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Owing to an oversight, the co-authorship of Christiane Otto was not acknowledged in the print and final online versions of this paper.

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The authors apologise to readers for this mistake.

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Target-dependent specification of the neurotransmitter phenotype: cholinergic differentiation of sympathetic neurons is mediated in vivo by gp130 signaling

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Sympathetic neurons are generated through a succession of differentiation steps that initially lead to noradrenergic neurons innervating different peripheral target tissues. Specific targets, like sweat glands in rodent footpads, induce a change from noradrenergic to cholinergic transmitter phenotype. Here, we show that cytokines acting through the gp130 receptor are present in sweat glands. Selective elimination of the gp130 receptor in sympathetic neurons prevents the acquisition of cholinergic and peptidergic features (VAChT, ChT1, VIP) without affecting other properties of sweat gland innervation. The vast majority of cholinergic neurons in the stellate ganglion, generated postnatally, are absent in gp130-deficient mice. These results demonstrate an essential role of gp130-signaling in the target-dependent specification of the cholinergic neurotransmitter phenotype.

KEY WORDS: Cytokine, IL6/IL-6, Cholinergic, Sympathetic, VIP, VAChT

INTRODUCTION

The assembly of neuronal circuits requires developmental programs that ensure appropriate neuronal subtype differentiation and synaptic connections. This involves extrinsic signals acting on proliferating precursor cells to specify neuronal subtype identity, but also signals encountered later, during target innervation and from the target itself. One of the best-studied lineages leads from neural crest cells to sympathetic neurons and is characterized by successive differentiation steps that are controlled by signals derived from the environment. The initial specification towards an autonomic neuron phenotype is elicited by bone morphogenetic proteins (BMPs) acting on neural crest precursor cells that aggregate to ganglion primordia (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996; Schneider et al., 1999). BMPs induce a group of transcription factors, including MASH1 (ASCL1 – Mouse Genome Informatics), PHOX2A, PHOX2B, HAND2 and GATA2/GATA3, that, in turn, control the expression of autonomic neuron-specific features (reviewed by Goridis and Rohrer, 2002). Subsequently, glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) produced by intermediate and final targets are essential for sympathetic neuron migration, the development of axon projections and proper target innervation (Honma et al., 2002; Enomoto et al., 2001; Hiltunen and Alraksinen, 2004). Neurotrophins control final stages of target organ innervation, as well as target-dependent survival of sympathetic neurons (Glebova and Ginty, 2004; Francis and Landis, 1999).

Target-derived, retrogradely acting signals not only affect axon outgrowth and neuron survival but also mediate the acquisition of neuronal traits, such as the expression of distinct neurotransmitters. The best understood example for target-dependent control of the neurotransmitter phenotype is the sympathetic innervation of sweat glands in the footpads of rats and mice (reviewed by Ernsberger and Rohrer, 1999; Francis and Landis, 1999). Sweat glands are innervated by noradrenergic sympathetic axons shortly after birth. Because of signals derived from this target, adrenergic traits such as catecholamine production are downregulated, whereas the induction of cholinergic features, like choline acetyl transferase (ChAT), vesicular acetylcholine transporter (VAChT) and the co-expressed neuropeptide vasoactive intestinal peptide (VIP), leads to a functionally cholinergic sweat gland innervation. The importance of the target tissue for this transmitter phenotype switch to occur has been firmly established by sweat gland transplantation, by replacement by parotid gland (Schotzinger and Landis, 1990) and by analysis of the tabby mouse mutant, which is devoid of sweat glands (Guidry and Landis, 1995). Cholinergic sympathetic neurons innervate, as additional target tissues, the skeletal muscle vasculature and the periosteum, the connective tissue covering the bone. For the periosteum of the sternum and ribs it has been demonstrated that the initial innervation loses catecholaminergic markers and starts to express cholinergic properties and VIP (Asmus et al., 2000). The sternum can induce cholinergic differentiation of sympathetic neurons both in vitro and in vivo, upon transplantation to regions of hairy skin (Asmus et al., 2001; Asmus et al., 2000).

The interaction between sympathetic neurons and the sweat gland also includes the induction and maintenance of the secretory responsiveness of sweat glands (Francis and Landis, 1999; Landis, 1999). Secretory responsiveness, the ability of glands to produce sweat after nerve stimulation or cholinergic agonist administration, does not develop in the absence of sweat gland innervation (Stevens and Landis, 1987). Both catecholaminergic and cholinergic neurotransmission are required for the induction of secretory responsiveness during development (Tian et al., 2000; Grant et al., 1995), reflecting the developmental change in neurotransmitter phenotype. In addition to eliciting sweat secretion in adult rodents, cholinergic transmission is required for the maintenance of secretory responsiveness (Grant et al., 1995). The cholinergic differentiation

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factor(s) produced by the developing sweat gland thus seems to trigger indirectly, by inducing cholinergic neurotransmission, an essential step in the differentiation of this target tissue. However, the molecular identity of the target-derived signal(s) has remained unclear.

The first factor that was observed to induce cholinergic differentiation of cultured sympathetic neurons was leukemia inhibitory factor (LIF) (Yamamori et al., 1989). LIF belongs to the IL6 family of cytokines (Taga and Kishimoto, 1997), which includes interleukin 6 (IL6), IL11, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin 1 (CT1; CTF1 – Mouse Genome Informatics), cardiotrophin-like cytokine (CLC; CLCF1 – Mouse Genome Informatics) (Elson et al., 2000) [also known as novel neurotrophin 1/B-cell stimulating factor 3 (Shi et al., 1999; Senaldi et al., 1999)] and neuropoietin/cardiotrophin 2 (NP; CTF2 – Mouse Genome Informatics) (Derouet et al., 2004). CLC interacts with the soluble receptor cytokine-like factor 1 (CLF; CRLF1 – Mouse Genome Informatics), or with soluble CNTFRα, to form a functional ligand for the CNTF receptor complex (Elson et al., 1998; Elson et al., 2000). All family members analysed to date were shown to induce ChAT and VIP, and to reduce noradrenergic gene expression in cultured sympathetic neurons (Yamamori et al., 1989; Saadat et al., 1989; Geissen et al., 1998; Rao et al., 1992a). This can be explained by their common mechanism of action, activating receptor complexes that share the signaling receptor subunit gp130 (IL6ST – Mouse Genome Informatics), leading to the alternative term gp130 cytokines for IL6 cytokine family members (Taga and Kishimoto, 1997; Heinrich et al., 2003). The gp130 receptor family can be subdivided into receptors that contain, as signaling subunits, either gp130 homodimers or heterodimers, composed of gp130/LIFRβ or gp130/OSMR. Additional ligand-binding α-receptor subunits can associate with the core signaling receptors to form tripartite or even more complex (CLC/CLF) receptors (reviewed by Heinrich et al., 2003). In addition to IL6 cytokine family members, other signals were also found to induce cholinergic sympathetic differentiation in vitro, including the TGFβ family member activin (Fann and Patterson, 1995), the GFL family member GDNF (Brodski et al., 2002) and the neurotrophin NT3 (Brodski et al., 2000).

