Development/Plasticity/Repair

p38α and p38β Mitogen-Activated Protein Kinases Determine Cholinergic Transdifferentiation of Sympathetic Neurons

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Although the p38 mitogen-activated protein kinases are active in many neuronal populations in the peripheral and central nervous systems, little is known about the physiological functions of p38 in postmitotic neurons. We report that p38 activity determines *in vitro* and *in vivo* the switch from noradrenergic to cholinergic neurotransmission that occurs in sympathetic neurons on exposure to the neuropoietic cytokines CNTF and LIF. This transdifferentiation serves as a model for the plastic mechanisms that enable mature neurons to change some of their central functions without passing through the cell cycle. We demonstrate that in postmitotic neurons, p38 and STAT pathways are concurrently activated by neuropoietic cytokine treatment for at least 12 h overlapping with changes in neurotransmitter marker gene expression. Inhibition of p38 blocks the upregulation of the nuclear matrix protein Satb2 and of cholinergic markers by CNTF without affecting STAT3 phosphorylation. Conversely, overexpression of p38α or β in the absence of cytokines stimulates cholinergic marker expression. The neurotransmitter switch *in vitro* is impaired in neurons isolated from p38$\beta^{-/-}$ mice. Consistent with these *in vitro* results, a substantial loss of cells expressing cholinergic properties is observed *in vivo* in the stellate ganglion of mature mice deficient in the p38β isoform.

Introduction

The p38 mitogen-activated protein kinases (MAPKs) are activated during pathophysiological processes including inflammation and cytotoxic stress. In the nervous system, p38 kinases are mostly studied as mediators of excitotoxic and apoptotic pathomechanisms after injury or during neurodegeneration (Semenova et al., 2007). Little is known about the physiological roles of p38 in intact neurons, although p38 activity is remarkably high in normal brain. A small number of studies implied p38 activity in neuroplasticity processes such as development of addictive behavior (Zhang et al., 2011) or synaptic long-term depression (Bolshakov et al., 2000).

The process whereby fully functional postmitotic sympathetic noradrenergic neurons change into cholinergic neurons is referred to as "cholinergic switch" (Wolinsky and Patterson, 1983). The switch *in vivo* depends on target tissue-derived cytokines activating gp130/LIFRβ heterodimeric receptors (Habecker et al., 1997; Geissen et al., 1998; Stanke et al., 2006). The capacity to undergo the cholinergic switch is a plastic potential intrinsic to most sympathetic neurons *in vivo*, since postnatal noradrenergic sympathetic neurons are capable of cholinergic transdifferentiation after establishing contact with transplanted sweat gland tissue (Schotzinger and Landis, 1988). In cell cultures, addition of ciliary neurotrophic factor (CNTF) (Saadat et al., 1989) or leukemia inhibitory factor (LIF) (Yamamori et al., 1989) in defined medium triggers cholinergic transdifferentiation in all postganglionic sympathetic neurons. This process extends over days to weeks and affects a large number of gene loci (Chun and Patterson, 1977; Fann and Patterson, 1998; Apostolova et al., 2007).

Previous research on cholinergic sympathetic development was focused on extracellular signaling determinants with the aim of identifying growth factors and signaling receptors which stimulate cholinergic differentiation (Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Apostolova and Dechant, 2009). By contrast, the intracellular signaling pathways that orchestrate the complex transcriptional changes have been investigated in few studies only (Dziennis and Habecker, 2003). Since this work mainly addressed regulation of noradrenergic markers (Pellegrino et al., 2011), the mechanisms underlying cholinergic specification remained by and large elusive. Recently we showed that the nuclear matrix protein Satb2 binds to the cholinergic locus and drives expression of cholinergic marker genes. *In vivo* Satb2 is specifically expressed in cholinergic sudomotor neurons during the switch (Apostolova et al., 2010).

In the present study, we investigated the acute receptor-mediated signaling occurring in primary sympathetic neurons after neuropoietic cytokine addition to regulate Satb2 and subse-

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quently the neurotransmitter phenotype. Neuropoietic cytokines activate two major types of signaling pathways: STAT and MAPK modules (Heinrich et al., 2003). STAT activation is generally observed in all sensitive cells, whereas MAPK activation is variable and appears to be cell-type and cytokine specific. While activation of ERK, JNK, and p38 MAPK in response to neuropoietic cytokines has been described in other cell types, only the activation of the ERK pathway has so far been reported for primary neurons (Dziennis and Habecker, 2003). Here we show that neuropoietic cytokines activate p38 in neurons and that cholinergic differentiation depends on the p38 pathway *in vitro* and *in vivo*.

Materials and Methods

Animals. Sprague Dawley rats and C57BL/6 mice were obtained from Harlan Winkelmann. The C57BL/6 p38$\beta^{-/-}$ mouse line was described previously (Beardmore et al., 2005). Animals were of either sex and were killed in accordance with relevant regulations.

Primary neuronal cell culture. Neuronal cultures were established as described previously (Apostolova et al., 2010). Rat superior cervical ganglia (SCGs) were dissected from postnatal day P0–P2 pups and mouse SCG neurons from P5–P7 mice, which is the earliest developmental stage during which robust marker gene regulation in response to CNTF was observed *in vitro* (data not shown). Cultures were established in 5 ng/ml NGF (Peprotech), the next day the medium was changed, to 25 ng/ml CNTF (Peprotech) and 5 ng/ml NGF for cholinergic differentiation or 20 ng/ml NGF for maintenance of the noradrenergic phenotype. LIF (1000 U/ml, Millipore), Neurturin (25 ng/ml, Peprotech), Activin A (25 ng/ml, Peprotech), or Interferon γ (25 ng/ml, Invitrogen) replaced CNTF in some experiments. All treatments were performed at day 1 *in vitro* if not indicated otherwise. For the investigation of p38 activation in long-term cultures, neurons were maintained for 4 d in 20 ng/ml NGF.

In vitro *inhibitor studies.* Unless indicated otherwise, SCG neurons were incubated with inhibitors 1 h before CNTF treatment. All substances were dissolved in DMSO. Control cultures were treated with equal volumes of DMSO since short-term (<1 h) p38 activation was observed after DMSO treatment in test experiments (data not shown). DMSO had no effect on marker gene expression at any time point examined (data not shown). Substances were tested at several concentrations for their toxicity (see Fig. 3 and data not shown). The following nontoxic concentrations were applied: 10 μM SB202190, 50 μM SKF-86002, 10 μM PD169316, 100 nM and 1 μM JAK inhibitor, and 1 and 10 μM SB202474 (all from Calbiochem).

