Hand transcription factors are required for neonatal sympathetic neuron survival
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Supplementary Methods
Neuron cultures and nucleofection

SCG neurons, purified by percol gradient centrifugation (> 95% pure) from newborn (P0) CD1 mice, were grown in defined medium in polyornithine/laminin coated tissue culture dishes as described previously (Doxakis et al, 2004). The neurons were nucleofected with either stealth siRNA alone or stealth siRNA plus expression plasmids according to the manufacturer’s instructions (Amaxa Biosystems). Briefly, 200,000 freshly dissociated P0 SCG neurons were centrifuged and the resulting pellet was resuspended in 100 µl Mouse Neuron Nucleofector™ solution at room temperature with 1.2 µl of 100 µM stealth siRNAs (scramble, Hand1, Hand2 or Hand1/2) with or without expression plasmids (3 µg pmaxGFP, 4.5 µg of pcDNA3-TrkA or empty pcDNA3 control plasmid). The mixture was transferred to a 2 mm Amaza electroporation cuvette, inserted in the Nucleofector™ II device and electroporated using program G-013. Immediately after transfection, 500 µl of pre-warmed culture medium was added to the cell suspension, and the neurons were plated in either 35 mm tissue culture dishes for survival assays or 24-well plates for RT-PCR assays. The latter cultures received the irreversible caspase inhibitor Q-VD-OPH (25 µM, Calbiochem) to prevent neuronal death and ensure mRNA levels were measured in similar numbers of neurons in all experimental conditions. The medium was changed in all cultures 3 hours after plating. For survival experiments involving plasmid nucleofection (where the transfection efficiency is around 20%), nucleofected neurons were recognized by the expression of GFP. Neuronal survival was quantified by counting the number of labeled neurons 12 hours after plating and again at 48 hours and expressing the latter counts as a percentage of the 12 hour counts. For survival experiments involving siRNA nucleofection (where transfection efficiency is close to 100%), neuronal survival was quantified by counting the number of neurons 6 hours after plating and again at 24, 48 and 96 hours and expressing the latter counts as a percentage of the 6 hour counts. Statistical significance was assessed using the student's paired t-test. The Stealth siRNA (Invitrogen) oligo sequences were:
scramble 1, GCUUGACACAUCGCUCAUCUCAUGA;
scramble 2, CCAACAUCCUACACUCACUCUAGGG;
Hand1 siRNA, GCUACCAGUUACAUCCUCUACUUGA;
Hand2 siRNA, CCACCAGAUACAUCGCCUACCUCAU;
Hand1/2, GCCGACACCAAACUCUCCAAGAUCA.
Scramble 1 and 2 were used interchangeably.

**Immunocytochemistry**

SCG neurons were plated on poly-L-lysine/laminin-coated (SIGMA) borosilicate glass coverslips (VWR) in Neurobasal media (Invitrogen) supplemented with 10 ng/ml NGF (Calbiochem) for 48 hours. Cells were fixed with fresh 4% (w/v) paraformaldehyde (SIGMA) for 13 minutes, followed by 1 minute in 100% methanol (Fisher Scientific). The cells were subsequently washed once with PBS (Biochrom), permeabilized for 3 minutes with 4% Triton X-100 (SIGMA), washed twice with PBS and were blocked with PBS containing 4% of secondary serum for 1 hour before incubated overnight at 4°C with primary antibodies (rabbit anti-Hand1, 1:200, Chemicon; goat anti-N-terminal Hand2, 1:50, Santa Cruz; goat anti-C-terminal Hand2, 1:50, Santa Cruz; rabbit anti-TrkA, 1:100, Upstate; mouse anti-βIII-tubulin (Tuj1), 1:400, Covance). The cells were then washed 3 times with PBS and incubated for 1 hour with either Alexa Fluor secondary antibodies (1:400 for Hand1 and TrkA, Invitrogen) or Santa Cruz fluorescence secondary antibodies (1:200 for Hand2 and βIII-tubulin) in the presence of the DNA stain 4′,6-diamidino-2-phenylindole (DAPI). Cells were then washed four times with PBS and mounted with Vectashield (Invitrogen) media. Photos were taken with a Leica TCS SP5 confocal microscope.