The role of IL6 cytokines in the cholinergic differentiation of sweat gland innervation has been investigated by expression analysis and by loss-of-function approaches (Rohrer, 1992; Rao and Landis, 1993; Habecker et al., 1995a; Francis et al., 1997). These studies excluded all known cytokines acting singly as sweat gland-derived cholinergic differentiation factors. Neither was cholinergic sweat gland innervation affected by the combined elimination of LIF and CNTF (Francis et al., 1997). IL6 cytokines are also implicated in the cholinergic differentiation of periosteum innervating neurons, as antibodies against LIFRβ prevented ChAT induction in sympathetic neurons co-cultured with periosteal cells (Asmus et al., 2001). Although tissue homogenates from rat footpads and supernatants of cultured sweat glands or sternum were shown to contain a cholinergic differentiation activity with properties of a LIF-related cytokine (Habecker et al., 1997), the relevance of these findings for the in vivo situation is unclear, as production and response to cytokines in neural cells is rapidly induced upon in vivo lesioning or in tissue culture (Freidin et al., 1992; Rao and Landis, 1993; Zigmond, 1996; Yao et al., 1997).

To address the physiological importance of cytokine signaling for target-dependent cholinergic sympathetic differentiation, we have selectively eliminated gp130 in noradrenergic cells by crossing mice carrying a floxed *gp130* allele (Betz et al., 1998; Hirota et al., 1999) with a mouse line that expresses Cre recombinase under the control of the dopamine β-hydroxylase (*DBH*) promotor. The observed complete lack of cholinergic fibers in mutant sweat glands, the massive reduction in the number of cholinergic neurons in the stellate ganglion and the maintenance of noradrenergic sweat gland innervation demonstrates an essential function of Il6 cytokines for target-dependent cholinergic differentiation. The co-expression of candidate cytokines *CNTF*, *CLC/CLF*, *CT1* and *NP* observed in sweat gland tissue suggests that several factors may act together in this process, and explains the lack of effects of single knockouts. As sweat glands show a normal secretory response in mice displaying noradrenergic instead of cholinergic innervation, cholinergic neurotransmission seems not to be required for the acquisition and maintenance of secretory responsiveness.

MATERIALS AND METHODS

Generation of animals

The generation of *gp130$^{fl/fl}$* mice and the ROSA26R mice has been described elsewhere (Betz et al., 1998; Soriano, 1999). *DBH-iCre* mice were generated by introducing the *iCre* sequence into a P1-derived bacterial artificial chromosome (PAC) that harboured the gene for mouse *DBH* (R. Parlato et al., unpublished). Using homologous recombination in *E. coli*, the PAC was modified to carry the improved coding sequence of Cre recombinase (*iCre*) (Wintermantel et al., 2002). Gp130-deficient mice were generated by backcrossing *gp130$^{fl/fl}$* animals and *DBH-iCre* animals with C57Bl/6 mice to the fourth generation. Resulting animals were intercrossed and bred to homozygosity.

Tissue preparation

Animals were killed and immediately dissected to collect front feet, stellate and superior cervical ganglia. Tissues were deep frozen on blocks of dry ice and stored at –20°C. Prior to immunohistochemistry and in situ hybridization, tissues were cryosected and 14 μm sections were collected on glass slides.

β-galactosidase staining

Embryos or tissue sections were fixed in 0.4% glutaraldehyde for 2-4 hours or 15 minutes, respectively. Staining was carried out overnight in a solution containing 0.1% sodium deoxycholate, 0.2% Nonidet NP-40, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml X-Gal.

Immunohistochemistry and in situ hybridization

For in situ hybridization and immunohistochemistry, slices were postfixed for 15 minutes in 0.1 M NaH_2PO_4 with 4% paraformaldehyde and rinsed in PBS. For antibody staining on sections, slices were pre-incubated for 1 hour in PBS with 5% fetal calf serum and 0.2% Triton X-100 (antibody buffer). Primary antibodies in antibody buffer were incubated overnight at room temperature and slices were washed in PBS with 0.2% Triton X-100 (PBT). Secondary antibodies in PBT with 0.1% DAPI were incubated for two hours and washed off.

The following antibodies were used: mouse monoclonal anti-neuronal class III β-tubulin (Tuj1; Hiss Diagnostics, Freiburg, Germany), rabbit polyclonal anti-porcine VIP (PROGEN, Heidelberg, Germany), rabbit polyclonal anti-VAChT serum (Phoenix Pharmaceuticals, Belmont, CA, USA), rabbit polyclonal anti-ChT1 (kindly provided by T. Okuda, Tokyo, Japan), rabbit polyclonal anti-TH (BioTrend, Cologne, Germany).

In situ hybridization was performed according to established protocols (Ernsberger and Rohrer, 1997). Slices were incubated with Digoxigenin (DIG)-labeled antisense RNA probes at 68°C overnight in hybridization buffer with 1×SSC and 50% formamide. Alkaline phosphatase-coupled anti-DIG antibody was applied overnight and the staining reaction was carried out using NBT/BCIP as substrate. Antibody staining following hybridization was performed following the standard protocol given above.

Antisense probes for *CLC*, *CLF* and *CT1* were generated using IMAGE Consortium cDNA clones CloneID 3411865, CloneID 3710164 and CloneID 315363, respectively (RZPD, Berlin, Germany). The antisense probe for *NP* was generated using a cDNA encompassing the coding sequence.

Morphometric analysis

Pictures of immunostained sweat glands were taken at a Zeiss Axioplan microscope stand using a Visitron SPOT CCD camera and 20× and 40× objective magnification. The pictures of sweat glands were imported into the NIH-imaging software ImageJ to determine the number of pixels covering the area of the sweat glands and the percentage of pixels covering immunopositive fibers within that area.

For stellate ganglia sections, pictures were taken likewise with 10× objective magnification. The number of pixels covering the area of the cross sections was measured with ImageJ and calibrated to metric values. Immunoreactive cells containing DAPI-stained nuclear profiles in the sections plane were counted manually using 40× objective magnification. Student's *t*-test was performed to test for statistical significance of differences.

Functional sweat gland test

Mice were collected at postnatal days 52 to 58. The animals were anesthetized by intraperitoneal (ip) injections of a mixture of ketamine (0.2 mg per animal) and xylazine (0.08 mg per animal). To induce sweat response, animals received 3 μg pilocarpine per gram body weight (ip). As soon as the animals showed robust salivation, both hind feet were wiped with ethanol and coated with Coltexfine dental paste (Coltene/Whaledent AG Altstaetten, Switzerland). After polymerization of the material, the moulds were removed and the process was repeated once. At the end of the experiment all animals were killed by decapitation and tail tissue was collected for genotyping.