Electroporation, RNA isolation and real-time PCR. For overexpression experiments, 1×10^6 to 1.5×10^6 freshly dissociated rat SCG neurons were resuspended in 100 μl of Nucleofector Solution (Amaxa Rat Neuron Nucleofector Kit) and mixed with expression plasmids—either 3.5 μg of pmaxGFP (Lonza) and 3 μg of pCMV5-Flaq-p38 (Raingeaud et al., 1996), 3 μg of pcDNA3-Flag-p38β2 and 3 μg of pcDNA3-HA-MKK6b(E) (Jiang et al., 1996), or as control 3.5 μg of pmaxGFP, 5 μg of pcDNA3.1 vector, and 5 μg of pCMV5A (both Invitrogen). Transfected cells were identified by cotransfection with a GFP plasmid. GFP-positive cells were collected by aspiration through a glass capillary 2 d after transfection. All subsequent steps were performed as described previously (Apostolova et al., 2010). Primers used for RT-PCR amplification listed in 5′-3′orientation: rat Gapdh-For CTGGAGAAACCTGCCAAGTATG, rat Gapdh-Rev ACAACCTGGTCCTCAGTGTAG; rat VAChT-For CTCACCACTTGTAACATTCCCC, rat VAChT-Rev AAACCATGCCCATCTCCCAC; rat Net-For CAGCACCATCAACTGTGTTACC, rat Net-Rev GGCTTCTGGATACAGGACAAATAC; rat VIP-For CACGCCCTATTATGATGTGTCC, rat VIP-Rev TCCGAGATGCTACTGCTGATTC; rat Chat-For CAGCCAATTGGGTCTCTGAATAC, rat Chat-Rev GAGGCAGGCTGCAAATCTTAG; rat NF160-For AGCATTGAGCTCGAGTCGGTG, rat NF160-Rev CTGCTGGATGGTGTCCTGGTAG; rat TH-For TCCGAGCCTTTGACCCAGA, rat TH-Rev CATACTTCCTGAGCTTGTCCTTG; rat Gch11-For GAGACCATCTCAGATGTCCTG, rat Gch11-Rev CTACAGTGTACCCTTCCCAC; rat Vmat2-For GCAATTGGGATGGTGGACTC, rat Vmat2-Rev CAGCAGAGGGACCGATAGCATA; rat Dbh-For GAATGCTGTGACTGTCCACC, rat Dbh-Rev GTAGTTGACGCACATCTCCTC; mouse Gapdh-For AGGGCTCATGACCACAGTC, mouse Gapdh-Rev CAGCTCTGGGATGACCTTG; mouse Chat-For GCTTACTACAGGCTTTACCAG, mouse Chat-Rev CAGCAGCTGCAGTTTCTCAG; mouse VAChT-For TGTGAGGACGACTACAACTAC, mouse VAChT-Rev TAGGGTACTCATTAGAGGAGG; mouse VIP-For GCTGGAGAAATGATGGGAAGAG, mouse VIP-Rev GACATGTAGAACTCATAATGTAATC; mouse Net-For GAGACAGTTCCAGTTGCGG, mouse Net-Rev AGGAATCCCAAGGCGGTATC; mouse TH-For AAGGACAAGCTCAGGAACTATG, mouse TH-Rev GCATTTAGCTAATGGCACTCAG; mouse Tau-For GGAGGCAGTGTGCAAATAGTC, mouse TH-Rev TATTATCCAAGGAGCCAATCTTC; mouse p38α-For GAAAGCAGGGACCTTCTCATAG, mouse p38α-Rev GACAGGTTCTGGTATCGTTCG.

Immunoblotting. Cultures were growth factor-deprived for 1 h in defined medium before acute stimulation in the same medium. Lysates in 2× Roti-Load sample buffer (Roth) were subjected to SDS-PAGE and processed as described previously (Apostolova et al., 2010). Unless otherwise indicated, antibodies were obtained from Cell Signaling Technology and diluted 1:1000: monoclonal rabbit phospho p38 MAPK (Thr180/Tyr182) (D3F9) XP, monoclonal mouse-phospho-p44/42 MAP Kinase (Thr202/Tyr204) E10, monoclonal phospho-Akt (Ser473) (D9E) XP (1:2000), rabbit polyclonal phospho-SAPK/JNK (Thr183/Tyr185), rabbit polyclonal phospho-STAT1(Tyr701) (1:500), rabbit polyclonal phospho-STAT3(Tyr705), rabbit polyclonal phospho-STAT3(Ser727) (1:500), and mouse anti-α-tubulin (1:100 000, Sigma). The Satb2 antibody was used at 1:100 (SATBA4B10, Abcam). After incubation with HRP-coupled secondary antibodies for 1 h (anti-rabbit from Cell Signaling Technology, anti-mouse from BD Biosciences), blots were developed using ECL reagent (GE Healthcare) and scanned. Pictures were quantified using ImageJ software according to the following protocol: http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html. Relative values for phospho proteins are represented as arbitrary units after normalization to α-tubulin.

Tissue preparation, immunohistochemistry, and immunocytochemistry. Stellate ganglia from 60-d-old wild-type or p38$\beta^{-/-}$ mice were dissected and sections were stained with rabbit anti-Vacht (1:2000, Sigma) as previously described (Apostolova et al., 2010). Pictures for Vacht staining were taken with an ApoTome Imaging System based on Axiovert 200M (Zeiss) using AxioVision software. Exposure times were kept constant for all pictures taken. DAPI-stained neuronal nuclei and Vacht-positive cells were counted using MetaMorph software (Molecular Devices) by personnel blinded to the genotype of the cells. Primary SCG neurons were prepared for immunostaining as described previously (Apostolova et al., 2010). Cells were incubated overnight at 4°C with primary antibodies (mouse anti-βIII-tubulin, 1:500, Promega) and monoclonal rabbit phospho p38 MAPK (Thr180/Tyr182) (D3F9) XP (1:200, Cell Signaling Technology) diluted in 1% BSA in PBST.

Statistical analysis. Statistical analysis was conducted by using a two-tailed Student's t test. Data represent mean ± SEM of at least three independent biological experiments. The level of significance was taken as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

Acute CNTF or LIF treatment causes sustained p38 MAPK activation

First we analyzed the activation of candidate signaling pathways 30 min after CNTF treatment in cultures of rat SCG sympathetic neurons (Fig. 1*A*). Cytokine treatment was performed at day *in vitro* 1 (DIV1) in the continuous presence of NGF, which is an essential survival factor for sympathetic neurons. Cells maintain their noradrenergic phenotype in the presence of NGF when no cytokine is added. CNTF addition caused hyperphosphorylation of STAT1, STAT3, and p44/42 MAPK. No activation was observed for Akt or JNK. Although activation of p38 has not been reported after treatment with neuropoietic cytokines (Dziennis and Habecker, 2003) this pathway was tested in our experiments based on previous reports that NGF can activate p38 in PC12 cells (Morooka and Nishida, 1998). Unexpectedly, we found that p38