**Measurement of Hand1, Hand2, TrkA, DβH, and TH mRNAs**

Semi-quantitative (for Hand1 and Hand2) or quantitative RT/PCR assays (for TrkA, DβH, TH and GAPDH) were used to assess the levels of Hand1, Hand2, TrkA and DβH mRNAs in cultured neurons and freshly isolated and purified neurons. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel). The RNA was reverse transcribed and then amplified as previously described (Doxakis and Davies, 2004; Wyatt and Davies, 1993). The forward primers were as follows: Hand1, 5′-GGTGCTAATTGGGCTGAC-3′; Hand2, 5′-
GCAGGACTCAGAGCATCAAC-3'; TrkA, 5’-CGTCATGGCTGCTTTATGG-3'; DβH, 5’-CAGCAAGACTACCAGCTGCTC-3'; GAPDH, 5’-TCCAGTATGACTCCACTCAC-3’. The reverse primers were: Hand1, 5’-GTGTGAGTGAGTGATGATGG-3'; Hand2, 5’-TGAGGTAGGCGATGTAGCTG-3'; TrkA, 5’-ACTGGCAGAAGGAGACAG-3'; DβH, 5’-CACTAGATGGACAGTGTGTCATCC-3'; GAPDH, 5’-TCCTGGAAGATGTTGATGG-3’. The levels of Hand1, Hand2, TrkA and DβH mRNAs were calculated relative to GAPDH mRNA.

**Chromatin Immunoprecipitation (ChIP)**

ChIP was performed on cultured Neuro2A cells (ECACC) stimulated with or without 50 ng/ml NGF for 30 mins. Following formaldehyde cross-linking (1% for 10 mins) and cell lysis, DNA was sonicated on ice into lengths between 200 and 1000 basepairs using a Diogenode Bioruptor at medium power for 5 seconds with 20 second intervals over a 10 minute time course. The protocol was carried out according to manufacturer’s guidelines (Upstate) using Hand2 immunoprecipitating antibody (Santa Cruz) at 1:500 dilution. Quantitative real-time PCR was performed using Stratagene reagents for Mx3000 equipment at 40 cycles of amplification. The forward primers were as follows: TrkA, 5’ CCCTCTCCCTTTCTTACTA 3’; DβH, 5’ TGGAGGACATCAGCCACTC 3; GAPDH, 5’ TCCACTCTTCCACCTTC 3’. The reverse primers were as follows: TrkA, 5’ ACTTAAGAGGGTGTGCATGT 3’; DβH, 5’ CCAGGCTGCACCTAATCA 3; GAPDH, 5’ CTGTAGCCGTATTTCATTGTC 3’. The relative proportions of immunoprecipitated fragments were determined based on 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, where: (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 Figures

Supplementary Figure S1. Hand, DβH and TrkA mRNA levels in SCG neurons transfected with Hand siRNAs.

Gels showing the products of RT-PCR reactions amplified with primers specific for Hand1, Hand2, DβH, TrkA and GAPDH transcripts in cultured P0 SCG neurons 48 hours after transfection with Hand1, Hand2, Hand1/2 or scrambled siRNAs. The amplification products of two separate RT/PCR reactions are shown in each case.
Supplementary Figure S2. ChIP assay showing association of Hand2 with the TrkA minimal enhancer and DβH promoter.

ChIP analysis was performed on Neuro2A cells treated + / - 50 ng/ml NGF for 30 mins, followed by amplification of the TrkA minimal enhancer, the DβH promoter, and coding regions of GAPDH and β-actin (negative controls). Input DNA represents sheared chromatin not subjected to immunoprecipitation protocols. All reactions performed over 32 cycles of amplification.

Whilst these findings are consistent with association of Hand2 with the TrkA minimal enhancer, Hand2, like other bHLH transcription factors, is promiscuous and can bind various E-box sequences. This affinity may differ according to the Hand2 binding partner and the presence or absence of Hand2 DNA binding regulators like Jab1 (Dai et al., 2004). It is therefore possible that several other conserved E-box sequences in the TrkA promoter may be Hand2 binding targets. Hand2 may also function without direct DNA binding as has been shown for digit development in limb bud (McFadden et al., 2002). NGF does not significantly affect Hand2 mRNA expression in SCG neurons (not shown), suggesting that NGF signalling leads to enhanced direct or indirect DNA binding of pre-existing Hand2.
**Supplementary Figure S3.** Photomicrographs of P0 SCG neurons cultured with 10 ng/ml NGF for 24 hours after transfection with a GFP expression plasmid together with either a control pcDNA3.1 plasmid or TrkA expression plasmid.
Supplementary References


