkA plasmid (overexpression of TrkA). The mean and s.e.m. of six separate experiments are presented. * $P < 0.05$, *** $P < 0.001$, statistical comparison between control-transfected and TrkA-transfected (t -test). Brackets indicate statistical comparisons. ChIP, chromatin immunoprecipitation; DβH, dopamine β-hydroxylase; GAPDH, glyceraldehyde phosphate dehydrogenase; NGF, nerve growth factor; P, postnatal day; SCG, superior cervical ganglion; siRNA, short interfering RNA; TrkA, tropomyosin-related kinase A.

overexpressing TrkA (Fig 5D). Transfected neurons are shown in supplementary Fig S3 online. These results indicate that HAND transcription factors sustain the survival of postnatal sympathetic neurons with NGF by enhancing the expression of TrkA.

In summary, we have discovered an unexpected role for HAND transcription factors in sustaining the survival of neonatal sympathetic neurons with NGF. NGF signalling promotes the binding of HAND2 to the TrkA minimal enhancer, leading to the

increased expression of TrkA and improved survival with NGF. In this way, HAND2 forms part of a feed-forward loop that enhances the sensitivity of the neurons to NGF. In neonatal mice, the survival of sympathetic neurons is crucially dependent on a supply of NGF from their targets. As the production of NGF is not sufficient to support the survival of all of the neurons, many die by apoptosis (Glebova & Ginty, 2005). The operation of an NGF/HAND/TrkA feed-forward loop might have a function in facilitating the selection of neurons that survive and die under conditions of limiting NGF supply. Neurons for which axon terminals are well placed to procure an adequate supply of NGF will express higher levels of TrkA leading to a reduced requirement for NGF and continued survival, whereas neurons for which axon terminals are less well placed will express lower levels of TrkA leading to an escalating requirement for NGF and rapid demise. This idea is supported by the finding that small changes in the levels of expression are decisive for the ability of immature sympathetic neurons to survive in the presence of NGF (von Holst *et al*, 1997). NGF-promoted upregulation of TrkA has also been recently proposed to be an important component of the competition between sympathetic neurons for survival during development (Deppmann *et al*, 2008). Another possible function for the NGF/HAND/TrkA feed-forward loop is to compensate for ligand-mediated receptor downregulation, as binding of NGF to TrkA is followed by internalization and enhanced lysosomal degradation of TrkA (Jullien *et al*, 2002). In future work, it will be interesting to ascertain how NGF signalling enhances binding of HAND2 to the TrkA minimal enhancer and whether similar feed-forward mechanisms are also present in neuronal populations devoid of HAND2.

METHODS

Neuron cultures and nucleofection. Dissociated, purified SCG neurons from newborn mice of the CD-1 outbred line were grown at low density in defined neurobasal medium on a polyornithine/laminin-coated substratum in 35-mm tissue culture dishes for survival assays or 24-well plates for reverse transcription-PCR assays. The neurons were nucleofected with stealth siRNA (scrambled, HAND1, HAND2 or HAND1/HAND2) with or without expression plasmids (pmaxGFP, pcDNA3-TrkA or empty pcDNA3 control plasmid). For details and oligonucleotide sequences, see supplementary information online.

Immunocytochemistry. SCG neuron cultures were fixed with paraformaldehyde and methanol followed by permeabilization with Triton X-100. After blocking with serum, the cultures were incubated overnight at 4 °C with primary antibodies. After washing, the cultures were incubated for 1 h with an appropriate secondary antibody in the presence of the DNA stain DAPI (4',6-diamidino-2-phenylindole). Cells were then washed, mounted with Vectashield (Invitrogen, Carlsbad, CA, USA) medium and examined by confocal microscopy. See supplementary information online for further details.

Measurement of HAND1, HAND2, TrkA, DBH and tyrosine hydroxylase mRNAs. The levels of mRNAs were assessed by semiquantitative (HAND1 and HAND2) or quantitative (TrkA, DBH, tyrosine hydroxylase and GAPDH) reverse transcription-PCR in total RNA extracted from purified SCG neurons or from neuronal cultures. See supplementary information online for further details and primer sequences.

ChIP. ChIP was performed on cultured Neuro2A cells stimulated with or without 50 ng ml⁻¹ NGF for 30 min. Following formaldehyde crosslinking (1% for 10 min) and cell lysis, DNA was sonicated into 200–1000 bp lengths, and quantitative real-time PCR was performed on HAND2-immunoprecipitated fragments (see supplementary information online for further details and primer sequences). The relative proportions of immunoprecipitated fragments were determined on the basis of the threshold cycle (C_T) value for each PCR reaction. The relative fold difference between amplified fragments was calculated relative to a 'minus antibody' control using the $\Delta\Delta C_T$ method: (HAND2 ChIP relative to control (no Ab (= 1)) ChIP) = $2^{(\Delta C_T(\text{control}) - \Delta C_T(\text{HAND2}))}$ where $\Delta C_T = C_T(\text{immunoprecipitated sample}) - C_T(\text{input})$.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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