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The bHLH transcription factor *Hand2* is essential for the maintenance of noradrenergic properties in differentiated sympathetic neurons

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Abstract

The basic helix-loop-helix transcription factor *Hand2* is essential for the proliferation and noradrenergic differentiation of sympathetic neuron precursors during development. Here we address the function of *Hand2* in postmitotic, differentiated sympathetic neurons. Knockdown of endogenous *Hand2* in cultured E12 chick sympathetic neurons by siRNA results in a significant (about 60%) decrease in the expression of the noradrenergic marker genes *dopamine-β-hydroxylase (DBH)* and *tyrosine hydroxylase (TH)*. In contrast, expression of the pan-neuronal genes *TuJ1*, *HuC* and *SCG10* was not affected. To analyze the *in vivo* role of *Hand2* in differentiated sympathetic neurons we used mice harboring a conditional *Hand2*-null allele and excised the gene by expression of Cre recombinase under control of the *DBH* promoter. Mouse embryos homozygous for *Hand2* gene deletion showed decreased sympathetic neuron number and TH expression was strongly reduced in the residual neuron population. The *in vitro Hand2* knockdown also enhances the CNTF-induced expression of the cholinergic marker genes *vesicular acetylcholine transporter (VAcHT)* and *choline acetyltransferase (ChAT)*. Taken together, these findings demonstrate that the *Hand2* transcription factor plays a key role in maintaining noradrenergic properties in differentiated neurons.

Keywords

Hand2; siRNA knockdown; *TH*; *DBH*; *ChAT*; *VAcHT*; sympathetic neuron survival; proliferation; sympathetic ganglia

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Introduction

The development of sympathetic neurons is initiated by bone morphogenetic proteins (BMPs) acting on neural crest cells that have migrated to the dorsal aorta. BMPs control the expression of a group of transcription factors, including *Ascl1*, *Phox2b*, *Phox2a*, *Hand2* and *Gata2/3* that play important roles in sympathetic neuron specification and differentiation (reviewed in (Goridis and Rohrer, 2002; Howard, 2005; Rohrer, 2003)). Sympathetic neuron development is completely prevented in the absence of *Phox2b*, which is essential for development of the autonomic nervous system (Pattyn et al., 1999). *Ascl1* mainly affects the timing of sympathetic neuron development, as shown by delayed sympathetic neuron differentiation in *Ascl1*-deficient mouse embryos (Pattyn et al., 2006). A similar role has been demonstrated for the transcription factor *Insm1*, which in addition also affects sympathetic neuron proliferation (Wildner et al., 2008). Whereas the knockouts of *Phox2b*, *Ascl1* and *Insm1* displayed pleiotropic functions on noradrenergic and generic neuronal differentiation, more selective effects on the expression of noradrenergic marker genes were observed in the absence of *Gata2/3* and *Hand2*, in particular during early development. In the absence of *Gata3* a selective decrease of *TH* expression was found at E10.5, followed by impaired neuronal differentiation and cell death (Lim et al., 2000; Tsarovina et al., 2004). *TH* and *DBH* are strongly reduced in developing sympathetic ganglia of the *Hand2* zebrafish mutant *hands off* (Hendershot et al., 2008; Lucas et al., 2006) and in conditional *Hand2* mouse knockouts where the *Hand2* gene was excised in neural crest cells, using *Wnt1-Cre* (*Hand2^{Wnt1Cre}*) (Hendershot et al., 2008; Morikawa et al., 2007). HuC, TuJ1 and NF160 are less affected and reduced to a variable extent in different model systems analyzed (Hendershot et al., 2008; Morikawa et al., 2007; Lucas et al., 2006). Interestingly, *Hand2* is also required for the proliferation of sympathetic neuron precursors and immature sympathetic neurons, resulting in a massive decrease in neuron numbers in the conditional *Hand2^{Wnt1Cre}* knockout (Hendershot et al., 2008).

The knockouts demonstrate that *Hand2* is important for the proliferation and noradrenergic differentiation in sympathetic ganglia. The marked decrease in *TH* and *DBH* expression implies an essential function for *Hand2* in the initial onset of noradrenergic differentiation (Hendershot et al., 2008; Morikawa et al., 2007; Lucas et al., 2006). However, *Hand2* is not required for the initial expression of *TH* and *DBH* in young parasympathetic ciliary ganglion neurons that are devoid of *Hand2* (Müller and Rohrer, 2002). *TH* and *DBH* are expressed during normal ciliary neuron development only transiently, but the loss of *TH* and *DBH* could be prevented by ectopic *Hand2* expression. We thus proposed that the function of *Hand2* in sympathetic neurons includes the maintenance of noradrenergic differentiation (Müller and Rohrer, 2002).

To address the role of *Hand2* in sustaining noradrenergic gene expression, a siRNA knockdown approach was used in cultures of postmitotic, differentiated neurons from E12 chick sympathetic ganglia. The *Hand2* knockdown resulted in a strongly decreased expression of *TH* and *DBH*, without affecting neuron number and the expression of pan-neuronal genes like TuJ1, HuC and *SCG10*. In parallel, we analyzed sympathetic ganglia of a mouse line where *Hand2* has been conditionally eliminated in differentiated, *DBH*-expressing neurons (*Hand2^{DBHCre}*). Sympathetic ganglia of E11.5 and E14.5 *Hand2^{DBHCre}* mouse embryos show decreased neuron numbers and a significantly diminished *TH* expression in the residual neuron population, confirming the results of the in vitro *Hand2* knockdown. Thus, we conclude that *Hand2* is essential for the maintenance of the noradrenergic phenotype of sympathetic neurons.

Materials and methods

Construction of plasmids

pCAGGS-mHand2 and *pCAGGS-zHand2* PCR technology was used to insert a Kozak sequence linked to a *NheI* site and a *Clal* site flanking the coding sequence of *pcDNA3-mus*

musculus Hand2 (kindly provided by E. Doxakis) and *pCS2+-zebrafish Hand2* (kindly provided by P. D. Henion). *mHand2*-Primer: sense: 5'-AAG CTA GCA CCA CCA TGA GTC TGG TGG GGG GC-3'; antisense: 5'-TTA TCG ATT CAC TGC TTG AGC TCC AGG G-3'; *zHand2*-Primer: sense: 5'-AAG CTA GCA CCA CCA TGA GTT TGA TTG GAG GGT TTC-3'; antisense: 5'-TTA TCG ATT CAT TGC TTC AGT TCC AAT GCC-3' (sense primer: bold, *NheI* site; underlined, Kozak sequence; bold + underlined, Start) (antisense primer: bold, *Clal* site; bold+underlined, Stop). The PCR product was then cloned directly into the *pCAGGS* vector.

Primary culture preparation and electroporation

Chick sympathetic neurons were prepared by dissociation of paravertebral lumbosacral sympathetic chain ganglia, dissected at E7/E12 (Ernsberger et al., 1989a; Ernsberger et al., 1989b). Cells were cultivated in the presence of either nerve growth factor (NGF, 15ng/ml, (Peprotech)) or of both NGF and ciliary neurotrophic factor (CNTF, 30ng/ml, (Peprotech)). For *Hand2* knockdown experiments, 200.000 cells were mixed with either 3µg siRNA against *Hand2* or control siRNA and electroporated with the Amaxa Nucleofector device according to the Amaxa nucleofection protocol for primary chicken neurons (Program G13). For rescue experiments, cells were transfected by electroporation as described above with 3µg siRNA plus expression plasmids (1µg control siRNA plus 1µg *pCAGGS-eGFP* (*GFP*) for controls, 1µg control siRNA plus 1µg *pCAGGS-eGFP* and 1µg *pCAGGS-z/mHand2* for overexpression, 1µg siRNA against *Hand2* plus 1µg *pCAGGS-eGFP* for knockdown and 1µg siRNA against *Hand2* plus 1µg *pCAGGS-eGFP* and 1µg *pCAGGS-z/mHand2* for rescue). 200.000 cells per electroporation were plated on a 35mm 4-well dish or 35mm dish precoated with poly-DL-ornithine (Sigma) and Laminin (Invitrogen). The cultures were incubated for 2 days in MEM media containing 5% FCS, 10% HS, 1% glutamine and 1% Penicillin/Streptomycin at 37°C and 5% CO₂. Cells were harvested after 4 days and total RNA was isolated using the QIAGEN RNeasy Mini Kit, following the manufacturer's protocol. The siRNAs against *ggHand2* target the following sequence: 5'-CACAGTTAGCAGCAGCGATAA-3' (*Hand2* siRNA1; Qiagen) and 5'-GAAGAGGAAGAAGGAGCTGAA-3' (*Hand2* siRNA2; Qiagen). *Hand2* siRNA1 does not target mouse and zebrafish *Hand2* due to 3 and 5 nucleotide mismatches, respectively (Dahlgren et al., 2008). The control siRNA against *GFP* (*siGFP*; Qiagen) targets the sequence 5'-GCAAGCTGACCCTGAGTTC-3'. The control siRNA against *NP25* is non-functional and targets the sequence 5'GCACCTCTGTCTGTAGAGA.