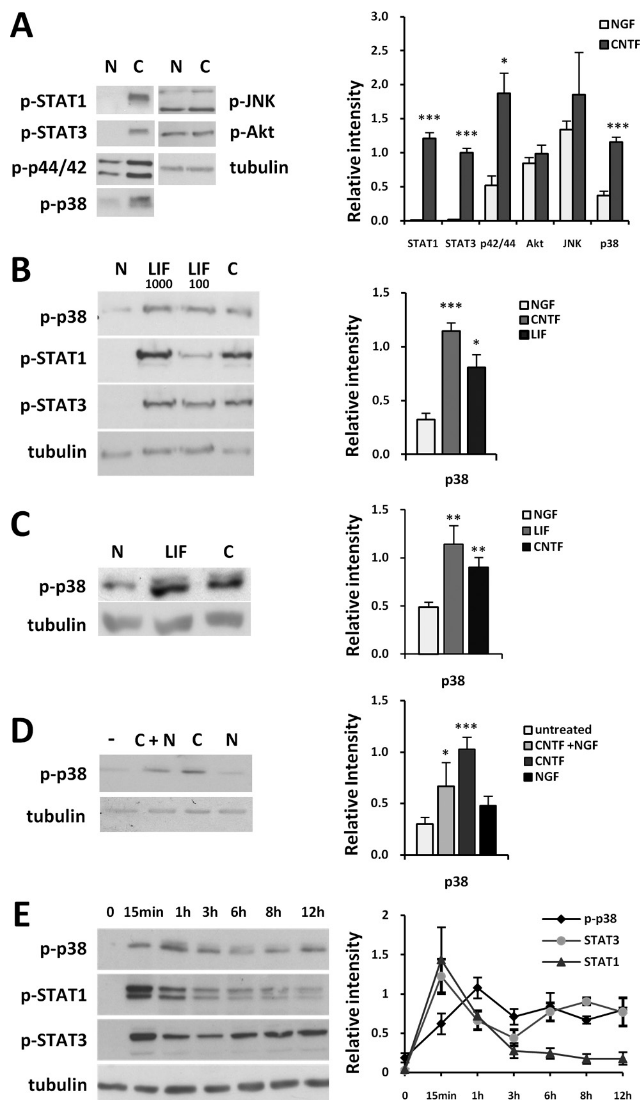

Figure 1. CNTF and LIF cause sustained hyperphosphorylation of p38 MAPK. ***A***, Acute CNTF (C) treatment for 30 min leads to STAT1, STAT3, p42/p44 ERK, and p38 phosphorylation in SCG neurons compared to NGF (N)-treated cultures at DIV1 ($n = 3$). No difference is observed among levels of phosphorylated Akt and JNK. Tubulin was used as a loading control. Images were quantified with ImageJ. Values for phosphorylated proteins were calculated relative to tubulin levels and are represented as arbitrary units. ***B***, CNTF and LIF (1000 or 100 U) treatment cause similar activation of STAT1, STAT3, and p38 (representative image of Western blot) in comparison with NGF treated cultures at DIV 1 ($n = 3$). ***C***, p38 is also activated after acute CNTF or LIF stimulation of long-term cultures of SCG neurons (DIV4) ($n = 3$). ***D***, Representative Western blot and quantified results showing the absence of p38 hyperphosphorylation after acute NGF treatment (30 min) compared to untreated controls. CNTF activates p38 in the absence and presence of NGF ($n = 3$). ***E***, Quantification of Western blot results (representative image shown as left panel) reveals sustained p38 and STAT3 activation after CNTF treatment extending over at least 12 h. STAT1 is strongly activated only during the first 60 min ($n = 3$–7).

was robustly activated upon CNTF treatment in cultures of SCG neurons (Fig. 1*A*). The activation of p38 by pro-cholinergic cytokines was also confirmed after stimulation with LIF and no quantitative difference was observed between the LIF and CNTF effects at the concentrations tested (Fig. 1*B*). p38 is activated by cellular stress and might be influenced by the dissection procedures and axotomy of neurons during establishment of primary cultures. To test whether p38 activation by neuropoietic cytokines is restricted to freshly established cultures or also occurs in long-term cultures, we used neuronal cultures maintained for 4 d (DIV4) in 20 ng/ml NGF. Both CNTF and LIF activated p38 compared with NGF when DIV4 cultures were stimulated for 30 min with either 25 ng/ml CNTF or 1000 U LIF (Fig. 1*C*). Therefore, p38 activation by neuropoietic cytokines occurs similarly in short as well as long-term SCG cultures. Acute NGF treatment of NGF-deprived cultures in the absence of neuropoietic cytokines had no effect on p38 phosphorylation, as compared to untreated controls (Fig. 1*D*). When the time course of p38, STAT1 and STAT3 activation was compared after CNTF addition (Fig. 1*E*) phosphorylation of all three proteins occurred within 15 min after CNTF treatment. Both STAT3 and p38 remained activated for at least 12 h, whereas STAT1 phosphorylation peaked between 15 min and 1 h and returned to background levels after 3–8 h.

p38 activation occurs in neurons and is jak dependent

Since neuronal cultures contain small contaminating populations of glia and fibroblasts, we investigated in which cell type p38 is activated by CNTF (Fig. 2*A*). Phospho-p38 (p-p38) immunoreactivity was restricted to neurons, since all βIII tubulin-positive cells were also positive for p-p38, whereas the non-neuronal βIII-negative cells were devoid of detectable p-p38 immunoreactivity. Active p38 is predominantly located in neuronal nuclei after CNTF treatment. To exclude the possibility that p38 may be activated by a CNTF receptor-independent mechanism as a stress kinase, we compared STAT3 and p38 activation following CNTF-stimulation in the presence or absence of an inhibitor of jak kinases. p38 activation by CNTF depends on jak activity, since p38 phosphorylation was prevented in a dose-dependent manner by JAK inhibitor 1 (Fig. 2*B*). Similar doses of JAK inhibitor 1 blocked p38 and STAT3 activation. Next, we compared the effects of CNTF and LIF on p38 activation with the effects of other differentiation-promoting growth factors, known to act on sympathetic neurons through gp130/LIFRβ-independent mechanisms (Fig. 2*C*). Activation of p38 was not observed with IFNγ, which like neuropoietic cytokines activates STAT1 signaling in sympathetic neurons but does not trigger the switch (data not shown). The TGFβ homolog activin A and the GDNF family member neurturin (NRTN), both known to promote sympathetic differentiation (Fann and Patterson, 1994; Hiltunen and Airaksinen, 2004), did not have an effect on p38 activation either. Thus, of all tested factors, the pro-cholinergic neuropoietic cytokines CNTF and LIF, which resemble the activity of the classical sweat gland-derived cholinergic differentiation factor, selectively activated p38 in sympathetic SCG neurons.

Inhibition of p38 signaling prevents cholinergic gene regulation

After cytokine treatment initial changes in *Satb2* transcript levels are observed within 2 h (Apostolova et al., 2010) overlapping with the time points when p38 activation can be detected. To correlate neurotransmitter marker changes during neurotransmitter differentiation with receptor-mediated signaling mechanisms, we determined the time point of first robust changes in neurotransmitter marker gene expression. The following genes were chosen as cholinergic markers: choline acetyltransferase (*Chat*), the vesicular acetylcholine transporter (*Vacht*) and vasoactive intestinal peptide (*VIP*), which in the sympathetic nervous system is coexpressed with *Chat* and *Vacht* (Ernsberger and Rohrer, 1999). Noradrenergic properties were investigated using the tyrosine hydroxylase (*Th*), dopamine-β-hydroxylase (*Dbh*), high-affinity norepinephrine transporter (*Net*), vesicular monoamine transporter 2 (*Vmat2*), and GTP cyclohydrolase 1 (*Gch*) genes as markers (Habecker et al., 2002). Cytokine-treated neurons were

compared with parallel cultures maintained with NGF alone, i.e., under noradrenergic conditions. We found robust and significant changes in gene expression of cholinergic markers 12 and 24 h after cytokine addition (data not shown). No qualitative or quantitative difference on marker transcript levels was observed between LIF and CNTF treatments (data not shown).