The moulds were analyzed under stereoscopic magnification. Imprints of sweat droplets were counted for each of the two interdigital foot pads. The mean number of interdigital sweat glands was calculated from all counts derived from a single animal. The overall mean and its standard error were calculated for the groups of wild-type and mutant animals. Statistical significance of differences was tested for by Student's *t*-test.

Laser dissection of sweat glands and total RNA isolation

Freshly dissected tissue was frozen and embedded in tissue-Tec (Sakura, The Netherlands). Serial cryostate sections (10 μm) were cut and mounted on autoclaved polytarthalene (PEP) foil stretched on a metal frame (Leica). Sections were then fixed in ice-cold acetone (2 minutes), dried on a heater (40°C, 10 minutes) and stained with 1% Toluidine Blue (Merk, Darmstadt, Germany). After differentiation in 75% ethanol (3 minutes), the sections were dried (40°C, 10 minutes) and subjected to laser microdissection (LMD) (Burbach et al., 2003) using the AS LMD system from Leica Microsystems. Total RNA from LMD-isolated sweat gland coils was obtained by using the RNeasy Micro Kit (Qiagen, Hilden, Germany).

RT-PCR on sweat gland RNA

cDNA synthesis was performed using the Thermoscript RT-PCR System and oligo-dT primers (Invitrogen, Karlsruhe, Germany). For the detection of neuropoietic cytokines, the following primer combinations and the Hot Star Taq Master Mix Kit (Qiagen, Hilden, Germany) were used:

OSM, 5′-CAAGGAAGATGTCTGGCTCCCTTTAGCCC-3′ and 5′-GAAGGGCAGGCCTTCTGGGAACATGAC-3′;

LIF, 5′-GCCACCTGTGCCATACGCCACCC-3′ and 5′-CCACGTGGCCCACAGGTACTTG-3′;

CNTF, 5′-CCAGTGGCAAGCACTGATCGCTGGAG-3′ and 5′-GGCTCTCATGTGCTGAGATTCCCATG-3′;

CLC, 5′-CTCTGCCCAGGGCCACGGTCAAC-3′ and 5′-GGGTAGCCAAGCGTCGCCATGAC-3′;

CLF, 5′-GCGCCCAGTGACGCGCGTGAGG-3′ and 5′-GCCAGGGCCAGGGCCAGGGTG-3′;

CT-1, 5′-CTGGTGCCAGGGGGCGTCGCC-3′ and 5′-CCATCCAGAGCTATATGGGTGAGACCCTGTCTC-3′;

NP, 5′-GGAAGGAGCCAAGGAGGGAGG-3′ and 5′-CCCTGGGCTCGGCTTAGCC-3′;

GAPDH, 5′-CCAGGAGCGAGACCCCACTAACATC-3′ and 5′-CGCAGGAGACAACCTGGTCCTCAG-3′.

For all reactions, a standardized PCR protocol was used: 40 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 30 seconds at 72°C.

Detection of recombined gp130 allele

Primers used for detection of the recombined *gp130* allele (Betz et al., 1998) were 5′-TTTCAAGTACCCTGGGGATGG-3′ (forward) and 5′-TGAGGCAGAAACACACTCATGC-3′ (reverse), and are expected to produce a PCR product of >4 kb for $gp130^{fl/fl}$ and about 800 bp for the recombined *gp130* allele in noradrenergic cells of $gp130^{DBHcre}$ mice. The PCR protocol used was 42 cycles of 1 minute at 94°C, 30 seconds at 58°C and 2 minutes at 68°C.

Sympathetic neuron culture

Cultures of embryonic day (E) 7 chick sympathetic neurons were prepared and maintained as described previously (Ernsberger et al., 1989). Cytokines were added immediately to induce the expression of VIP (GPA, 2 ng/ml; CNTF, 1.5 ng/ml; CLC/CLF, 100 ng/ml; NP, 500 ng/ml). After 4 days, the cells were stained for VIP, as described previously (Ernsberger et al., 1989).

RESULTS

Generation of a specific *gp130* knockout in sympathetic neurons

To investigate whether cytokines induce cholinergic differentiation of sympathetic neurons during sweat gland innervation, we aimed to eliminate the cytokine receptor subunit gp130 that is essential for the action of all IL6 cytokine family members. As mice deficient for gp130 display embryonic lethality and defects in diverse embryonic organs, a conditional knockout was generated in noradrenergic neurons, using *gp130* floxed allele mice (Betz et al., 1998). This floxed *gp130* allele contains two loxP sites flanking exon 16, which encodes the transmembrane domain of gp130. Recombination leads to the loss of gp130 from the plasma membrane, resulting in a complete lack of responsiveness to IL6 cytokine family members (Betz et al., 1998; Hirota et al., 1999). To mutate this allele in sympathetic neurons, we have used a transgenic mouse line carrying the Cre recombinase under the control of the *DBH* gene, expressed selectively in noradrenergic neurons (R.P., C. Otto and G. Schütz, unpublished). As previous plasmid-based transgenesis using a 5.8 kb *DBH* promotor did not fully reproduce the normal DBH expression pattern (Hoyle et al., 1994; Mercer et al., 1991), Cre recombinase was expressed under the control of the *DBH* gene, embedded in a 150 kB P1-derived bacterial artificial chromosome.

The transgenic mouse line carrying the *DBH-Cre* PAC (DBH-Cre) shows Cre expression exclusively in noradrenergic neurons of the peripheral (PNS) and central (CNS) nervous system, as revealed by immunohistological analysis of Cre expression (data not shown) and by analysing Cre activity using the ROSA26 reporter mouse line (Soriano, 1999). In the PNS, Cre-mediated recombination leading to *lacZ* expression was restricted to *DBH*-expressing cells, including paravertebral sympathetic ganglia (Fig. 1A), and was observed as early as at E10.5 (data not shown). On cross sections of E16.5 sympathetic ganglia, virtually all ganglion neurons seemed to be *lacZ*-positive (Fig. 1B). Peripheral targets of cholinergic sympathetic neurons, i.e. sweat glands in the fore- and hindlimb footpads, did not show any recombination (Fig. 1C), as expected from the endogenous *DBH* expression pattern.