In situ hybridization in cell culture

Non-radioactive in situ hybridization in cell culture and preparation of digoxigenin-labelled riboprobes for *SCG10*, *DBH*, *TH* and *Hand2* were carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999).

Immunocytochemistry in cell culture

Cell culture dishes were washed once with PBS and cells were fixed using 4% paraformaldehyde in 0.1M sodium phosphate buffer for 15min. Cells were washed with PBS and incubated for 15min in staining buffer (PBS, 5% FCS, 0.2% Triton-X-100). Primary antibody for *Hand2* (Santa Cruz) was diluted 1/100, for *TH* (Rohrer et al., 1986), for βIII-Tubulin (Tuj1 antigen, HISS Diagnostics, MMS-435P), for *HuC/HuD* (Molecular Probes) were diluted 1/1000 in staining buffer and incubated for 1h at RT. Cell culture dishes were washed twice with PBST (PBS, 0.2% Triton-X-100). Fluorophore coupled secondary antibody (in PBST + DAPI (1µg/ml)) was incubated for 30 min at RT and washed off twice with PBS. Sections were covered with AquaPolyMount covering media (Polysciences, Inc.) and glass cover slips. Neurons counts were obtained by examining phase-bright or fluorescent neurons in randomly selected fields of the culture dish. At least 10 visual fields were counted for each

well. Two to four wells of a dish were examined for each parameter in each experiment. The number of independent experiments analyzed is indicated by *n*. The results are given as the mean number per dish \pm s.e.m. of at least five experiments analyzed. Student's *t*-test was used for statistical analysis.

Semiquantitative RT- PCR

cDNA synthesis on total RNA from sympathetic ganglia cells was performed using the M-MLV Reverse Transcriptase Kit (Invitrogen). For detection of *ChAT*, *VACHT*, *SCG10* and *GAPDH* mRNA levels, the following primer combinations and the Taq Polymerase Kit (Invitrogen) for PCR reaction were used: gg*ChAT*-forward (for) 5'-CAACATTAGGTCTGCTACGGCG-3', gg*ChAT*-reverse (rev) 5'-GCAACTGTGTGGCTTCTTCTTG-3', gg*VACHT*-for 5'-TTTCTGGCAGGTCATCATCCC-3', gg*VACHT*-rev 5'-GGTGTCTAGAGTCCCTTAGGTCC-3'; gg*SCG10*-for 5'-TTTAATGCCCGGAGATTCTG-3', gg*SCG10*-rev 5'-TCAGCTTTTCTCTGCCATT-3' gg*GAPDH*-for 5'-CAGAGGTGCTGCCAGAA-3', gg*GAPDH*-rev 5'-GCAGGGGCTCCAACAAAG-3'.

All PCR reactions were performed within the linear range of amplification; this was determined empirically for each primer pair and cell preparation. *GAPDH* was analyzed after 20 and 22 cycles, *SCG10* after 27 and 29 cycles, *VACHT* after 29 and 31 cycles and *ChAT* after 31 and 33 cycles. The PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide. Quantification was done with the ScionImage Software version 1.63 (Scion Corporation). *ChAT*, *VACHT*, *SCG10* mRNA level were normalized to *GAPDH* mRNA level. In some cases *ChAT* and *VACHT* mRNA levels were referred to *SCG10* mRNA levels. Quantification for each experiment and gene was done in triplicate. Data shown represent mean \pm s.e.m. of at least 4 independent experiments. Statistical analysis was done using paired Student's *t*-test.

Transfection of stable cell lines expressing Hand2-myc (Hand2-myc-DF1)

Chicken fibroblasts of the DF1 cell line were transfected with 1 μ g *RCAS(B)-Hand2-myc* in 10 cm dishes according to the Qiagen Effectene protocol and incubated in DMEM with 10% FCS and 1% Penicillin/Streptomycin at 37°C and 10% CO₂. Expression levels of Hand2 were assessed by western blotting of 2-day-cultures.

Protein Blotting

Hand2-myc-DF1 cells were harvested and pelleted by centrifugation (100 \times g, 10min). Pelleted cells were lysed with cold lysis buffer (50mM Tris/HCl pH7.4, 150mM NaCl, 40mM NaF, 5mM EDTA, 5mM EGTA, 1mM Na₃VO₄, 1% Triton-X100, 0.1% Na-deoxycholat, 0.1% SDS, 1mM PMSF, 10 μ g/ml Aprotinin) for 10min on ice. After centrifugation (10.000 \times g, 10min, 4°C) the supernatant was collected and protein content determined with the DC protein assay (BioRad). After conventional SDS-PAGE and semi-dry blotting to PVDF membrane (BioRad), the membrane was blocked with 5% instant milk powder in TBST (10mM Tris/HCl pH8, 150mM NaCl, 0.05% Tween20) for 1h before antibody incubation. The anti-myc- (Abcam) and anti-actin-antibodies (Dianova, MS-1295) were diluted 1/2500 in blocking solution and incubated overnight at 4°C. After washing 3 times with TBST the appropriate HRP-coupled secondary antibody (1/5000 in TBST) was incubated for 1h at RT and washed twice with TBS. The blots were developed using standard ECL according to manufacturer's instructions (Pierce, SuperSignal). For quantification of protein bands the software ScionImage version 4.03.2 from (Scion Corporation) was used with the macro "gel blot 2".

Mouse Strains

All animal care, breeding procedures, and experimental protocols were approved by the Medical University of Ohio (renamed University of Toledo Health Sciences Campus) animal care and use committee. A description of our strategy for targeting *Hand2* is published elsewhere (Hendershot et al., 2007). To generate *Hand2^{DBHCre}* mice, *DBHCre⁺⁰;Hand2^{wt/del}* males were mated to *Hand2^{fl/fl}* females. The *DBHCre* mouse line has been described previously (Matsushita et al., 2004). Plug date was counted as E0.5. Because targeted deletion of *Hand2* is embryonic lethal at around E11 we used a pharmacological approach to rescue embryos making it possible to assess effects of *Hand2* deletion in embryos older than E10-12. For rescue, pregnant dams received water containing a cocktail of catecholamines (200 µg/ml L-phenylephrine, 100 µg/ml isoproterenol, and 2 mg/ml ascorbic acid) beginning at E8.

Immunocytochemistry and cell counts of tissue sections

Preparation of tissue sections for cell counts and immunostaining were carried out using our established methods (Hendershot et al., 2008; Hendershot et al., 2007). Following fixation in 4% paraformaldehyde, pH 7.4, embryos are washed in PBS and stored in 30% sucrose. Embryos are fixed from 4 hours (E10) to overnight (E12 and E14) at 4°C. For cell counts, embryos were embedded in Cryo-gel™ (Instrumedics, St. Louis, MO) and 20µm serial sections were cut on a Leica CM 3050 S freezing microtome. Cell counts were compiled from trunk sympathetic ganglia on serial sections spanning the region from just above the forelimb and inclusive to the hindlimb. For immunostaining, tissue sections were blocked (0.1M Tris, pH 7.4, 1.5% NaCl, 0.3% Triton-X 100 and 10% horse serum) for 30 minutes and then incubated in primary antibodies (0.1M Tris, pH 7.4, 1.5% NaCl, 0.3% Triton-X 100 and 4% horse serum) overnight at 4°C. After washing with 0.1M Tris pH 7.4, 15% NaCl, sections were incubated at room temperature for 3 hours in secondary antibodies (0.1M Tris, pH 7.4, 1.5% NaCl, 0.3% Triton-X 100 and 4% horse serum). Sections were washed 3 × 5 minutes (0.1M Tris, pH 7.4, 15% NaCl) and mounted in Vectashield (Vector Laboratories, Burlingame, CA) or Fluoromount-G (Southern Biotech, Birmingham, AL). The anti-TH antibody (Pel Freez, Rogers, AK) was used at a dilution of 1:200. The anti-Hu antibody (1:50,000) was the generous gift of Vanda A. Lennon (Mayo Clinic, Rochester, MN). Species specific secondary antibodies used were goat anti-rabbit FITC (TH) (Abcam, Cambridge, MA) and Donkey anti-human TRITC (Hu) (Jackson ImmunoResearch, Inc. West Grove, PA). Data of cell counts are presented as the mean ± s.e.m. Statistical significance was determined using ANOVA and Bonferroni post hoc test.

Results

siRNA knockdown of *Hand2*

Two *Hand2* siRNA oligonucleotides that reduce *Hand2* protein expression by 50–60% were selected using the chick DF-1 cell line previously transfected with *Hand2-myc* expressing RCAS virus (Fig. 1). Control siRNA were used that are directed either against the pan-neuronal gene *NP25* (Pape et al., 2008) or against *GFP*, which is not expressed in vertebrate cells. To assess the extent of *Hand2* knockdown in sympathetic neurons, E12 (Fig. 2A,B) and E7 (not shown) chick sympathetic ganglion cells were transfected with control and *Hand2* siRNA before plating and analyzed by in situ hybridization for *Hand2* expression in 2-day-cultures. Whereas 93±2% of sympathetic neurons displayed an in situ hybridization signal for *Hand2* in *GFP* siRNA transfected control cultures, (Fig. 2) only 44±2% and 50±3% *Hand2*-positive cells were observed upon transfection with *Hand2* siRNA1 and *Hand2* siRNA2, respectively. In control hybridizations with sense *Hand2* RNA no labeled cells were observed. Antibody staining for *Hand2* was less sensitive, but revealed a similar extent of *Hand2* knockdown, from

about 60% neurons with nuclear Hand2-staining in *siGFP*-controls to 20% in *Hand2* siRNA transfections (not shown).