To explore the function of p38 in the neurotransmitter switch, pharmacological inhibitors were added to the cell culture medium before CNTF stimulation. The samples were then analyzed for marker gene expression 16 h later. First we applied the JAK inhibitor 1, which blocks both p38 and STAT3 activation (Fig. 2*B*). This substance completely blocked the differential expression of cholinergic marker genes (data not shown). Next we added the p38 inhibitor SB202190 to SCG cultures. No effect of 10 μM SB202190 was observed on morphology of cultured sympathetic neurons (Fig. 3*A*), nor on neuronal survival, Tau immunoreactivity or metabolic activity (data not shown). However the upregulation of the cholinergic markers *Chat*, *Vacht*, and *VIP* in response to CNTF treatment for 16 h was effectively blocked by SB202190 (Fig. 3*B*). Expression levels of the noradrenergic markers *Th*, *Net*, *Dbh*, *Vmat2*, *Gch*, or the panneuronal marker *Tau* were not affected by SB202190 (Fig. 3*B*). Since low levels of p-p38 are consistently observed in SCG cultures treated with NGF alone (Fig. 1), we tested the effect of SB202190 on marker gene expression in the absence of pro-cholinergic cytokines. A significant effect of SB202190 was observed on the expression of *VIP*, which is expressed at low level in noradrenergic neurons before cytokine stimulation (Fig. 3*B*), whereas the inhibitor had no effect on *Chat*, *Vacht*, *Vmat2*, or *Gch* and small effects (about twofold) on *Net* and *Dbh* expression levels (Fig. 3*B* and data not shown). Therefore inhibition of p38 activity in the absence of added cytokines reduces endogenous levels of *VIP* expression under noradrenergic conditions and effectively prevents upregulation of *Chat*, *Vacht*, and *VIP* by pro-cholinergic cytokines.

The effects of SB202190 on the cholinergic locus and VIP were dose dependent and were observed at low concentration of 300 nM (Fig. 3*D*). Since SB202190 does not inhibit p38γ or p38δ isoforms the effects on cholinergic gene expression depend on p38α and/or p38β. The activation of p38 is detected in sympathetic neurons for at least 12 h following CNTF stimulation (Fig. 1*E*). Hence we tested whether long-lasting p38 activation is required for the regulation of the cholinergic markers by CNTF. The addition of SB202190 6 h after CNTF treatment still blocked cholinergic gene expression 16 h after CNTF addition (Fig. 3*E*). Therefore cholinergic marker gene expression depends on sustained p38 activity. Similar to the effect of SB202190, the application of other p38 inhibitors including PD169316 or SKF-86002 prevented the upregulation of cholinergic markers (Fig. 3*F*) without affecting the downregulation of the noradrenergic marker genes. In contrast, SB202474, an inactive structural analog of SB202190, had no effect (Fig. 3*F*).

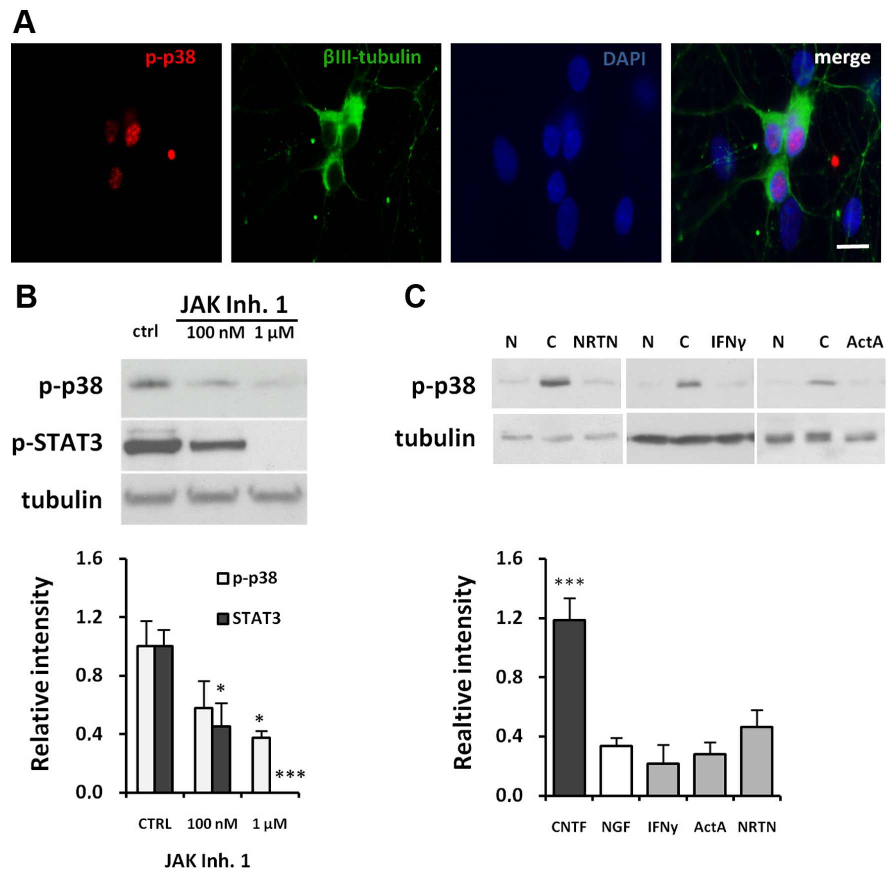

Figure 2. p38 activation occurs in neuropoietic cytokine-treated neurons. ***A***, Primary SCG neurons after acute CNTF stimulation were costained for βIII-tubulin and phospho-p38. Nuclei were stained with DAPI. p-p38 staining is not detected in βIII-tubulin-negative cells (scale bar 20 μm). ***B***, Immunoblots demonstrating a dose-dependent decrease in p38 and STAT3 phosphorylation after JAK Inhibitor 1 administration in comparison with neurons treated only with CNTF (Ctrl) ($n = 3$). Cells were lysed 4 h after CNTF treatment. ***C***, No activation of p38 is detected after neurturin (NRTN), interferon γ (IFNγ), or activin A (ActA) treatment compared to NGF- and CNTF-treated cultures ($n = 3$).

p38 inhibitor does not influence STAT phosphorylation but prevents Satb2 protein regulation

The pro-cholinergic effect of p38 activation can tentatively be explained by the stimulating effect of p38 on STAT protein activation (Xu et al., 2003). Hence we determined the level of STAT phosphorylation in response to CNTF in the presence or absence of jak or p38 inhibitors (Fig. 4*A*). While JAK inhibitor 1 prevented STAT3 and STAT1 phosphorylation on residues tyrosine 705 and tyrosine 701 in CNTF-treated cultures, no inhibition of STAT3 or STAT1 tyrosine phosphorylation was observed in the presence of p38 inhibitor SB202190 (Fig. 4*A*, quantified in Fig. 4*C*), nor was STAT3 phosphorylation on serine 727 influenced by SB202190 (Fig. 4*B*,*C*). These results indicate that p38 activation is not required for STAT1 or STAT3 phosphorylation after CNTF treatment. STAT3 phosphorylation on crucial tyrosine and serine residues is insufficient to initiate the upregulation of the cholinergic markers downstream of jak when p38 is inhibited. The chromatin architecture protein Satb2 is rapidly induced by CNTF and LIF and is a nuclear regulator of the target-dependent neurotransmitter switch (Apostolova et al., 2010). Application of SB202190 before CNTF addition effectively prevented Satb2 protein regulation by CNTF at any time point tested (Fig. 5).