Crossing of *DBH-Cre* with $gp130^{fl/fl}$ mice produced $DBHCre;gp130^{fl/fl}$ (abbreviated to $gp130^{DBHCre}$) offspring, born in normal Mendelian ratio, that were viable, with normal weight (25.5±0.5 g in *fl/fl* versus 23.8±0.4 g in $gp130^{DBHcre}$) and without any obvious impairments. The $gp130^{DBHcre}$ mice breed and can be maintained as homozygous line. The very high recombination efficiency in sympathetic ganglia observed in the ROSA26 mouse line implies that functional gp130 is eliminated in sympathetic neurons of $gp130^{DBHcre}$ mice. We were unable to analyse gp130

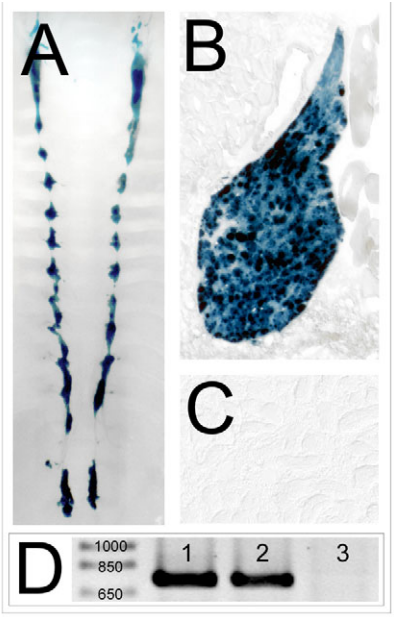

Fig. 1. Analysis of Cre expression in *DBH-Cre* mice. (**A-C**) Using the ROSA26 reporter mouse line, Cre-mediated *lacZ* expression was detected by β-galactosidase staining in sympathetic ganglia of E16.5 mouse embryos. *lacZ* expression is demonstrated for the sympathetic chain (A,B) and is absent in sweat gland tissue (C). (**D**) Cre-mediated elimination of exon 16 in $gp130^{DBHcre}$ mice is revealed by the amplification of an 800 bp band from sympathetic ganglia (1), adrenal gland (2) and the absence in non-adrenergic tissue (tail-cut, 3). (A) Whole-mount staining; (B,C) β-galactosidase staining of tissue sections.

protein expression in wild-type and mutant sympathetic ganglia, most likely due to low expression levels and the small amount of tissue available (data not shown). However, Cre-mediated elimination of exon 16 in *DBH*-expressing sympathetic ganglia and adrenal glands of $gp130^{DBHcre}$ mutant mice could be demonstrated at genomic level by PCR (Fig. 1D).

Sweat gland innervation in $gp130^{DBHcre}$ mice lacks cholinergic properties

The neurotransmitter phenotype of sweat gland innervation in adult $gp130^{DBHcre}$ and control mice was analysed by staining for the cholinergic markers VAChT and the high-affinity choline transporter (ChT1), and for the neuropeptide VIP. β-III-tubulin (TUJ1) and neurofilament (NF160) were used general neuronal markers.

Whereas VAChT, ChT1 and VIP were expressed by the sweat gland innervation in wild-type and control $gp130^{fl/fl}$ mice (Fig. 2C,I,P), cholinergic markers were virtually absent in the sweat gland innervation of $gp130^{DBHcre}$ mice (Fig. 2F,M,S). The extent of cholinergic innervation was quantified morphometrically, revealing a nearly complete loss of cholinergic features (VAChT, ChT1) and VIP, when compared with control mice. This effect was not due to a decreased sweat gland innervation or reduced terminal sprouting (Hiltunen and Alraksinen, 2004), as the expression of the general neuronal marker TUJ1 was not reduced, as shown in the double immunostainings (Fig. 2D,E,K,L,Q,R) and by the quantitative analysis of TUJ1 immunostaining (Fig. 3A-C). The expression of neurofilament immunoreactivity was investigated as an additional neuronal marker, revealing no significant change following the elimination of gp130 signaling (128±21%; mean±s.e.m., n=3-4) in $gp130^{DBHcre}$ when compared with controls. In addition, the expression of the adrenergic marker TH was not significantly different between $gp130^{DBHcre}$ mice and $gp130^{fl/fl}$ controls (Fig. 3D-F). Taken together, these results strongly suggest that in the absence of gp130 signaling noradrenergic properties are maintained, as in the wild type, and cholinergic characteristics are not acquired.

Elimination of *gp130* results in a reduced number of VAChT- and VIP-positive neurons in the sympathetic stellate ganglion

The lack of VAChT and VIP immunoreactivity in the sweat gland innervation of $gp130^{DBHcre}$ mice could be due to a general switch of the neurotransmitter phenotype or to a restricted effect on the distal processes. Selective effects on the cholinergic properties of sweat gland innervation have been observed in *Gfra2*-deficient mice (Hiltunen and Alraksinen, 2004). It has also been suggested that the number of cholinergic neurons in the stellate ganglion would be constant from the latter half of gestation into adulthood, with increasing expression levels of cholinergic markers in the sweat gland innervation (Schäfer et al., 1997). To address these issues, the number of cholinergic neurons was analysed in the stellate ganglion of wild-type and $gp130^{DBHcre}$ mice at postnatal day (P) 60 and P2. The stellate ganglion is the source of forelimb sweat gland innervation (Morales et al., 1995; Schäfer et al., 1998) and may also contribute to the cholinergic sympathetic innervation of the periosteum of sternum and ribs (Asmus et al., 2000).

We observed in stellate ganglia of P60 $gp130^{DBHcre}$ mice a strong reduction in the density of VIP-positive neuronal cell bodies (from 35.4±4.5 to 7.8±2.7 VIP^+ cells/mm^2; mean±sem, n=3-5; P=0.013; Fig. 4A-C). As the mean ganglion area was not significantly altered (90±10.6% in $gp130^{DBHcre}$) when compared with controls (P>0.5), we conclude that the reduced density of VIP-immunoreactive (VIP-IR) cells reflects a lower number of VIP-positive cells/ganglion. Also, the number of VAChT-positive cell bodies was reduced by 70% (from 25±2 to 7.5±1.5 $VAChT^+$ cell bodies/mm^2; mean±s.e.m., n=3-5, P<0.01). The observed lower density of VAChT-IR cells, when compared with VIP-IR cells can be explained by the lower intensity of VAChT-IR. The strong reduction in the number of cholinergic neurons may either reflect the fact that the majority of stellate cholinergic neurons innervate the forelimb sweat glands or that gp130 signaling controls cholinergic differentiation also in other peripheral targets.

The presence of residual cholinergic, VIP-expressing sympathetic neurons that are maintained in $gp130^{DBHcre}$ mice could be due to incomplete recombination or to cytokine-independent cholinergic differentiation. The latter explanation is supported by the finding that a population of cholinergic sympathetic neurons is generated during embryonic development (Ernsberger et al., 1997; Schäfer et al., 1997) by signals that do not involve the cytokine receptors LIFRβ and CNTFRα (Stanke et al., 2000). By analysing sympathetic stellate ganglia of $gp130^{DBHcre}$ and control mice at P2, we could show that the number of VIP-positive cells at P2 closely corresponds to the number of VIP-positive cells observed in the adult sympathetic ganglia of $gp130^{DBHcre}$ animals (compare Fig. 4C with 4D), and to the number of VIP- and VAChT-positive cells determined at P0 in our previous study (Stanke et al., 2000). These data indicate that the generation of VIP-positive neurons during embryonic development is not controlled by gp130 signaling (Fig. 4D). By contrast, gp130 signaling is essential for the postnatal, target-induced cholinergic differentiation, resulting in a fivefold increase in the number of cholinergic sympathetic neurons in the stellate ganglion.

