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Cholinergic neuronal differentiation factors: evidence for the presence of both CNTF-like and non-CNTF-like factors in developing rat footpad

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Summary

Catecholaminergic sympathetic neurons are able to change their transmitter phenotype during development and to acquire cholinergic properties. Cholinergic sympathetic differentiation is only observed in fibers innervating specific targets like the sweat glands in the rat footpad. A function for ciliary neurotrophic factor (CNTF) in this process has been implied as it is able to induce cholinergic properties (ChAT,VIP) in cultured chick and rat neurons. We show here that a CNTF-like, VIP-inducing activity is present in rat footpads and that it increases 6-fold during the period of cholinergic sympathetic differentiation. Immunohistochemical analysis of P21 rat footpads demonstrated CNTF-like immunoreactivity in Schwann cells but not in sweat glands, the target tissue of cholinergic sympathetic neurons. The expression of this factor in footpads seems to be dependent on the presence of intact nerve axons, as nerve transection results in a loss of CNTF-like cholinergic activity and immunoreactivity. Immunoprecipitation experiments with rat footpad extracts provided evidence for the presence of ChAT-inducing factors other than CNTF, which may independently or together with CNTF be involved in the determination of sympathetic neuron phenotype.

Key words: CNTF, sympathetic neuron, Schwann cell, sweat gland, cholinergic neuronal differentiation, rat, footpad.

Introduction

The cellular and molecular mechanisms leading to the different neuronal phenotypes of the nervous system during development are largely unknown. There is however evidence for the pluripotentiality of neuronal precursor cells and for fate-determining interactions of precursor cells with the environment (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988). In addition, it was shown that postmitotic peripheral neurons in developing and even adult animals are able to change transmitter phenotype depending on the innervated target (Schotzinger and Landis, 1988, 1990; McMahon and Gibson, 1987; for reviews see Landis, 1988, 1990). The best studied example is developing sympathetic neurons innervating sweat glands in the rat footpad. The sympathetic axons that reach the developing sweat glands during the first postnatal week express catecholaminergic characteristics like catecholamine histofluorescence and tyrosine hydroxylase immunoreactivity. During the second and third postnatal weeks most noradrenergic properties are lost and cholinergic characteristics like choline acetyltransferase (ChAT) activity and vasoactive intestinal peptide (VIP)-immunoreactivity appear (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis et al., 1988). A similar noradrenergic-cholinergic switch was previously observed in vitro when sympathetic neurons were cultured in the presence of certain non-neuronal cells or medium conditioned by these cells (Patterson and Chun, 1974; Furshpan et al., 1976; Patterson and Chun, 1977; Weber, 1981; Swerts et al., 1983; Raynaud et al., 1987a) or in the presence of human placental serum and/or embryo extract (Johnson et al., 1976; Ross et al., 1977; Iacovitti et al., 1981). Cultured cells from various tissues including heart, skin and gut were shown to release several VIP- and ChAT-inducing factors (Nawa and Patterson, 1990) and heart-cell-conditioned medium contains at least three distinct factors which induce a specific pattern of transmitters and neuropeptides (Nawa and Sah, 1990). The major cholinergic factor from heart-cell-conditioned medium has been identified, isolated and characterized as a 45×10^3 M_r cholinergic differentiation factor (CDF) (Fukada, 1985), which was recently found to be identical to leukemia inhibitory factor (LIF) (Yamamori et al., 1989). In addition, a membrane-associated factor (MANS) has been recently purified from rat spinal cord (Wong and Kessler, 1987; Adler et al., 1989) and a

heparin-binding activity was identified in brain extract (Kessler et al., 1986) which also induces cholinergic properties in cultured sympathetic neurons.