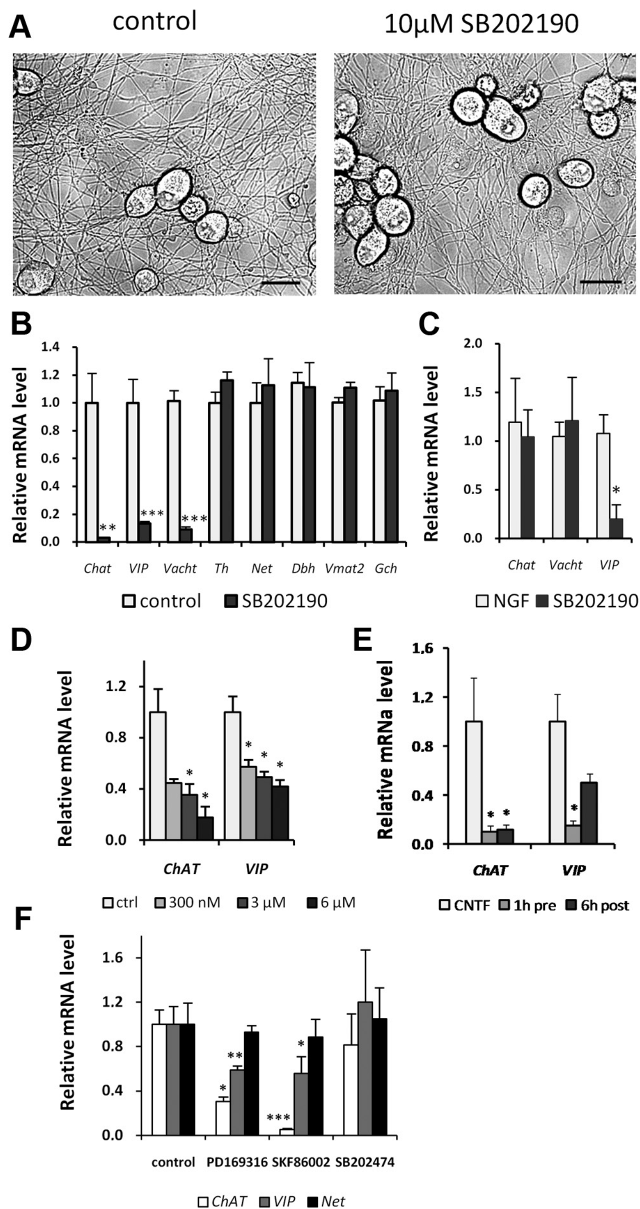

Figure 3. p38 Inhibitors block cholinergic differentiation. ***A***, Images of primary SCG neurons 16 h after plating in NGF- and CNTF-containing medium in the presence of 10 μM SB202190 compared to control cultures without inhibitor. No differences in cell morphology, neurite outgrowth or cell numbers were observed (scale bar 20 μm). ***B***, Quantitative RT-PCR experiments demonstrate a complete block of upregulation of *Chat Vacht* and *VIP* by 10 μM SB202190 in CNTF-treated SCG neurons. Expression levels of the noradrenergic markers *Th, Net, Dbh, Vmat2*, and *Gch* were not changed in comparison with CNTF-treated control cultures ($n = 3$– 6). ***C***, In the absence of neuropoietic cytokine stimulation SB202190 had no effect on trace levels of *Chat* and *Vacht* present in noradrenergic neurons and significantly reduced *VIP* levels ($n = 3$). ***D***, Effects of SB 202190 on *Chat* and *VIP* transcript levels in CNTF-treated SCG neurons are dose dependent. Comparison with CNTF-treated cultures as controls ($n = 3$). ***E***, Administration of 10 μM SB202190 6 h after CNTF treatment still inhibits upregulation of *Chat* mRNA and does also affect *VIP* ($p = 0.07$) in comparison with control CNTF cultures ($n = 4$). ***F***, Quantitative RT-PCR experiments demonstrating a significant decrease of *Chat* and *VIP* mRNA levels with p38 inhibitors PD169316 (10 μM) or SKF-86002 (50 μM), but not with the inactive compound SB202474 (10 μM) in comparison with CNTF-treated control cultures ($n = 3$– 4).

Overexpression of p38 stimulates cholinergic marker gene expression

To test whether activation of the p38 pathway is sufficient to stimulate cholinergic differentiation in the absence of neuropoietic cytokines, i.e., under noradrenergic conditions, plasmids encoding p38α or β isoforms were transfected into neurons together with the upstream kinase MKK6b(E), which is a constitutively active mutant of MKK6b, following a previously established protocol (Raingeaud et al., 1996).

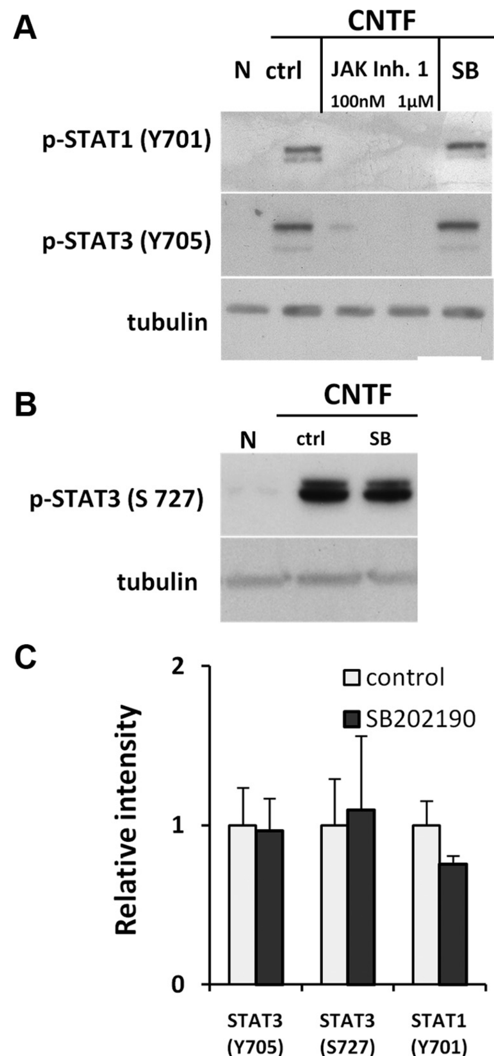

Figure 4. p38 inhibitors do not affect STAT protein phosphorylation. ***A***, Jak inhibitor 1 but not SB202190 (SB) reduces STAT3 tyrosine Y705 and STAT1 tyrosine Y701 phosphorylation after CNTF stimulation compared to control CNTF cultures and NGF-treated cultures (N). ***B***, STAT3 serine S727 phosphorylation is also not affected by SB202190 compared with CNTF (C). ***C***, Quantification of results shown in ***A*** and ***B*** ($n = 3$– 4).