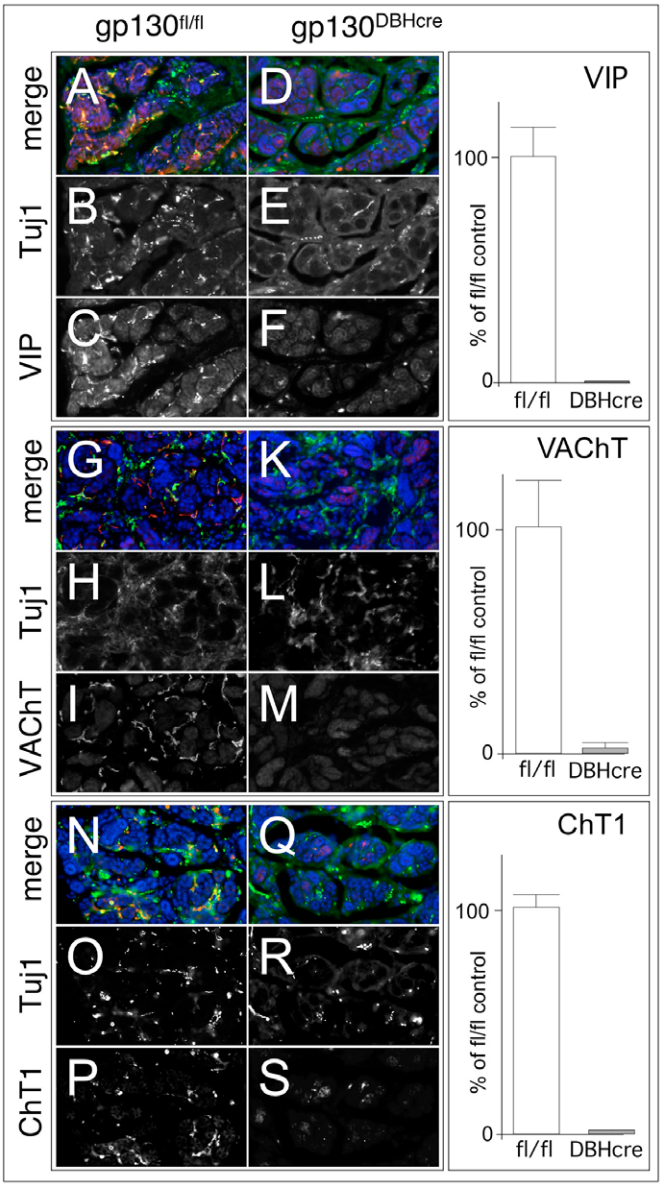

Fig. 2. Sweat gland innervation in *gp130*^{DBHcre} mice lacks cholinergic properties. Frozen sections from P60 mouse footpads of control *gp130*$^{fl/fl}$ and mutant *gp130*^{DBHcre} mice were co-stained for (**A-F**) TUJ1 and VIP, (**G-M**) TUJ1 and VAChT, (**N-S**) TUJ1 and ChT1. Nuclei were stained with DAPI. The expression of VIP-IR, VAChT-IR and ChT1-IR is virtually absent in sweat gland innervation of *gp130*^{DBHcre} mice, as is evident from the individual stainings (F,M,S) and the quantitative analysis (bar graphs, left). By contrast, TUJ1-IR fibers are not affected, as shown by the individual stainings (E,L,R) and the overlays (D,K,Q). For the quantitative analysis, sections were stained individually for VIP-IR, VAChT-IR and ChT1-IR (no co-staining with TUJ1). Quantitative data are expressed as mean±s.e.m. (n=3-8 for each group).

Il6 cytokine family members are expressed in sweat gland tissue

To identify cytokines expressed in the sweat gland proper rather than in footpad connective tissue, we used RT-PCR on secretory coils isolated by Laser-Capture-Microdissection (LCM) from footpad sections (Fig. 5A,B). Using optimized RT-PCR conditions, we could detect, in LCM-isolated sweat gland RNA, transcripts for *CNTF*, *CLC*, *CT1* and *NP* at P4 (Fig. 5C), as well as at P8 and P21 (not shown). *LIF* and *OSM* mRNA could not be amplified from this tissue but could be amplified from E15 mouse embryos (Fig. 5C). Thus, candidate cholinergic differentiation factors are expressed in sweat gland tissue during the period of cholinergic differentiation. To confirm these observations and to address the cellular identity of cytokine-producing cells, the localization of cytokine expression was analysed by in situ hybridisation. The expression of *CT1*, *CLC* and *CLF* could be clearly localized to sweat glands, whereas *NP* could not be detected by this technique (Fig. 5D-G). This correlates with the finding from the RT-PCR that *NP* is expressed at a lower level than the other factors (Fig. 5C). The in situ hybridisation signal is more intense at the circumference of the secretory coils, and is most pronounced for *CLF* (Fig. 5E). Although activation of gp130 signaling has been demonstrated for all IL6 family members, direct evidence for the induction of VIP or cholinergic properties in sympathetic neurons had not been shown for CLC/CLF and NP. We now demonstrate that treatment of cultured sympathetic neurons with NP and CLC/CLF induces a significant increase in the number of VIP-expressing cells (Fig. 5H). The high concentration required to elicit the effects of CLC/CLF or NP most likely reflects the low species cross-reactivity of these factors.

Secretory responsiveness of sweat glands develops in the absence of cholinergic innervation

The development of secretory responsiveness of sweat glands, i.e. sweat secretion in response to cholinergic agonists, depends on noradrenergic and cholinergic neurotransmission (Tian et al., 2000; Grant et al., 1995). The correlation between the timing of cholinergic sweat gland innervation and the development of secretory response, together with inhibitory effects of cholinergic antagonists in the adult, suggested an important function of cholinergic neurotransmission in sweat gland maturation and functional maintenance (Grant et al., 1995; Landis, 1999). As cholinergic innervation is lacking in *gp130*^{DBHcre} mice, it was expected that this would lead to an impaired secretory response to cholinergic agonists. Interestingly, only a small reduction in sweat gland activity was observed (Fig. 6). Whereas 25±0.6 (n=12) secretory sweat glands were stimulated by pilocarpine control footpads, 19±2.6 (n=6) sweat glands were observed in the footpads of *gp130*^{DBHcre} mice (P<0.05). These findings suggest that secretory responsiveness can be acquired and maintained in the absence of cholinergic neurotransmission.