We have previously shown that ciliary neurotrophic factor (CNTF) (Manthorpe and Varon, 1985; Stöckli et al., 1989; Lin et al., 1989) induces ChAT-activity and reduces tyrosine hydroxylase (TH) in cultures of rat sympathetic neurons (Saadat et al., 1989) and that chick sympathetic neurons respond to CNTF by an induction of VIP (Ernsberger et al., 1989a). In view of the multitude of cholinergic activities described in vitro, it is important to identify the factors present *in vivo*, to define their cellular localization and expression during normal development. We show here by a sensitive VIP-induction assay combined with immunoprecipitation and immunohistochemistry using anti-CNTF antibodies that CNTF is present in developing rat footpad and is localized in Schwann cells. However, only about half of the ChAT-inducing activity in footpad extract can be precipitated by anti-CNTF antibodies which indicates that other factors, not recognized by anti-CNTF antiserum, are also present in developing rat footpad.

Materials and methods

Cell culture

Chick sympathetic neurons were isolated from lumbosacral sympathetic chain ganglia of E7 chick embryos and cultivated on a polyornithine-laminin-coated substrate in Ham's F14 medium, supplemented with 10% horse serum and 5% fetal calf serum as described in detail previously (Ernsberger et al., 1989a,b). CNTF was purified from rat sciatic nerve as described previously (Saadat et al., 1989). Murine LIF was obtained from AMRAD, Kew Victoria, Australia and was used in chick sympathetic neuron cultures at a concentration, of 1000 units/ml. At this concentration, several batches of LIF were active as ES stem cell-maintenance factor and induced ChAT in cultures of rat sympathetic neurons. Basic FGF and aFGF were obtained from Progen (Heidelberg, FRG) and were used at a concentration of 5 ng/ml and 50 ng/ml respectively. Acidic FGF was assayed both in the absence and in the presence of heparin (2 μg/ml). Acidic FGF was shown to be active as an angiogenesis factor on chick chorioallantoic membrane and bFGF as a survival factor for chick motoneurons.

Rat sympathetic neurons were isolated from superior cervical ganglia of newborn rats using the procedure of Mains and Patterson (1973; Chun and Patterson, 1977a,b) using modifications described by Schwab and Thoenen (1985) and Saadat et al. (1989). Dissociated sympathetic neurons were plated on 35 mm Costar tissue culture dishes coated with polyornithine-laminin, and cultivated in serum-free Ham's F12 medium supplemented with transferrin (100 μg/ml), insulin (5 μg/ml), putrescin (100 μM), progesteron (20 nM), selenium (30 nM), glutamin (500 μg/ml), Hepes (5 mM), bovine serum albumin (0.01%) and nerve growth factor (NGF) (50 ng/ml). The medium was changed every 3-4 days. At the end of the culture period the cultures were washed twice with PBS to remove serum proteins, harvested in PBS with a rubber policeman, collected by centrifugation, and stored frozen at −20°C until further use.

Tissue extracts

All tissues were frozen as small pieces in liquid nitrogen immediately after dissection and kept frozen at −70°C until further use. The frozen tissue was first broken to smaller pieces using a tissue grinder cooled with liquid nitrogen and then homogenized in two volumes/wet weight of 30 mM NaCl, 10 mM phosphate buffer, pH 7.4, supplemented with protease inhibitors aprotinin (20 ki.u./ml), benzamidin (1 mM), leupeptin (100 mM), PMSF (0.1 mM) and EDTA (1 mM), using a glass-glass homogenizer. After a low-speed centrifugation, the supernatant was centrifuged at 100 000 *g* for 1 hour. Supernatants were sterilized by centrifugation through 0.2 μm filters (Spin X; Costar) and stored frozen as small aliquots. For the immunoprecipitation experiments, footpad extracts were concentrated after the 100 000 *g* centrifugation, using centricon10 microcentrators (Amicon), sterilized and stored frozen as small aliquots. Footpad extracts were routinely made from 25 rats (21-day-old), which resulted in 3-4 g tissue (wet weight). Skeletal muscle was dissected from the hindlimb and care was taken to remove the sciatic nerve trunk as completely as possible. For the preparation of skin extracts, an area was shaved before dissecting the skin.