Overexpression of p38α or β isoforms in combination with MKK6b(E) caused a robust upregulation of transcripts of the cholinergic locus and *VIP* compared to control mock-transfected cells (Fig. 6*A*). Whereas hyperactivation of endogenous p38 by overexpression of the upstream kinase MKK6b(E) alone, had no effect on *Vacht* expression, a significant stimulatory effect was observed on *VIP*. Together with the finding that *VIP* but not *Chat* or *Vacht* expression is reduced by SB202190 in the absence of cytokines (Fig. 3*C*), this result points to a higher sensitivity of VIP expression for activity of the p38 pathway compared to the cholinergic locus. No effect was observed on the expression levels of *Net* or neurofilament *NF160* (Fig. 6*B*). Hence activation of the p38 pathway in neurons stimulates cholinergic differentiation in the absence of neuropoietic cytokines.

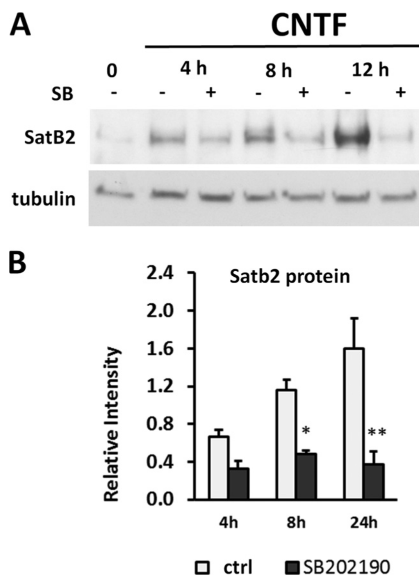

Figure 5. p38 inhibitor prevents Satb2 protein regulation. ***A***, Representative Western blot of Satb2 protein in the absence (−) and presence (+) of SB202190 determined 4, 8, and 12 h after stimulation with CNTF. ***B***, Quantification of the results. The upregulation of Satb2 protein in response to CNTF is effectively blocked by the p38 inhibitor at any time point tested.

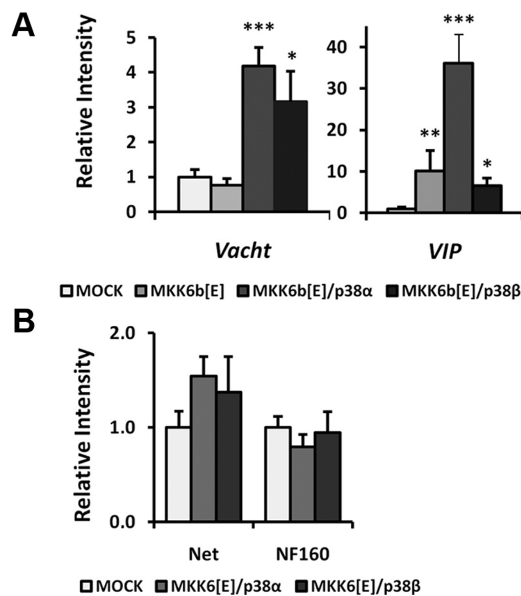

Figure 6. Neurotransmitter markers are regulated after p38 overexpression. ***A***, Quantitative RT-PCR experiments demonstrate a marked increase in *Vacht* and *VIP* transcript levels in NGF-treated SCG neurons coexpressing MKK6b[E] and p38 isoforms in the absence of neuropoietic cytokines. Stimulation of endogenous p38 by overexpression of the p38-MAPKK MKK6b[E] alone has no effect on *Vacht* levels but significantly increases *VIP* mRNA levels. ***B***, The mRNA levels of *Net* and *NF160* remain unaltered in all overexpression experiments. Approximately 80 GFP-positive nucleofected neurons were analyzed per experiment and compared with pCMV and pCDNA3-transfected neurons (Mock).

Cholinergic gene regulation is impaired in p38β-deficient neurons

We next tested whether p38α or β are necessary for cholinergic transdifferentiation in response to neuropoietic cytokines in a loss of function gene targeting model. For our experiments, we chose the p38β-deficient mouse strain since p38β is the predominantly expressed isoform in neurons compared with p38α (Beardmore et al., 2005) and (http://biogps.gnf.org/#goto=genereport&id=5600, http://biogps.gnf.org/#goto=genereport&id=1432). This finding was confirmed for sympathetic neurons in our cultures (data not shown). Furthermore, p38β-deficient mice are viable and fertile (Beardmore et al., 2005). In contrast, the p38α phenotype is lethal and homozygous mutant animals die prenatally before reaching the postnatal stages at which the neurotransmitter switch can be studied. We confirmed that the switch occurs similarly in response to neuropoietic cytokines in rat and wild-type mice (data not shown). The SCG of $p38\beta^{-/-}$ animals showed no obvious anatomical or size differences to age-matched wild-type animals (Fig. 7*A*). p38β-deficient SCG neurons survived and extended long neurites in the presence of NGF in culture without a detectable difference to wild-type neurons (Fig. 7*B*). p38 is activated by CNTF in wild-type mouse sympathetic neurons similarly to rat sympathetic cultures (Fig. 7*C*). These findings allowed us to analyze the effect of p38β deficiency on neurotransmitter marker gene regulation in mouse sympathetic cultures. Parallel cultures were established from C57BL/6 wild-type and C57BL/6 p38β mutant mice under noradrenergic conditions. Following stimulation by CNTF, *Vacht*, *Chat*, and *VIP* levels were significantly reduced in p38β-deficient cells compared with wild-type neurons (Fig. 7*D*). No difference was observed between wild-type and $p38\beta^{-/-}$ neurons in the expression levels of *Net* and *Tau*. Hence the lack of p38β alone is sufficient to inhibit cholinergic differentiation in primary neurons despite the presence of p38α, which is expressed at unchanged levels in the $p38\beta^{-/-}$ neurons (Fig. 7*D*).