DISCUSSION

The development of cholinergic sweat gland innervation represents a classical example for neuron-target interactions that control neuronal differentiation and are essential for the development of functional circuits (Ernsberger and Rohrer, 1999; Francis and Landis, 1999). Here, we demonstrate that IL6 cytokines acting through gp130 receptors are required for the cholinergic differentiation of sympathetic neurons innervating sweat glands in mouse footpads. Candidate IL6 cytokines were found to be expressed in the target tissue during the critical developmental time period. gp130 signaling controls the differentiation of the vast

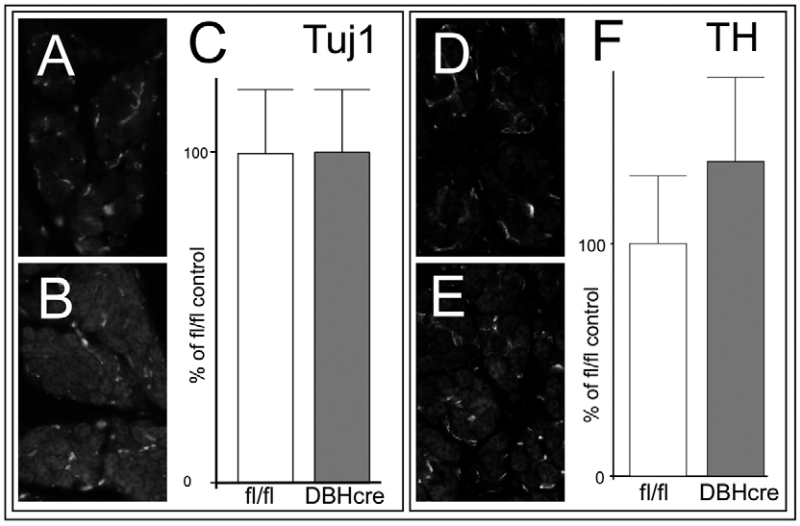

Fig. 3. β-III-tubulin (TUJ1) and TH expression in *gp130*^{DBHcre} sweat gland innervation. Sections from P60 mouse footpads of control *gp130*$^{fl/fl}$ and mutant *gp130*^{DBHcre} mice were stained for TUJ1 and TH. (**A**-**C**) The expression of TUJ1-IR is not reduced in *gp130*^{DBHcre} (B) sweat glands compared with *gp130*$^{fl/fl}$ (A). Quantitative analysis of TuJ1-IR is shown in (C). (**D**-**F**) Sweat glands in mutant *gp130*^{DBHcre} mice (E) display a slightly stronger TH-IR signal than control tissues do (D), which is also reflected in the quantitative analysis (F). Data are expressed as the mean±s.e.m. (n=8-9 for TUJ1; n=3-4 for TH). The differences between *gp130*$^{fl/fl}$ and *gp130*^{DBHcre} are not significant.

majority of cholinergic neurons in the stellate ganglion, which occurs during postnatal development. These results implicate target-derived IL6 cytokines in the specification of virtually all cholinergic sympathetic neurons that differentiate postnatally.

gp130 signaling in cholinergic differentiation

Cytokines of the IL6 family have been implicated in the cholinergic differentiation of sympathetic neurons, as they induce cholinergic properties in cultured sympathetic neurons and in parallel reduce noradrenergic features (Yamamori et al., 1989; Saadat et al., 1989; Geissen et al., 1998; Rao et al., 1992a). Also, in vivo, cholinergic differentiation could be elicited by LIF overexpression (Bamber et al., 1994). A role for IL6 cytokines in sweat gland innervation is supported by the demonstration of a cholinergic differentiation activity in footpad homogenates and in the supernatants of cultured footpads that acts through LIFRβ and activates JAK/STAT pathways (Habecker et al., 1997). It has remained unclear, however, whether these activities reflect the physiological factor(s), as the expression of cytokines and cytokine responsiveness can be rapidly induced in culture (Freidin et al., 1992; Rao and Landis, 1993; Zigmond, 1996; Yao et al., 1997). In addition, factors like CNTF that are secreted from cells only at very low levels (Stöckli et al., 1989; Lin et al., 1989) may represent the major active components of tissue homogenates (Rohrer, 1992). The difficulty to draw valid conclusions from in vitro studies for the in vivo situation is also illustrated by the finding that noradrenergic neurotransmission, which is essential for the expression of the cholinergic differentiation factor in sweat gland cultures (Habecker et al., 1995b), seems not to be relevant in the in vivo situation (Tian et al., 2000).

The present findings demonstrate that cytokines acting through gp130 in sympathetic neurons are essential for the cholinergic differentiation of sweat gland innervation, affecting the expression of VAChT, ChT1 and VIP. The reduced number of VIP- and VAChT-positive cholinergic neurons in the stellate ganglion supports the conclusion that retrograde gp130 signaling from the target controls the neurotransmitter phenotype of sympathetic neurons. In addition, the large, 70-80% decrease indicates that cholinergic differentiation of neurons innervating other targets may also depend on gp130 cytokines. For the periosteum, there is indeed in vitro evidence that this target tissue also produces a cholinergic differentiation signal acting through LIFRβ (Asmus et al., 2001). Whether skeletal muscle vasculature receives cholinergic sympathetic innervation is controversial in rodents (Schäfer et al., 1998; Guidry and Landis, 2000; Dehal et al., 1992), but there is clear evidence for other species, such as cat, guinea pig and chick (see Ernsberger and Rohrer, 1999). Previous knockdown studies in the chick have also shown essential roles of gp130 (Geissen et al., 1998) and LIFRβ (Duong et al., 2002) for VIP expression in cholinergic sympathetic neurons. VIP expression in chick sympathetic neurons, in contrast to ChAT and VAChT expression, is observed at late stages of development and is thought to be induced by signals produced by the innervated vascular targets (Geissen et al., 1998; Duong et al.,

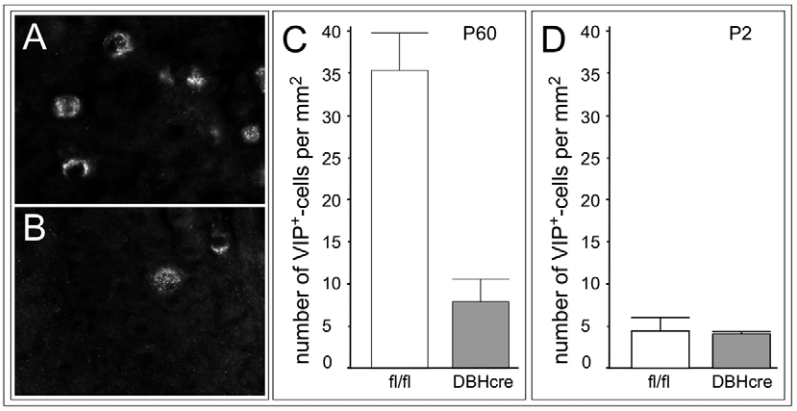

Fig. 4. The number of VIP-IR neurons is reduced in *gp130*^{DBHcre} stellate ganglia. (**A**,**B**) VIP-staining of sections from P60 stellate ganglia of *gp130*$^{fl/fl}$ (A) and *gp130*^{DBHcre} (B) mice demonstrate a strong reduction in the density of VIP-IR neurons. (**C**) Quantitative analysis of VIP-IR neurons per ganglion. Data are expressed as mean±sem (n=3-5). The differences between *gp130*$^{fl/fl}$ and *gp130*^{DBHcre} mice are significant (P<0.05, unpaired, two-tailed t-test with Welch-correction). (**D**) VIP-staining of sections from P2 stellate ganglia of *gp130*$^{fl/fl}$ and *gp130*^{DBHcre} mice show no significant difference.