Hand2 knockdown results in a strong decrease in TH and DBH expression

Hand2 knockdown by siRNA affected *TH* mRNA expression in both E12 (Fig. 3) and E7 (not shown) sympathetic neuron cultures. Whereas $79\pm 2\%$ of neurons express *TH* in E12 cultures transfected with control siRNA, only $39\pm 3\%$ and $40\pm 4\%$ *TH*-positive cells were present in cultures with *Hand2* siRNA1 and *Hand2* siRNA2, respectively (Fig. 3). A sense probe for *TH* did not produce a signal in the in situ hybridization. The *Hand2* knockdown also reduced the proportion of TH-immunoreactive neurons from $62\pm 2\%$ in E12 cultures transfected with control siRNA, to $31\pm 3\%$ TH-immunoreactive in cultures transfected with *Hand2* siRNA1 or *Hand2* siRNA2 (Supplementary Fig. 1; not shown). To exclude the possibility that the response to *Hand2* siRNA treatments are due to off-target effects, rescue experiments were done using parallel transfections with *Hand2* siRNA1 and mouse and zebrafish *Hand2* that are not targeted by the chick *Hand2* siRNA1. The transfection of both mouse and zebrafish *Hand2* completely rescued the effect of chick *Hand2* siRNA on the expression of *TH* (Supplementary Fig. 1), demonstrating that the effects of chick *Hand2* siRNA are due to reduced *Hand2* levels. The best studied target gene of *Hand2* in the sympathoadrenal lineage is *DBH*, where DNA binding and transactivation have been characterized in detail (Rychlik et al., 2003; Xu et al., 2003). Therefore, we analysed the proportion of *DBH*-expressing cells to assess the role of *Hand2* in the maintenance of noradrenergic differentiation. *DBH*-expressing neurons with strong in situ hybridization signal could be clearly distinguished from *DBH*-negative satellite cells and fibroblasts in E12 (Fig. 4) and E7 cultures (not shown). The specificity of the *DBH* in situ signal is demonstrated by the complete lack of hybridization with a *DBH* sense probe. In control *GFP* and *NP25* siRNA transfections of E12 ganglion cells, about 85% of cells with neuronal morphology were distinctly *DBH*-positive (Fig. 4A,B,G). *Hand2* siRNA transfections resulted in a strong decrease in the proportion of *DBH*-positive neurons to $40\pm 2\%$ and $35\pm 1\%$ for *Hand2* siRNA1 and *Hand2* siRNA2, respectively (Fig. 4C,D,G).

Taken together, these results demonstrate that *Hand2* knockdown produces a strong decrease in the proportion of noradrenergic, *TH*- and *DBH*-expressing neurons.

Hand2 knockdown does not affect sympathetic neuron survival

The decreased proportion of noradrenergic neurons observed upon *Hand2* knockdown could either be due to the selective survival of non-noradrenergic neurons or due to a general decrease in *TH* and *DBH* expression which leads in some cells to levels below the detection limit. An effect of *Hand2* knockdown on chick sympathetic neuron survival was expected, since *Hand2* has been shown to control *trkA* expression and NGF-dependent survival in cultures of postnatal mouse sympathetic neurons (Doxakis et al., 2008). To investigate effects of *Hand2* knockdown on neuron survival, the number of sympathetic neurons per culture, identified as cells with neuronal morphology and DAPI-positive nucleus, was determined in 2-day-cultures. As shown in Fig. 5, the same neuron density was observed in cultures transfected either with control or *Hand2* siRNA in E12 neuron cultures. This result excludes survival effects of the *Hand2* knockdown under the culture conditions used, thus supporting the notion that the levels of *TH* and *DBH* expression in differentiated sympathetic neurons are *Hand2* dependent. The lack of survival effects, in contrast to findings in mouse neuron cultures (Doxakis et al., 2008), is most likely due to the shorter culture period, different culture conditions and the knockdown of *Hand2* rather than of both *Hand2* and *Hand1*. As sympathetic neuron proliferation has ceased in the chick embryo at E12, neuron numbers in these cultures are also not affected by the strong antiproliferative effects of *Hand2* elimination observed at earlier stages (Hendershot et al., 2008).

The expression of generic neuronal genes in differentiated E12 chick sympathetic neurons does not depend on *Hand2*

In the conditional mouse *Hand2* knockouts and the zebrafish *Hand2* mutant, discrepant results were observed with respect to the expression of pan-neuronal genes (Hendershot et al., 2008; Lucas et al., 2006; Morikawa et al., 2007). Thus, it was of considerable interest to investigate to which extent *Hand2* controls generic neuronal gene expression in differentiated sympathetic neurons. The expression of three pan-neuronal gene products was analyzed in chick sympathetic ganglion neurons in vitro: HuC and TuJ1 by immunostaining and *SCG10* by in situ hybridization (Fig. 6). Interestingly, all neuronal markers were unaffected by the *Hand2* knockdown, which demonstrated that the siRNA-mediated reduction of *Hand2* expression levels is essential for *TH* and *DBH* expression but has no functional relevance for the expression of HuC, TuJ1 and *SCG10*. To directly demonstrate the correlation between *Hand2*, noradrenergic and pan-neuronal gene expression, *Hand2* siRNA-transfected cells were analyzed for *Hand2*, *TH* and *TUJ1* (Supplementary Fig. 2A–D). Only a minority (8±1%) of *Hand2*-negative sympathetic neurons was *TH*-positive, whereas virtually all expressed *TuJ1* (Supplementary Fig. 2E). In contrast, *Hand2*-positive sympathetic neurons, reflecting incomplete knockdown, displayed both *TH* and *TuJ1* (Supplementary Fig. 2F). Together, these results implicate a selective role of *Hand2* in the sustained expression of noradrenergic but not pan-neuronal genes in postmitotic neurons.

Epistatic relationship of *Hand2* and *Gata2*

Hand2 acts in a network of cross-regulatory transcription factors (Goridis and Rohrer, 2002; Howard, 2005). Previous studies of sympathetic neuron development in the zebrafish *Hand2* mutant *hands off* revealed a progressive loss of *Gata2* expression (Lucas et al., 2006), suggesting that *Hand2* might maintain *Gata2* expression and that the reduced *Gata2* levels participate in mediating effects of *Hand2* on noradrenergic gene expression. An action of *Gata2* downstream of *Hand2* was implied from the finding that *Gata2* is expressed after *Hand2* during chick sympathetic ganglion development (Tsarovina et al., 2004). Whereas in chick sympathetic neurons *Gata2* but not *Gata3* is expressed, *Gata3* is essential for sympathetic neuron differentiation in the mouse (Tsarovina et al., 2004). In the conditional *Hand2* knockouts in the mouse, *Gata3* was found to be reduced in one mouse line (Hendershot et al., 2008) but not affected in the other (Morikawa et al., 2007). Is *Gata2* expression dependent on *Hand2* in differentiated chick sympathetic neurons? *Hand2* knockdown produced only a very minor reduction of *Gata2* expression (Supplementary Fig. 3). In view of the delayed decrease of *Gata2* expression in zebrafish, *Gata2* levels were analyzed also at a later time point. However, in 4-day-old cultures no significant effects on *Gata2* expression were found (Supplementary Fig. 3). As *Gata3* responds much less to a reduction in *Hand2* expression levels than *TH* and *DBH* (Hendershot et al., 2008; Lucas et al., 2006) it seems very unlikely that *Hand2* effects on noradrenergic differentiation are mediated through effects on *Gata2/3* expression. However, *Gata2/3* may be required, at least in part, to mediate other downstream effects of *Hand2*.