The number of cholinergic sympathetic neurons is reduced in p38β-deficient mice

We next asked whether the cholinergic differentiation of sympathetic neurons *in vivo* is affected in the $p38\beta^{-/-}$ mice. To this end, the number of cholinergic neurons was determined in the stellate ganglion in adult wild-type and p38β-deficient animals using Vacht immunoreactivity as a marker (Schäfer et al., 1998). The stellate ganglion innervates the sweat glands of the forepaws, which are the classical target tissue of neurons undergoing the cholinergic switch and 4–5% of all neurons express cholinergic properties after having completed the process (Masliukov and Timmermans, 2004). In our experiments, 4.1% of wild-type mouse stellate ganglion neuronal somata expressed detectable Vacht immunoreactivity (Fig. 7*E*). In stellate ganglia of p38β-deficient animals, only 1% of all neurons expressed detectable Vacht immunoreactivity. No difference was observed in the intensity of the vesicular Vacht staining in preganglionic cholinergic terminals (Fig. 7*E*). These results confirm that cholinergic differentiation of postganglionic sympathetic neurons depends on p38β activity *in vivo*.

Discussion

The sympathetic neurotransmitter switch serves as a textbook model for how postmitotic neurons can become reprogrammed by target-derived signals. Here we demonstrate that this impressive variant of neuronal plasticity depends on long-lasting p38 MAPK activation which occurs in parallel with STAT activation and overlaps with the initial time points of marker gene regulation. One of the molecular targets depending on p38 activity is the nuclear matrix protein Satb2 which links receptor-mediated signaling to chromatin modifications. Consistent with our *in vitro* results, a substantial loss of cholinergic cells is observed *in vivo* in the mature stellate ganglion of mice deficient in p38β. This provides initial evidence for a specific role of p38β in neurons.

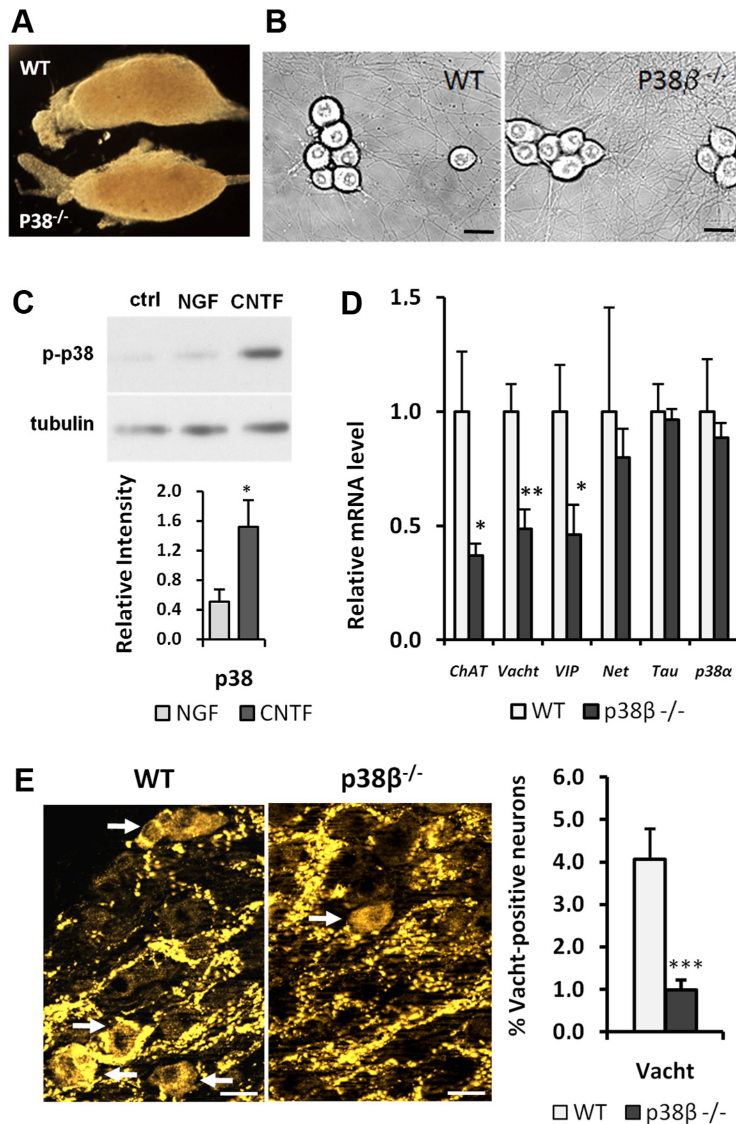

Figure 7. Cholinergic differentiation is impaired in p38β-deficient neurons *in vitro* and *in vivo*. ***A***, Representative photograph of mouse SCGs at postnatal day 6 from either wild-type (WT) or p38β-deficient mice. ***B***, Images of primary mouse SCG neurons from WT and p38$\beta^{-/-}$ mice 48 h after plating in the presence of CNTF and NGF. No differences in cell morphology, neurite outgrowth, or cell number was observed depending on the genotype (scale bar, 20 μm). ***C***, Representative Western blot and quantified results showing a significant increase in p38 phosphorylation in CNTF-treated mouse SCG neurons in comparison to NGF-treated cultures. Tubulin was used as a loading control ($n = 3$). ***D***, 38β deficiency in CNTF-treated mouse SCG neurons greatly reduces *Chat*, *Vacht*, and *VIP* transcript levels, without affecting *Net*, *Tau*, and *p38α* levels in comparison with CNTF-treated wild-type cultures after 48 h in culture ($n = 4$). ***E***, Sections from postnatal day 60 mouse stellate ganglia were immunostained for Vacht in wild-type and p38$\beta^{-/-}$ mice. Vacht-positive cell somata are indicated by white arrows (scale bar 20 μm). Quantitative analysis of Vacht-immunoreactive neurons per ganglion demonstrates that the number of Vacht-positive cells is greatly reduced in p38β mutant ganglia. The percentage of Vacht-IR neurons per ganglion was calculated as Vacht/DAPI-positive neuron ratio in section where nuclei were stained with DAPI. Data are expressed as mean ± SEM ($n = 5$; 5 animals per group i.e., 10 ganglia were analyzed per genotype).

Signaling of CNTF and LIF at early stages of the switch includes p38 MAPK

Our finding of rapid, robust and sustained hyperphosphorylation of p38 MAPK in neurons in response to neuropoietic cytokines was unexpected (Takeda and Ichijo, 2002). No evidence for p38 activation had previously been found in sympathetic neurons, both after LIF treatment (Dziennis and Habecker, 2003) and NGF withdrawal (Eilers et al., 1998). The seeming contrast between our study and the lack of p38 activation in response to LIF reported earlier is explained by detection of phospho-p38 with increased sensitivity. Receptor-mediated p38 activation downstream of gp130/LIFRβ heteromeric complexes was not described before in any cell type with the exception of a single report (Crowe et al., 2008). In our experiments, long-lasting p38 and STAT3 activation rather than STAT1 activation was detected as overlapping with first gene expression changes in neurotransmitter markers. This sustained activation is functionally important since the delayed addition of p38 inhibitor is still sufficient to block upregulation of cholinergic marker transcripts.