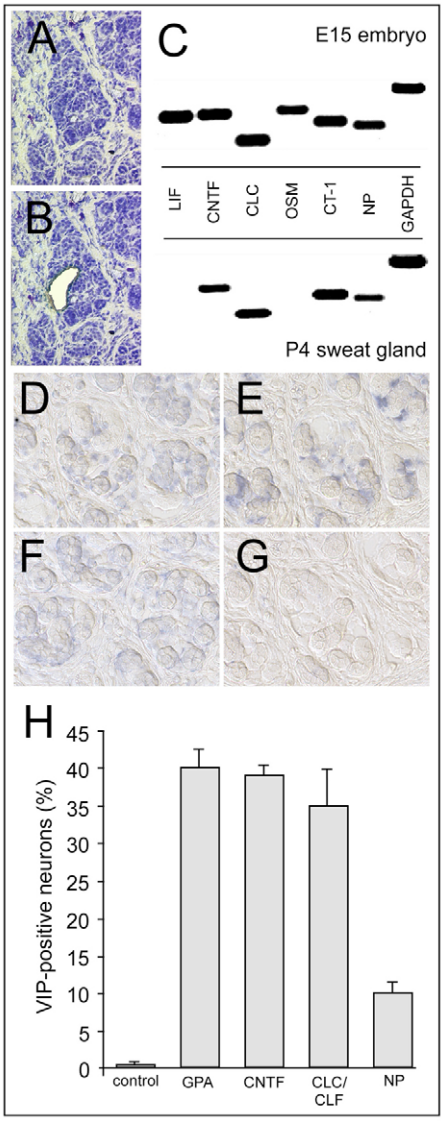

Fig. 5. IL6 cytokine expression in mouse sweat gland tissue. (**A**,**B**) Individual sweat gland coils were dissected from P4 Toluidine-stained sections by LCM. (A) Before and (B) after dissection of sweat gland coil. (**C**) RT-PCR detected the expression of *CNTF*, *CLC*, *CT1* and *NP*, but not of *OSM* and *LIF*, in P4 sweat glands, whereas all cytokines could be detected in total RNA from E15 mouse embryos. (**D-F**) In situ hybridisation of P10 sweat glands revealed expression of *CLC* (D), *CLF* (E) and *CT1* (F). The in situ hybridization signal is mostly localized lateral to the secretory cells, and is most pronounced for *CLF* (E). (**G**) No signal was detected for *NP*, correlating with the minor band in the RT-PCR (see C). (**H**) GPA (2 ng/ml), CNTF (1.5 ng/ml), CLC/CLF (100 ng/ml) and NP (500 ng/ml) induce the expression of VIP in cultures of chick sympathetic neurons.

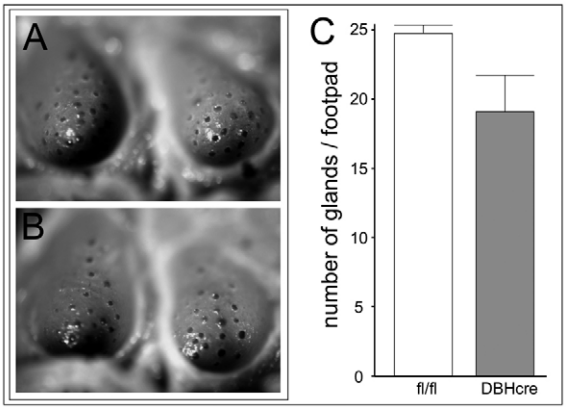

Fig. 6. Secretory responsiveness develops in the absence of cholinergic innervation. Sweating response in P60 mice after intraperitoneal injection of pilocarpine. (**A**,**B**) The glands in both control $gp130^{fl/fl}$ (A) and $gp130^{DBHcre}$ (B) footpads respond robustly to the pilocarpine treatment. Each pore, which appears as dark spot, represents the activity of a single gland. (**C**) Quantification of the number of active glands/per interdigital footpad. Data are presented as mean±s.e.m. of 12 control and six $gp130^{DBHcre}$ mice. The difference between $gp130^{fl/fl}$ and $gp130^{DBHcre}$ mice is significant ($P<0.05$; unpaired, two-tailed *t*-test).

2002). Taken together, these findings suggest that all known targets of cholinergic sympathetic neurons, i.e. sweat glands, periosteum and vasculature may control the neurotransmitter phenotype of their innervation through gp130 cytokines.

How are cholinergic sympathetic neurons generated that do not depend on gp130? The presence of cholinergic sympathetic neurons could be explained by an incomplete elimination of gp130 during DBH-Cre-mediated recombination. This possibility is very unlikely in view of the virtually complete loss of cholinergic properties in sweat gland innervation and the massive *lacZ* expression in the sympathetic ganglia of ROSA26 reporter mice. Cholinergic properties are expressed in a low number of sympathetic neurons during early embryonic development, most likely without target contact (Schäfer et al., 1997; Ernsberger et al., 1997; Stanke et al., 2000). VIP is transiently expressed in the embryonic rat superior cervical ganglion in up to 30% of the cells (Tyrrell and Landis, 1994). As the number of VIP- and VAChT-expressing cells at P0 is not affected in mice deficient for gp130, LIFRβ or CNTFRα, embryonic cholinergic differentiation seems not to be controlled by IL6 cytokines (this study). A similar conclusion was reached from the LIFRβ and gp130 knockdown in the chick, which did not affect the early expressed cholinergic markers ChAT and VAChT (Geissen et al., 1998; Duong et al., 2002).

The contribution of cholinergic sympathetic neurons generated either during embryonic development or postnatally to the population of cholinergic neurons in adult sympathetic ganglia has been unclear (Ernsberger and Rohrer, 1999; Francis and Landis, 1999). The present results demonstrate that the majority of cholinergic sympathetic neurons in the stellate ganglion are generated postnatally and by a gp130-dependent mechanism. This correlates with the timing of the transmitter phenotype switch during sweat gland innervation. The postnatal increase in the number of VIP- and VAChT-positive cells suggests that the cholinergic sympathetic neurons have not already become committed during embryonic development (Schäfer et al., 1997), and supports the notion that the gp130-dependent population is generated by target-induced differentiation (Guidry and Landis, 1998). Conversely, the gp130-independent population represents cells that acquired cholinergic properties during embryonic stages, most likely independently of target innervation. Differentiation factors of the GFL family, acting through the RET tyrosine kinase receptor, are not essential for the initial expression of VAChT and ChAT in vivo, but are required for the maturation of cholinergic sympathetic neurons during prenatal development (Burau et al., 2004). It will be interesting to analyse whether NT3 (Brodski et al., 2000) and activins (Fann and Patterson, 1995) influence embryonic cholinergic sympathetic differentiation in vivo.