Hand2 and cholinergic differentiation in sympathetic neurons

During early development, sympathetic and parasympathetic neurons transiently co-express noradrenergic and cholinergic markers (Bureau et al., 2004; Ernsberger et al., 1997; Ernsberger and Rohrer, 1999; Huber and Ernsberger, 2006; Lee et al., 2003; Müller and Rohrer, 2002). Subsequently, *ChAT* and *VACHT* expression are lost in most, if not all sympathetic neurons and, vice versa, *TH* and *DBH* expression disappears from most parasympathetic ganglia. Interestingly, *Hand2* is not expressed in the parasympathetic ciliary ganglion, but ectopic *Hand2* expression maintains noradrenergic properties (Müller and Rohrer, 2002). The correlation between *Hand2* expression and reduced cholinergic differentiation raised the

question whether *Hand2* not only induces noradrenergic properties but may also reduce the expression of cholinergic markers in sympathetic (autonomic) neurons. To address this issue, we have investigated the expression of *ChAT* and *VACht* in sympathetic neurons treated with *Hand2* siRNA. Semiquantitative RT-PCR analysis of *ChAT* and *VACht* expression in E12 sympathetic neurons, maintained under standard culture conditions, showed unaltered *ChAT* and *VACht* expression upon *Hand2* knockdown (not shown). We then investigated whether *Hand2* knockdown would enhance the expression of cholinergic marker genes stimulated by CNTF (Ernsberger et al., 2000; Ernsberger et al., 1989b). *ChAT* and *VACht* expression levels increased in response to CNTF to $160\pm 27\%$ and $230\pm 28\%$ of controls without CNTF (not shown). *Hand2* knockdown in the presence of CNTF further increased *VACht* and *ChAT* expression (Fig. 7). These results suggest that *Hand2* in parallel maintains noradrenergic and antagonizes cholinergic gene expression in differentiated sympathetic neurons. This notion is supported by the finding that *Hand2* expression levels in mature, E20 sympathetic ganglia are very low in the subpopulation of *VACht*-expressing cholinergic sympathetic neurons as shown by double-in situ hybridisation for *Hand2* and *VACht* (Supplementary Fig. 4A, B). Double-in situ hybridization for *Hand2* and *SCG10* was carried out as control, demonstrating similar *SCG10* expression levels in *Hand2*-positive and *Hand2*-negative sympathetic neurons (Supplementary Fig. 4C, D).

Hand2 is required in vivo for neuron proliferation and the maintenance of noradrenergic properties in sympathetic neurons

To investigate the in vivo role of *Hand2* in differentiated sympathetic neurons, *Hand2* was selectively eliminated in *DBH*-expressing neurons of *Hand2^{DBHCre}* mouse embryos. *Hand2^{DBHCre}* mice were generated by cross-breeding *Hand2^{fl/fl}* (Hendershot et al., 2008) and *DBHCre* mice (Matsushita et al., 2004). Although *DBH* (and *DBHCre*) is expressed very early, from E10.5 onwards in sympathetic ganglia, *Hand2* is eliminated after noradrenergic and generic neuronal gene expression has proceeded (Goridis and Rohrer, 2002; Howard, 2005). This allowed us to investigate the function of *Hand2* in the maintenance rather than the initial induction of noradrenergic and generic neuronal gene expression.

The analysis of sympathetic ganglia in *Hand2^{DBHCre}* embryos at E10.5 revealed no significant difference in the number of HuC- and TH-positive cells as compared to controls (data not shown). This is in contrast to the reduced number of TH- and HuC-positive cells in *Hand2^{Wnt1Cre}* embryos (Hendershot et al., 2008) but is expected due to the delayed onset of *Cre* expression in *DBH*-expressing cells in primary sympathetic ganglia (*Hand2^{DBHCre}*), as compared to migrating neural crest cells before the onset of differentiation (*Hand2^{Wnt1Cre}*). However, at E11.5, TH is strongly reduced, with a smaller effect on Hu expression (Fig. 8A,B). Quantification shows that the number of Hu-positive neurons is lowered in *Hand2^{DBHCre}* to about 84% of controls and only about 43% of these remaining cells express TH-immunoreactivity (Fig. 8C). At E14.5 the number of Hu-positive neurons is reduced to 12% of controls and only 41% of this residual neuron population express TH (Fig. 8D). Thus, less than half of the sympathetic neurons display TH-immunoreactivity in ganglia where *Hand2* has been eliminated in *DBH*-expressing cells, whereas virtually all Hu-positive sympathetic neurons co-express TH in control ganglia (Fig. 8D). This result confirms the conclusions from our in vitro analysis and demonstrates that TH expression depends on *Hand2* not only during initial differentiation but also in *DBH*-expressing sympathetic neurons.

The strongly reduced sympathetic ganglion size in the *Hand2^{Wnt1Cre}* conditional knockout is mainly due to reduced proliferation, reflected by a massive decrease in the proportion of Ki67-expressing cells (Hendershot et al., 2008). Also in the *Hand2^{DBHCre}* conditional knockout the proliferation of immature sympathetic neurons is affected. At E14.5, about 31% of Hu-positive

cells express Ki67 in controls, but almost no Hu-positive cells are dividing in sympathetic ganglia of *Hand2^{DBHCre}* embryos (Fig. 8E).

Discussion

The bHLH transcription factor *Hand2* is expressed in developing and mature sympathetic neurons. During specification and early differentiation of sympathetic neurons, *Hand2* has been shown to play important roles in the control of the noradrenergic marker genes *DBH* and *TH* and in the proliferation of progenitors and immature sympathetic neurons. Here, we provide evidence that *Hand2* is also required for the continued expression of *DBH* and *TH* in cultured avian postmitotic, differentiated sympathetic neurons, whereas the expression of the generic neuronal marker genes HuC, TuJ1 and *SCG10* in these cells does not depend on *Hand2*. In parallel, *Hand2* seems to interfere with cholinergic differentiation of sympathetic neurons, as *Hand2* knockdown facilitates the expression of cholinergic marker genes. By eliminating *Hand2* in *DBH*-expressing noradrenergic neurons of *Hand2^{DBHCre}* mice the important role of *Hand2* for the maintenance of TH-expression was confirmed in vivo. This analysis also revealed a continued *Hand2* requirement for sympathetic neuron proliferation.

Hand2 in the induction and maintenance of noradrenergic gene expression

In contrast to the transient expression of the proneural gene *Ascl1*, all other members of the network of transcription factors controlling sympathetic neuron specification and differentiation are also expressed in differentiated sympathetic neurons. Their function in differentiated neurons, in particular whether these functions are similar to their role in early development, is not known. We focus here on *Hand2* since we previously had suggested a function for *Hand2* in the maintenance of the noradrenergic phenotype of sympathetic neurons (Müller and Rohrer, 2002). This notion was deduced from our finding that *Hand2* overexpression in parasympathetic ciliary neurons was able to sustain the normally only transiently expressed *TH* and *DBH* (Müller and Rohrer, 2002). The analysis of the *Hand2* knockout in zebrafish (Lucas et al., 2006) and the conditional *Hand2* knockouts in mouse neural crest cells (Hendershot, 2008; Morikawa, 2007) revealed impaired *TH* and *DBH* expression, suggesting a major role for *Hand2* in the initiation of noradrenergic differentiation. In addition, proliferation in the sympathetic neuron lineage was virtually completely blocked, which results in ganglia with only few residual neurons (Hendershot et al., 2008). In *Hand2^{Wnt1Cre}* sympathetic ganglia Hu-positive neurons, most without TH-expression, are still present at E12 (Hendershot et al., 2008; Morikawa et al., 2007) but virtually no neurons are detectable at E18 (Hendershot et al., 2008). The interpretation of effects observed with a considerable delay after the elimination of *Hand2* is difficult, due to eventual secondary or compensatory effects. Thus, to address the function of *Hand2* in differentiated neurons we analyzed i.) the effect of *Hand2* knockdown in short-term cultures of postmitotic, differentiated neurons from E12 chick sympathetic ganglia and ii.) the effect of the conditional *Hand2* knockout in noradrenergic neurons of *Hand2^{DBHCre}* mice.

Using a *Hand2* siRNA knockdown protocol which strongly reduces Hand2 protein and *Hand2* mRNA expression in sympathetic neurons, we observed a selective inhibition of *TH* and *DBH* expression, however no effect on *SCG10*, TuJ1 and HuC. A similar reduction in *DBH* expression has recently been obtained upon *Hand2* knockdown in cultured sympathetic neurons from newborn mouse superior cervical ganglia (Doxakis et al., 2008). Also in vivo, TH expression was detectable only in about 40% of E11.5 and E14.5 mouse sympathetic neurons after eliminating *Hand2* in *DBH*-expressing cells, which supports the concept that noradrenergic marker gene expression in differentiated sympathetic neurons continues to depend on *Hand2*. The major effect observed in vivo is, however, the strongly reduced neuron number due to lack of proliferation. The effects of excising the *Hand2* gene in *DBH*-expressing

sympathetic neurons are very similar to the effects observed previously when the *Hand2* gene was eliminated in *Wnt1*-expressing neural crest cells, using *Wnt1Cre* (Hendershot, 2008; Morikawa, 2007). This demonstrates that the function of *Hand2* is not restricted to the onset of sympathetic neuron differentiation, e.g. the initiation of *TH* and *DBH* expression in sympathetic precursors, but continues in *DBH*-expressing cells. In addition, it is concluded that *Hand2* is important for the maintenance of *TH* expression and continued proliferation of noradrenergic, *DBH*- and Hu/TuJ1-expressing sympathetic neurons.