The selective activation of p38 in our experiments by neuropoietic cytokines but not by neurturin belonging to the families of GDNF family ligands (GFLs) helps to distinguish the classical cytokine-dependent cholinergic switch of sudomotor neurons from other modes of cholinergic sympathetic differentiation. The switch occurs after target contact in fully functional noradrenergic neurons and depends on target-derived retrograde signals downstream of gp130/LIFRβ receptors. This transdifferentiation involves a specific signaling module that causes phospho p38 and Satb2 levels to increase in the nucleus. A different mode of cholinergic differentiation seems to take place at earlier stages of development before target contact is established in bimodal embryonic neuronal precursors or early neurons (for review, see Apostolova and Dechant, 2009). Evidence is accumulating that this early cholinergic differentiation but not the classical sudomotor switch depends on signaling of the GFL receptor ret. Our findings that GFLs fail to activate p38 and to stimulate Satb2 expression in noradrenergic SCG neurons *in vitro* correlates well with the inability of these growth factors to trigger cholinergic transdifferentiation.

The cholinergic locus and VIP are regulated by p38 activity

Our study of the role of p38 has benefitted from the availability of imidazole-based inhibitors, such as SB202190. Although widely used, the specificity of p38 inhibitors has been challenged (Godl et al., 2003; Kunkel et al., 2004). Therefore we confirmed our conclusions derived from pharmacological experiments by complementary genetic approaches. Our studies with neuronal cultures from p38β-deficient mice are fully consistent with the results obtained with p38-antagonists. What is more, the results of our overexpression experiments demonstrated that gain of function of the p38 pathway leads to upregulation of cholinergic genes. Therefore, p38 activity can at least partially substitute for CNTF or LIF treatment. We cannot exclude and in fact we propose that these effects depend on basal levels of STAT3 activation.

Our results reveal a differential dependence of the cholinergic locus and the VIP gene on Satb2 and p38. While both loci are

regulated by the p38 pathway, the expression of the cholinergic locus but not of VIP depends on Satb2 (Apostolova et al., 2010). Low expression levels of cholinergic markers are detectable in noradrenergic sympathetic neurons. Therefore we tested how expression of these genes is influenced by p38 activity in the absence of added cytokines. *VIP* expression turned out to particularly sensitive for p38 activity since *VIP* but not *Chat* or *Vacht* mRNA levels are decreased when endogenous p38 activity is blocked in noradrenergic cells. Consistently activation of endogenous p38 by overexpression of MKK6b alone is sufficient to significantly raise *VIP* but not *Chat* or *Vacht* levels. Together these findings indicate a direct Satb2-independent influence of p38 on *VIP* gene expression, whereas regulation of the cholinergic locus necessitates p38 activity in combination with Satb2 regulation.

Intracellular signaling mechanisms that regulate the expression of the cholinergic locus and VIP in response to CNTF and LIF have been addressed in cell lines and embryonic neurons (Shimojo et al., 1998; Madziar et al., 2008). In the neuroblastoma cell line NBFL, CNTF drives VIP gene expression through the activation of STAT and AP-1 proteins, which bind to distinct sites within a 180 bp element in the VIP promoter, termed the cytokine response element (CyRE) (Symes et al., 1997). H7, a kinase inhibitor, which is effective on p38 (Xu et al., 2003), prevents CNTF-dependent VIP transcription (Symes et al., 1994; Rajan et al., 1998). It is an attractive hypothesis that p38 is the H7-sensitive kinase that regulates CyRE activity. In NBFL cells, the H7 effect does not depend on the STAT binding element, indicating that H7 is not acting via changing ser/thr phosphorylation of STAT proteins. Consistently, in our experiments p38 is unlikely to act via phosphorylation of STAT proteins, since STAT phosphorylation on both tyrosine and serine residues was unaffected by the p38 inhibitors under conditions where cholinergic marker gene regulation was completely inhibited.

With regard to noradrenergic markers, we consistently found that their expression was not influenced by manipulation of the p38 pathway under our experimental conditions. It is of note that the aim of the present study was to elucidate receptor-mediated events during the initial phases of the switch, i.e., minutes to hours after addition of cytokines. Therefore it was important for us to link signaling mechanisms to gene expression as directly as possible. Cholinergic markers turned out to be particularly useful as early readout of the switch since they are rapidly upregulated from hardly detectable levels before stimulation to already substantial (but not nearly maximal) levels within several hours. By contrast, the loss of noradrenergic markers, which are very abundantly expressed at the beginning of the treatment, occurs at a comparably slow timescale over days to weeks. Therefore, the observed lack of regulation for noradrenergic markers during the first hours can be explained by differences in timing between a swift upregulation of cholinergic and slow loss of noradrenergic markers. In fact, we predict that p38 regulates Net via Satb2 several days after the initiation of the switch (Apostolova et al., 2010). We did not extend our analyses to long-term neuronal cultures in the present study since long-lasting pharmacological treatment or recombinant overexpression of kinases carry substantial risk of accumulating unspecific effects in these primary cultures over time.

Indications of a specific role of p38β in neurons

The pyridinyl-imidazole-based inhibitors applied in our experiments affect p38α and β but not p38γ or δ (Coulthard et al., 2009). Both p38α and β isoforms appear to be involved in cholinergic transdifferentiation. Our overexpression studies revealed the potential of α and β isoforms to regulate the cholinergic locus in the absence of neuropoietic cytokines. Interestingly the effect of p38β overexpression was smaller on *VIP* transcript levels compared to p38α. This points to separate cellular functions of the two kinases, which might differentially control transcriptional and translational regulatory mechanisms in neurons. Several findings indicate a specific role of the p38β isoform in our paradigm and more generally in the nervous system: p38α and β were found to be differentially localized in brain regions (Lee et al., 2000), and p38β is the predominantly expressed isoform in the nervous system (Beardmore et al., 2005). Our results with sympathetic neurons derived from p38β-deficient mice now provide evidence that these cells are impaired in cholinergic differentiation *in vitro* and *in vivo*. Compensatory expression of p38α was not observed in our experiments with p38β-deficient animals, consistent with a previous report (Beardmore et al., 2005). The strongest argument for a specific function of p38β in neurons is the loss of Vacht expression in the stellate ganglion, which represents the second report of a p38β-specific phenotype (Greenblatt et al., 2010) and the first in the nervous system. Our result that regulation of the cholinergic marker genes is reduced in p38β-deficient cultures in comparison with wild-type cultures, but not completely blocked, suggests a role of the remaining p38α, which might also explain the lack of effect on noradrenergic markers.

Indications for a p38-Satb2 nuclear plasticity module in neurons

p38 has been linked to activity-dependent neuronal processes in the CNS (Thomas and Huganir, 2004). One form of hippocampal long-term depression that depends on mGluR activation is inhibited by the p38 inhibitor SB203580 (Bolshakov et al., 2000). The nuclear matrix protein Satb2 controls the neurotransmitter plasticity in primary sympathetic neurons (Apostolova et al., 2010). Satb2 affects transcription by binding to AT-rich sequences of matrix attachment regions, tethering them to the nuclear matrix, and forming distinct chromatin loops (Britanova et al., 2005; Szemes et al., 2006). Therefore, the signaling module that leads from p38 activation to Satb2 upregulation is an interesting candidate pathway for linking extracellular plasticity-promoting stimuli with long-range chromatin remodeling in the nervous system. This variant of nuclear plasticity might allow postmitotic neurons to adapt their transcriptome in response to changes in their environment under both physiological and pathophysiological conditions.

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