Whereas detectable stores of endogenous catecholamines disappear in neurons innervating sweat glands in rats and mice, TH- and DBH-IR were reported to decrease to low levels in rats (Landis et al., 1988) but to be maintained in mice (Rao et al., 1994; Guidry and Landis, 1995). The lack of catecholamine production in mouse sweat gland innervation was explained by a loss of the TH cofactor tetrahydrobiopterin and the tetrahydrobiopterin synthetic enzyme GTP cyclohydrolase (GCH) (Habecker et al., 2002). In the present study, we confirm that TH-IR is present in the adult mouse sweat gland innervation and that the extent of TH expression is not affected by the lack of gp130 signaling. This finding, together with the maintenance of TUJ1- and NF160-positive sweat gland innervation, demonstrates that IL6 cytokines do not affect target innervation or sympathetic neuron survival.

Candidate IL6 cytokines in cholinergic differentiation

What is the identity of the sweat gland-derived cytokine? The cytokines CNTF (Saadat et al., 1989), LIF (Yamamori et al., 1989), OSM (Rao et al., 1992a) and CT1 (Pennica et al., 1995b; Habecker et al., 1995a; Geissen et al., 1998) induce cholinergic function and VIP production while decreasing catecholamine content. The present study extends this list of cytokines by including CLC/CLF and NP as candidate cholinergic differentiation factors. Previous studies concluded that LIF (Rao et al., 1993) and OSM do not appear to be produced by sweat glands in vivo (Habecker et al., 1997). This is confirmed by the RT-PCR analysis of LCM-isolated sweat gland tissue. From the cytokines expressed in sweat gland tissue, CT1, NP and CLC/CLF are the most likely candidates, as, in contrast to CNTF, they are secreted proteins. In addition, CNTF expression is restricted to Schwann cells rather than sweat glands (Rohrer, 1992). CT1 and the newly discovered NP are both efficiently secreted (Pennica et al., 1995a; Derouet et al., 2004). CLC is also secreted when co-expressed with either the soluble receptor CLF (Elson et al., 2000; Lelièvre et al., 2001) or with CNTFRα (Plun-Favreau et al., 2001). CLC/CLF and NP act only on cells expressing the tripartite CNTF receptor (Elson et al., 1998; Elson et al., 2000; Derouet et al., 2004). CT1 binds to and activates a heterotrimeric receptor composed of LIFRβ, gp130 and an hypothetical CT1-specific α-receptor (Robledo et al., 1997). As the postulated CT1 α-receptor has not been identified so far, it is unclear whether it is expressed during sweat gland innervation. In view of the biological effects elicited by CT1 in cultured sympathetic neurons from different species (Pennica et al., 1995b; Habecker et al., 1995a; Geissen et al., 1998), it seems very likely that CT1α-receptors are present in vivo during target tissue innervation. As *NP* is expressed at much lower levels than *CT1* and *CLC/CLF*, apparent from both the RT-PCR and in situ hybridisation analysis, CT1 and CLC/CLF are the most likely candidates for the sweat gland cholinergic differentiation factor.

The co-expression of several secreted cytokines with cholinergic differentiation activity indicates an unexpected redundancy in the target-dependent control of this neurotransmitter phenotype. It should also be kept in mind that the involvement of additional, unknown cytokines cannot be excluded. To define the relevant factors would thus require the combined elimination of CT1, CLC, CLF and possibly NP, involving conditional knockouts, as mice deficient for CLF die around birth (Forger et al., 2003). The cytokine redundancy explains the difficulty in defining physiological relevant factors by loss-of-function approaches in sweat gland homogenates; for example, antibodies against CNTF and CT1 were unable to deplete the cholinergic activity of footpad homogenates (Rao et al., 1992b; Rohrer, 1992; Habecker et al., 1997).

The in situ hybridisation analysis suggests that *CT1* and *CLC/CLF* may be expressed at much higher levels by myoepithelial cells than by secretory cells. During development, both of these cells are generated from invaginating ectodermal cells and both cell types seem to express muscarinic acetylcholine receptors (Landis and Keefe, 1983; Grant et al., 1991). The myoepithelial cells of exocrine glands are highly contractile and have a central role in the ejection of liquids produced by the luminal secretory cells. They are located at the circumference of the glands, in direct contact with the basal lamina. This position is well suited for the production of factors that influence the innervating axons, which are present within a distance of about 1-2 μm of the basal lamina (Landis and Keefe, 1983).

Functions of cholinergic sympathetic innervation

Sweat secretion is elicited by cholinergic agonists, acting through muscarinic acetylcholine receptors of the M2 glandular (m3 molecular) subtype, in developing and adult sweat glands (Stevens and Landis, 1987). The morphological development of sweat glands, as well as the molecular and pharmacological properties of muscarinic cholinergic and adrenergic receptors of sweat glands are independent of innervation (Grant and Landis, 1991; Grant et al., 1991; Habecker et al., 1996). However, innervation is essential for a late step of sweat gland maturation, the development and maintenance of secretory responsiveness, i.e. the ability of glands to produce sweat after nerve stimulation or agonist treatment (Stevens and Landis, 1987; Stevens and Landis, 1988). Both catecholaminergic (Tian et al., 2000) and cholinergic (Grant et al., 1995) neurotransmission are required for the acquisition of secretory responsiveness during development. Cholinergic neurotransmission is also necessary for the maintenance of secretory responsiveness in adult sweat glands (Grant et al., 1995). These findings, showing an essential role of cholinergic neurotransmission for the functional maturation of sweat glands, predicted that mice devoid of cholinergic sweat gland innervation would not acquire and maintain secretory responsiveness. The present study demonstrates, however, that the sweating response of glands to cholinergic agonists is maintained in adult animals in the absence of cholinergic innervation. Adrenergic neurotransmission, essential to induce the sweating response during development (Tian et al., 2000), seems to be sufficient to keep the vast majority of sweat glands in a functional state that allows their response to experimentally administered cholinergic agonists. To explain the finding that the induction of the sweating response depends on both cholinergic and adrenergic neurotransmission (Tian et al., 2000), it has been suggested that a specific step in stimulus-secretion coupling, downstream of second messenger generation, would require acetylcholine and catecholamine signaling, and that both transmitters may control the same step. According to this notion, the lack of cholinergic neurotransmission in gp130-deficient mice would be compensated by the action of catecholamines. Such a compensation is not possible after the disruption of muscarinic neurotransmission in adult rodent sweat glands as their innervation is purely cholinergic (Grant et al., 1995).

In contrast to their ability to induce and maintain the secretory response, adrenergic agonists are very ineffective in eliciting sweat secretion (Stevens and Landis, 1987; Stevens and Landis, 1988). The inability of sweat glands to respond to adrenergic agonists requires the switch of the sympathetic innervation from adrenergic to cholinergic neurotransmission. The present findings demonstrate that IL6 cytokines are the essential, physiologically relevant signals for the specification of the appropriate cholinergic neurotransmitter phenotype during sweat gland innervation.

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