Hand2 in the control of sympathetic neuron numbers and pan-neuronal gene expression

Why is the number of Hu-expressing sympathetic neurons affected in the conditional *Hand2* knockout in vivo but not in the *Hand2* knockdown in vitro? The *DBHCre*-mediated *Hand2* elimination takes place in proliferating immature sympathetic neurons and neural precursor cells, at E10.5 in the mouse, corresponding to E3/4 in the chick, whereas the *Hand2* knockdown has been performed in postmitotic, mature E12 chick sympathetic neurons. The reduced proliferation of Hu-positive immature sympathetic neurons in *Hand2*^{*DBHCre*} embryos is the major cause for the low number of Hu-positive cells. In contrast, E12 chick sympathetic neurons do not proliferate in vitro and cell numbers are not affected by the in vitro *Hand2* knockdown.

The continued expression of TuJ1, *SCG10* and HuC in E12 chick sympathetic neurons with reduced *Hand2* levels due to siRNA treatment cannot be explained by an insufficient knockdown of *Hand2* as strong TuJ1 expression was observed in sympathetic neurons without detectable *Hand2* and *TH* expression. The expression of TuJ1, HuC and peripherin in E11/12 mouse embryos homozygous for neural crest *Hand2* deletion (Hendershot et al., 2008; Morikawa et al., 2007), demonstrates that initial generic neuronal differentiation also does not fully depend on *Hand2*.

The results of the present combination of in vitro and in vivo loss-of-function approaches support a role for *Hand2* in the continuation of noradrenergic but not generic neuronal gene expression. It is very likely that other aspects of sympathetic neuron function are also dependent on *Hand2* in mature sympathetic neurons. As recently shown, this includes the control of sympathetic neuron survival as *Hand2* affects the NGF-dependent expression of the NGF receptor *trkA* and NGF-dependent survival in cultured mouse sympathetic neurons (Doxakis et al., 2008).

Cholinergic versus noradrenergic differentiation in the autonomic nervous system

During development, noradrenergic and cholinergic marker genes are co-expressed in sympathetic neurons (Burau et al., 2004; Ernsberger et al., 1997; Ernsberger and Rohrer, 1999). The extrinsic signals that initiate *ChAT* and *VACHT* expression in embryonic sympathetic neurons are not known but the strongly decreased cholinergic differentiation in the *c-ret* knockout implicates members of the GFL family in the control of cholinergic differentiation (Burau et al., 2004). In mature mammalian ganglia only a small subpopulation of sympathetic neurons, innervating sweat glands and periosteum, display cholinergic properties that are dependent on cytokines released from the innervated targets (Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Stanke et al., 2006). In the chick, the proportion of cholinergic sympathetic neurons in mature ganglia is considerably larger than in rodents, with vasculature as the major target (Ernsberger and Rohrer, 1999). The generation of distinct subpopulations of cholinergic and noradrenergic neurons, including the downregulation of *ChAT* and *VACHT* in noradrenergic neurons, is thought to be regulated by signals from the innervated targets (Ernsberger and Rohrer, 1999; Francis and Landis, 1999).

Transcriptional control of the cholinergic locus is not understood in any detail and may involve different sets of transcription factors in different neuronal lineages. The present finding that *Hand2* knockdown facilitates cytokine-induced *VACHT* expression suggests that *Hand2* may participate, directly or indirectly, in the downregulation and subsequent repression of cholinergic marker genes in noradrenergic sympathetic neurons. This notion is also supported by the finding that *ChAT*-expressing neurons in mature sympathetic ganglia are characterized by low-level expression of *Hand2*, in contrast to noradrenergic sympathetic neurons. However, in the *Hand2^{Wnt1Cre}* embryos no significant effects on early cholinergic marker gene expression were observed (Hendershot et al., 2008). This is in agreement with the lack of effect observed upon *Hand2* knockdown without cholinergic stimulation by CNTF. A reduction of *Hand2* expression seems not to be sufficient to affect cholinergic gene expression but requires cytokine-mediated signaling, whereas *Hand2* knockdown is sufficient to reduce *TH* and *DBH* expression. In conclusion, the present results indicate a potential dual-function for *Hand2*, stimulating noradrenergic expression and antagonizing cytokine-mediated cholinergic gene expression.

Conclusion

The function of noradrenergic sympathetic neurons and adrenal chromaffin cells in the adult organism is subject to both short-term and long-term regulation (Kumer and Vrana, 1996; Mallet, 1996). TH, the rate-limiting enzyme in catecholamine biosynthesis represents the major target. Short-term regulation of TH-activity involves phosphorylation of the enzyme by a variety of kinases. Long-term increases in TH activity elicited by electrical stimulation (trans-synaptic induction) or environmental stress are, in contrast, due to increased *TH* mRNA and protein levels (Kumer and Vrana, 1996; Mallet, 1996). As important regulatory regions in the *TH* promoter were found to be essential for TH expression in adult but not in developing tissues, it was suggested that distinct mechanisms underlie the establishment and maintenance of the catecholaminergic phenotype (Trocmé et al., 1998). The present results, together with data from the *Hand2* knockouts (Hendershot et al., 2008; Morikawa et al., 2007; Lucas et al., 2006), argue against this notion and demonstrate that *Hand2* is important both for initial and sustained expression of *TH* and *DBH*. It will be of great interest to delineate in more detail the role of *Hand2* in the regulation of the mature noradrenergic transmitter phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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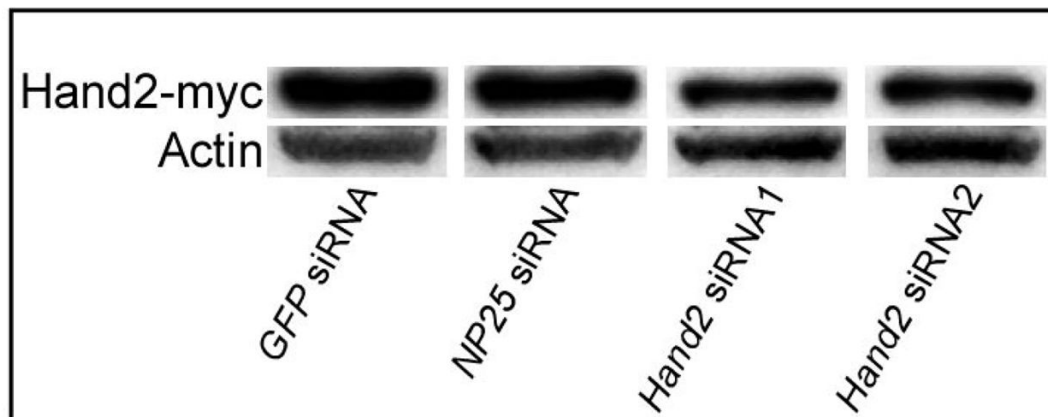
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A



B

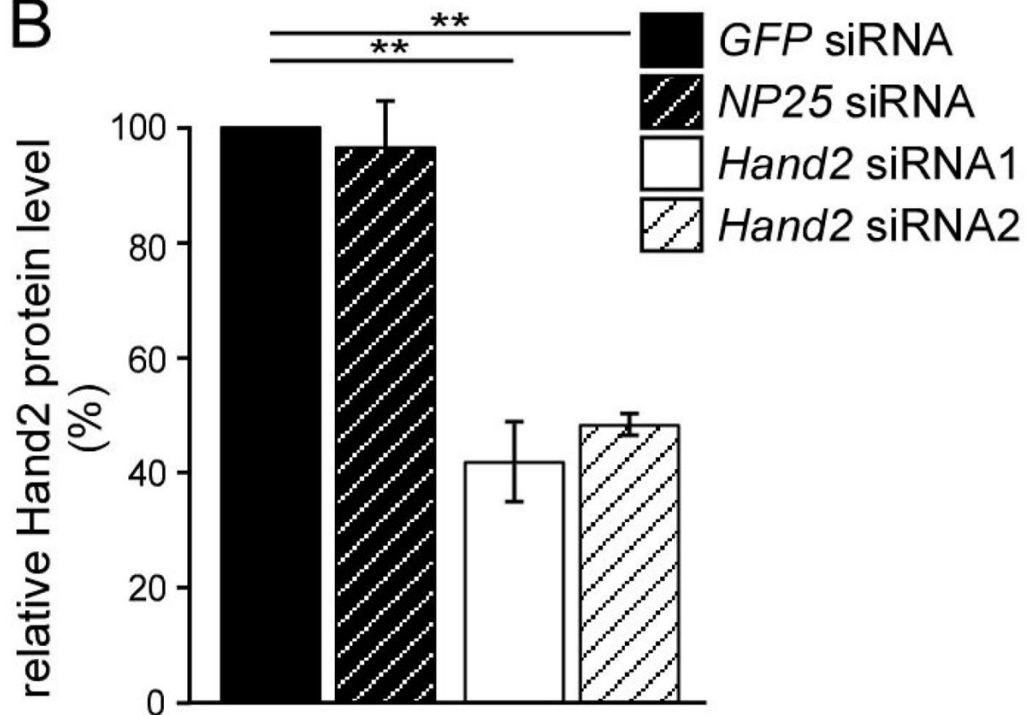


Fig. 1.

Knockdown of *Hand2* by siRNA. (A) Western blot analysis of Hand2 expression in a chick DF-1 cell line with permanent expression of Hand2-myc. DF-1 cells were transfected with control siRNA directed against *GFP* and *NP25* and with two different siRNAs directed against *ggHand2*. Protein extracts were subjected to PAGE electrophoresis and analyzed by western blotting for Hand2 and β -actin. (B) The intensity of Hand2 protein bands were quantified and normalized to β -actin. Hand2 expression levels were compared to *siGFP* control transfections. *Hand2* siRNA1 and *Hand2* siRNA2 reduced Hand2 protein levels to about 40% and 50% of control, respectively, whereas *NP25* siRNA had no effect. ** $P < 0,05$.

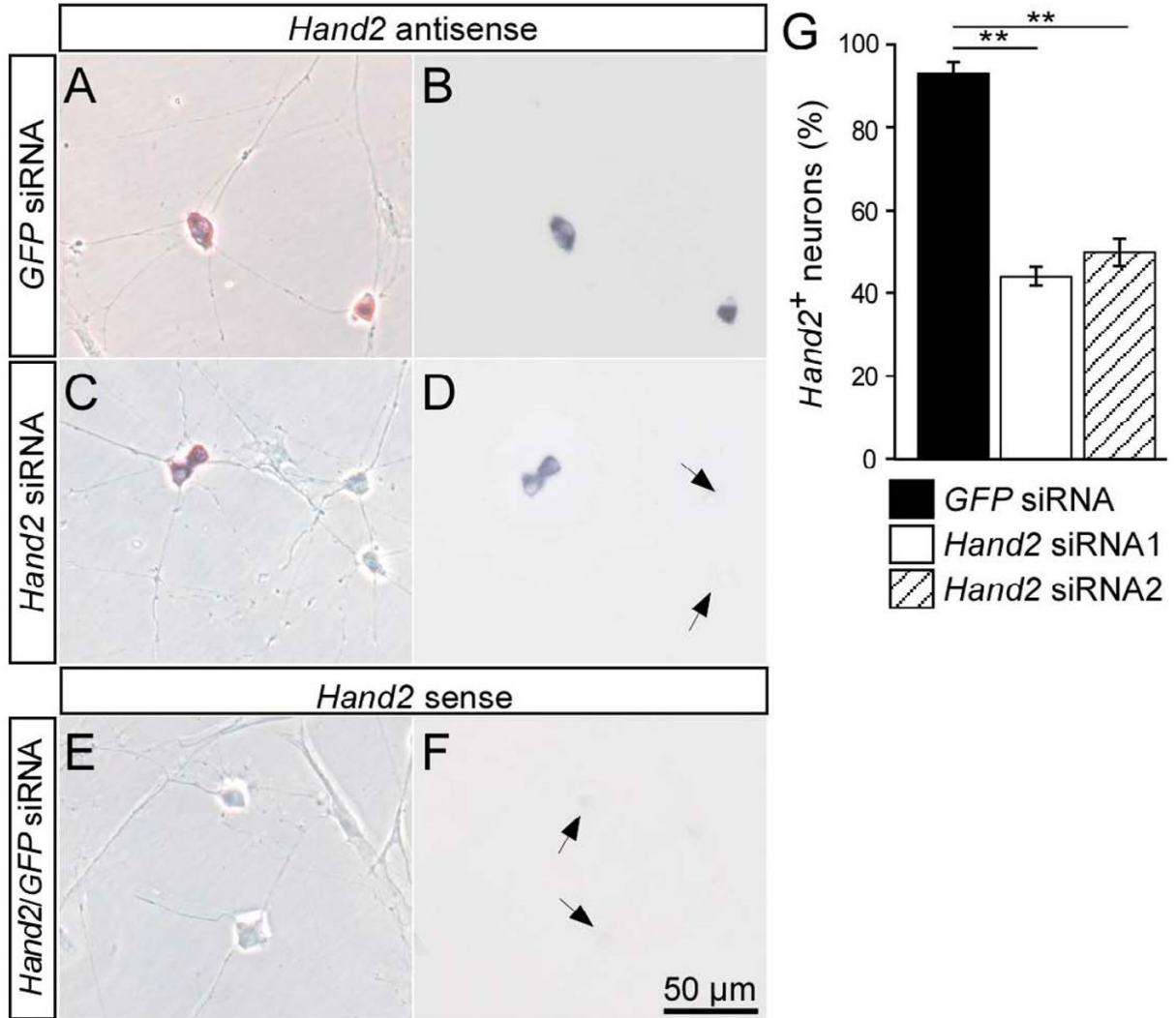


Fig. 2.

Knockdown of *Hand2* in cultured sympathetic neurons by siRNA. E12 sympathetic neurons were transfected with control *GFP* siRNA (A,B) or *Hand2* siRNA (C,D) and analyzed after 2 days in culture for the expression of *Hand2* mRNA by in situ hybridization. To control for the specificity of the signal obtained in the in situ hybridization also a sense RNA probe was used (E,F). Morphology of neurons and non-neuronal cells is shown in phase contrast (A,C,E), the in situ hybridization signal is shown in bright field optics (B,D,F). *Hand2*-positive neurons display a strong in situ hybridization signal (B,D), which is completely absent in the sense controls (F). Non-neuronal cells were also devoid of *Hand2*. In cultures treated with *Hand2* siRNA only 44–50% of neurons were *Hand2*⁺, whereas >90% *Hand2*⁺ neurons were observed in control transfections. Data shown in (G) represent mean \pm s.e.m. (n=3), **P<0,05.

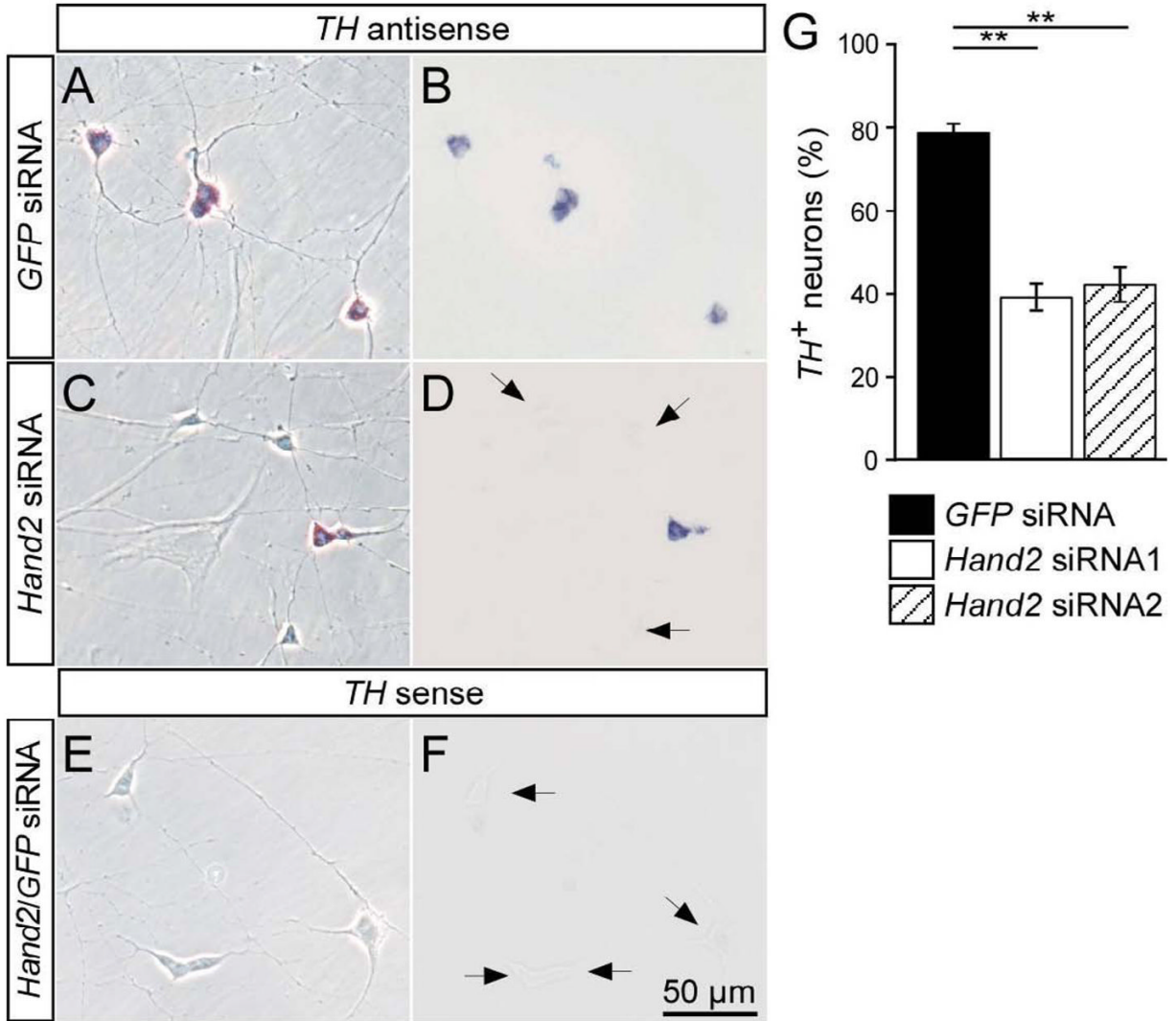


Fig. 3. Effect of *Hand2* knockdown on *TH* expression in cultured sympathetic neurons. E12 sympathetic neurons were transfected with control *GFP* siRNA (A,B) or *Hand2* siRNA (C,D) and analyzed after 2 days in culture for the expression of *TH* mRNA expression by in situ hybridization. A sense RNA probe was used to control for the specificity of the *TH* in situ hybridization signal (E,F). *TH*-positive neurons display a strong in situ hybridization signal (B,D), which is completely absent in non-neuronal cells and in neurons treated with the sense control RNA (F). In cultures treated with *Hand2* siRNA the proportion of *TH*-expressing sympathetic neurons decreased from 80% in control transfections to about 40% in cultures transfected with *Hand2* siRNA. (A,C,E) Phase contrast; (B,D,F) bright field optics. Data shown in (G) represent mean \pm s.e.m. (n=3), **P<0,01; ***P<0,001.

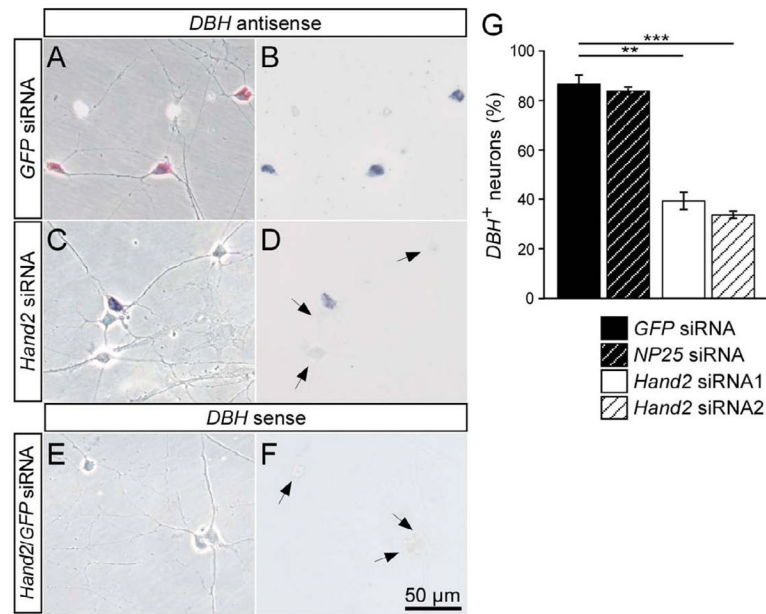


Fig. 4. Effect of *Hand2* knockdown on *DBH* expression in cultured sympathetic neurons. E12 sympathetic neurons were transfected with control *GFP* siRNA (A,B) or *Hand2* siRNA (C,D) and analyzed after 2 days in culture for the expression of *DBH* mRNA expression by in situ hybridization as described for *TH* in Fig. 3. In cultures treated with *Hand2* siRNA the proportion of *DBH*-expressing sympathetic neurons decreased from 83–87% in control transfections to 35–40% in cultures transfected with *Hand2* siRNA. (A,C,E) Phase contrast; (B,D,F) bright field optics. Data shown represent mean \pm s.e.m. (n=3), **P<0,01.

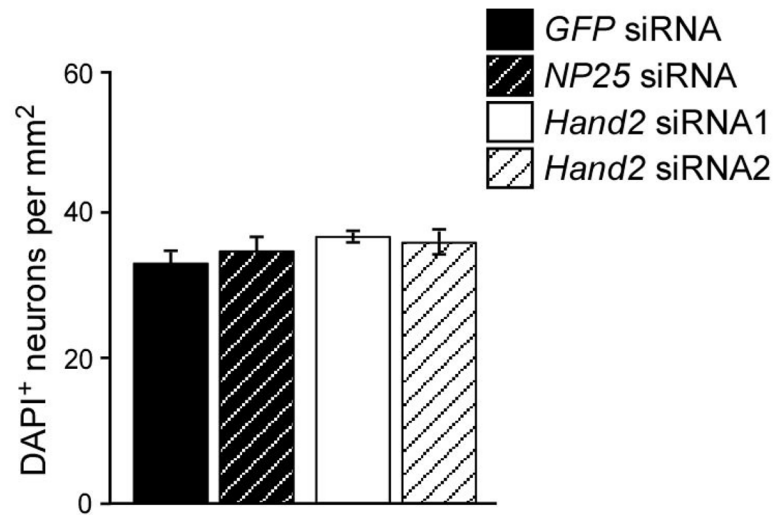


Fig. 5. Survival of E12 sympathetic neurons is not affected by *Hand2* knockdown. E12 sympathetic neurons were transfected with control *GFP* siRNA, *NP25* siRNA or *Hand2* siRNAs and analyzed after 2 days in culture for the number of sympathetic neurons. Neuron number was determined after fixation and nuclear staining for DAPI to distinguish individual neurons in small neuron groups. Data represent mean \pm s.e.m. (n=6).

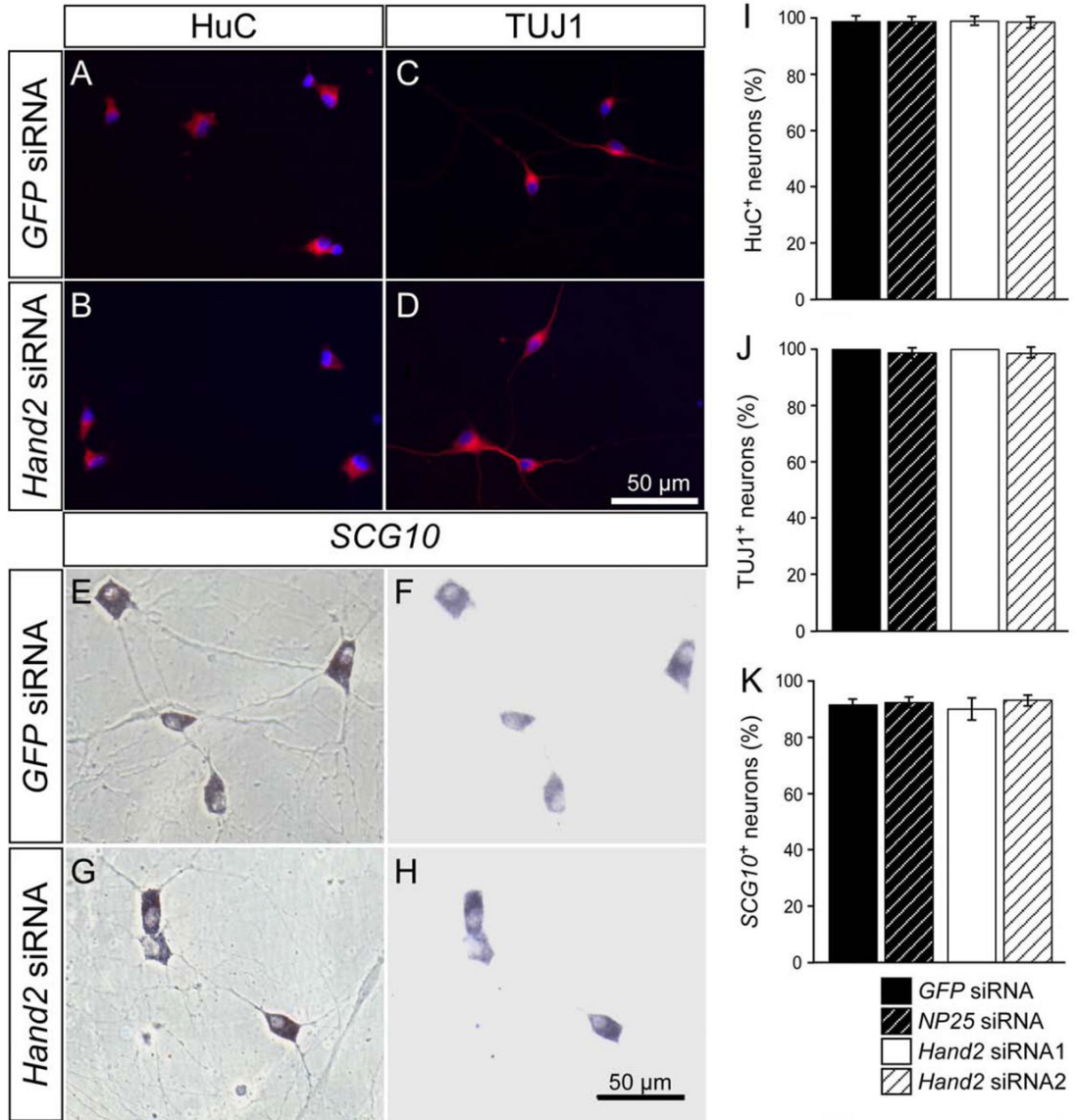


Fig. 6. Effect of *Hand2* knockdown on the expression of HuC, TuJ1 and *SCG10* in cultured sympathetic neurons. E12 sympathetic neurons were transfected with control *GFP* siRNA (A,C,E,F) or *Hand2* siRNA (B,D,G,H) and analyzed after 2 days in culture for the expression of HuC and TuJ1 by antibody staining (A–D) and for *SCG10* expression by in situ hybridization (E–H). In cultures treated with *Hand2* siRNA the proportion of HuC-, TuJ1- and *SCG10*-expressing sympathetic neurons was not significantly different from control transfections using *GFP* siRNA or *NP25* siRNA (I–K). (A–D) Immunofluorescence for HuC and TuJ1 (red) combined with nuclear DAPI-staining (blue). (E, G) phase contrast; (F,H) bright field optics. Data in (I–K) represent mean \pm s.e.m. (n=3).

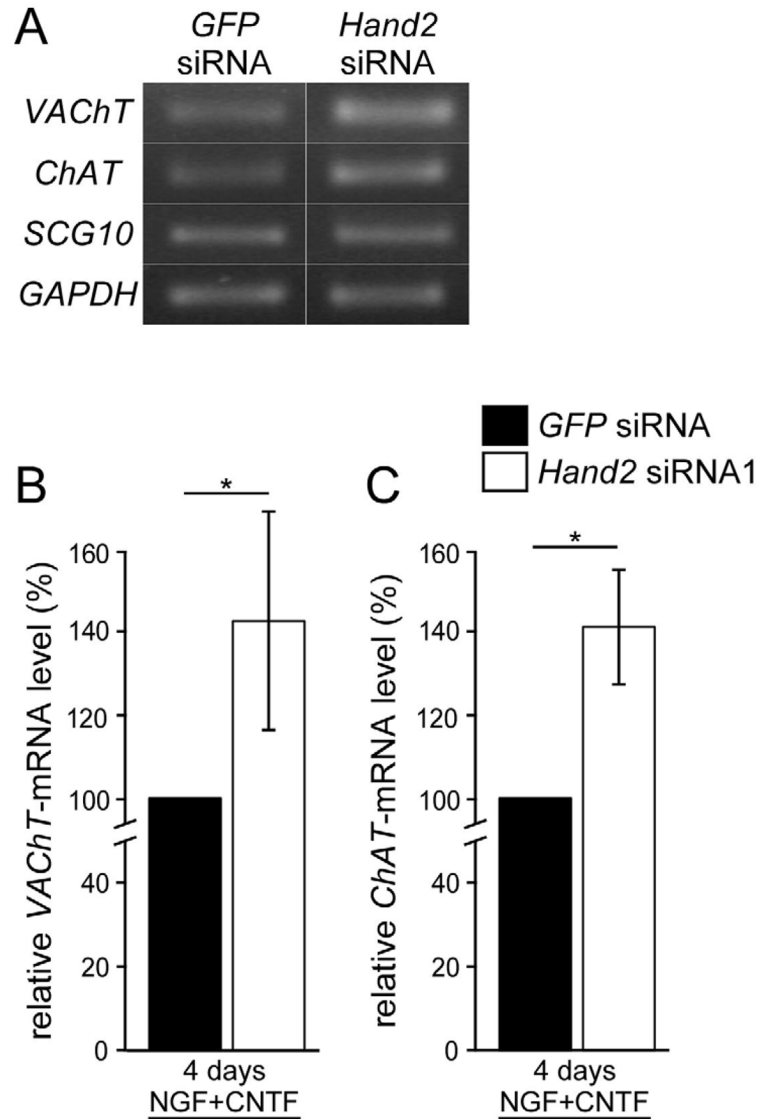
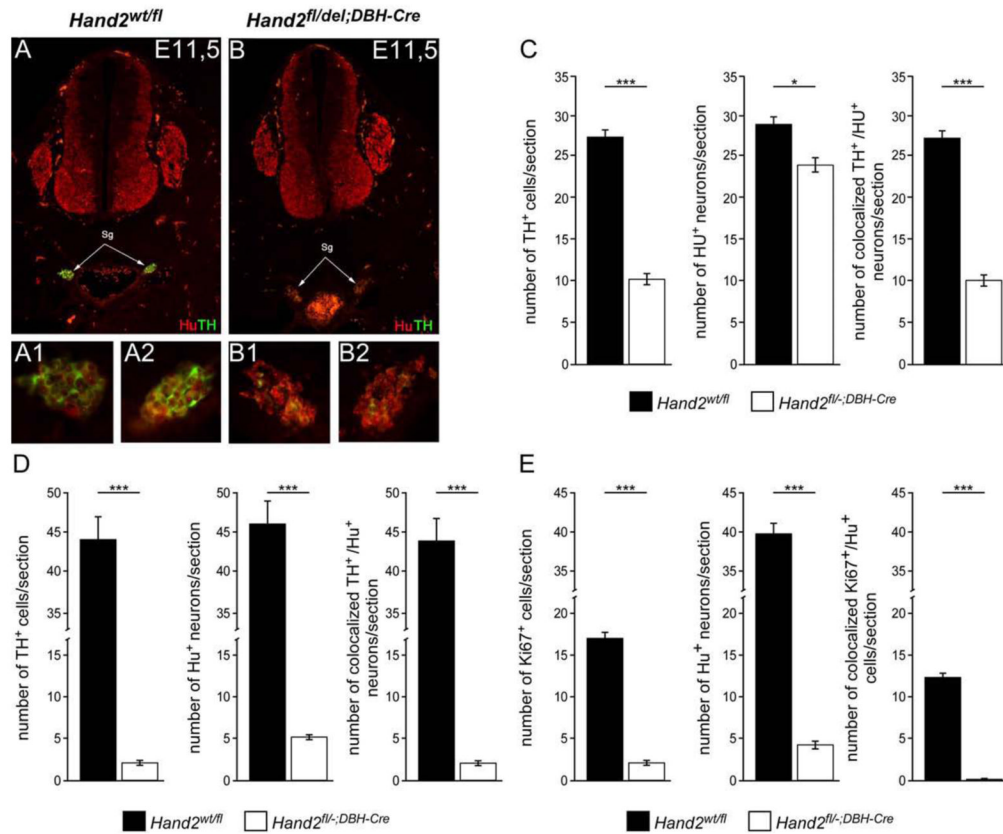


Fig. 7. Effect of *Hand2* knockdown on CNTF-mediated expression of *VACHT* and *ChAT*. E12 sympathetic neurons were transfected with control *GFP* siRNA or *Hand2* siRNA1, cultured with NGF plus CNTF and analyzed after 4 days for the expression of *VACHT*, *ChAT*, *SCG10* and *GAPDH* by semiquantitative RT-PCR. (A) RT-PCR bands for *VACHT*, *ChAT*, *SCG10* and *GAPDH* from cultures transfected with control *GFP* siRNA or *Hand2* siRNA (B,C) Quantitation of the levels of *VACHT* mRNA (B) and *ChAT* mRNA (C), normalized to *SCG10* mRNA. The knockdown of *Hand2* resulted in significantly increased *VACHT* and *ChAT* expression, as compared to control *siGFP*-treated cultures. * $P < 0,05$; (n=3).

**Fig. 8.**

Effect of conditional *Hand2* knockout on the number of TH-, Hu- and Ki67-positive cells in sympathetic ganglia of E11.5 and E14 mouse embryos. (A,B) Sections from E11.5 control *Hand2^{wt/fl}* (A) and *Hand2^{DBH-Cre}* (B) mouse embryos double-stained for Hu (red) and TH (green). Sympathetic ganglia (sg) are indicated by arrows and show strongly reduced TH expression in the conditional knockout (B). Enlargements of left (A1) and right (A2) sympathetic ganglia from control and conditional knockout (B1, B2). (C) Number of TH- and Hu-positive neurons/section of E11.5 *Hand2^{wt/fl}* (black bars) and *Hand2^{DBH-Cre}* (white bars) embryos. Double-labeling reveals that all TH-positive cells co-express TuJ1 in both control and *Hand2*-knockout animals (right graph). The number of TH-positive cells is significantly lower in *Hand2^{DBH-Cre}* ganglia as compared to *Hand2^{wt/fl}* controls ($P < 0.001$) (D) Number of TH- and Hu-positive neurons/section of E14.5 *Hand2^{wt/fl}* (black bars) and *Hand2^{DBH-Cre}* (white bars) embryos. The number of TH-positive cells is significantly lower in *Hand2^{DBH-Cre}* ganglia as compared to *Hand2^{wt/fl}* controls ($P < 0.001$). Double-labeling reveals that all TH-positive cells co-express TuJ1 in both control and *Hand2*-knockout animals (right graph). (E) Number of Ki67- and Hu-positive cells/section of E14.5 *Hand2^{wt/fl}* (black bars) and *Hand2^{DBH-Cre}* (white bars) embryos. Double-labeling reveals that none of residual Ki67-positive cells co-express Hu in *Hand2*-knockout animals whereas many Hu-positive neurons are Ki67-positive in control animals (right graph). *** significantly different from control $P < 0.001$. Data shown are the mean \pm s.e.m. ($n=3$).