To denervate rat footpad tissue, animals were anesthetized by ether inhalation. The sciatic nerve and the saphenous nerve were exposed in the region of the upper thigh of the left hind-limb of 14-day-old animals and pieces of about 2 cm were removed. In order to obtain complete denervation and to prevent reinnervation of the denervated glabrous skin by sprouting of the saphenous nerve, which innervates the dorsal surface of the foot (Mills et al., 1989), both sciatic and saphenous nerves were sectioned. After 7 days, the animals were killed and footpads of operated and unoperated contralateral hindlimbs were dissected. The absence of reinnervation was controlled by examination of the lesioned nerve. In addition, neurofilament-IR positive fibers were absent in operated footpads at P21 (staining according to Rohrer et al., 1988).

ChAT-assay

ChAT enzyme activity was assayed as described in detail previously (Saadat et al., 1989). Frozen cells were suspended in 100 μl of homogenization buffer (5 mM Tris-acetate, pH 7.4 and 0.1% Triton X-100) and homogenized by pipetting. Cell debris was pelleted by centrifugation (2 minutes at 10 000 *g*). 40 μl of the supernatant was used for ChAT enzyme activity assays (Fonnum, 1969; Raynaud et al., 1987b) and 30 μl for protein determination (Bradford, 1976) using ovalbumin as standard. The sensitivity of the enzyme assay was increased using subsaturating concentrations of acetyl-CoA as described by Raynaud et al. (1987b).

Antisera

Rabbit antisera were raised against oligopeptides that were synthesized according to the CNTF protein sequence. Rabbit antiserum I was raised against a peptide corresponding to amino acid 127-153 of the rat CNTF amino acid sequence, antiserum II was raised against the C-terminal part of CNTF (AA 186-199) (Stöckli et al., 1991). On western blots both antisera recognize purified rat CNTF up to serum dilutions of 1/25 000 and also identify CNTF when sciatic nerve extract is analysed on western blots. The mouse monoclonal antibody 4-65 was raised against recombinant rat CNTF (Stöckli et al., 1991).

Immunoprecipitation

Protein A-sepharose (100 μl of settled gel) was incubated for 3 hours at 4°C with 150-300 μl of anti-CNTF antiserum II or a control immune serum using a tube rotator. After the incubation the beads were washed 3 times with 500 μl buffer

(10 mM Tris-HCl, pH 8, with 150 mM NaCl) and then incubated with 300 μl tissue extract or extract dilutions for 3 hours at 4°C. The amount of tissue extract was adjusted to approx. 200 VIP-inducing units unless indicated differently. After the incubation of antibody-coated beads with the tissue extract, the beads were collected by centrifugation, the supernatant was sterilized by centrifugation through Spin-X filters and stored in small aliquots at −20°C.

Immunohistochemistry

Cultures of chick sympathetic neurons were stained for VIP-IR after 4 days in culture as previously described (Ernsberger et al., 1989). Cells were washed, fixed for 15 minutes with 4% paraformaldehyde in PBS, washed, permeabilized for 15 minutes with PBT1 (PBS supplemented with 1% BSA and 0.1% Triton X-100) and then incubated for 30 minutes with a rabbit anti-VIP antiserum (1:200) (Incstar Corp., Stillwater, Minnesota, USA). In some experiments, goat anti-VIP antiserum (a generous gift of Dr. Sharp, AFRC Edinburgh) was used, which gave identical results. After washing, biotinylated goat anti-rabbit antiserum (1:100, Amersham) was added for 30 minutes, followed by FITC streptavidin (1:100, Amersham) for 20 minutes. Then the cultures were washed and mounted in PBS/glycerol (1/1). The staining for VIP was completely abolished by preincubating the antiserum with 40 μg/ml VIP (Sigma) for 1 hour at room temperature. Stained cultures were viewed with a Zeiss Axiophot fluorescence microscope and the proportion of VIP-IR-positive neurons was determined. 300-600 neurons were analysed for each experimental point, depending on the proportion of labelled cells.

For immunohistochemistry with footpad, the tissue was dissected from 21-day-old rats, fixed for 2 hours at 4°C with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, washed and then kept overnight in 0.1 M phosphate buffer, pH 7.4, supplemented with 20% sucrose. The tissue was embedded in tissue-tec and 7 μm frozen sections were cut. Sections were dried on gelatine-coated glass slides and rehydrated in PGT (PBS supplemented with 0.1% gelatine and 0.2% Triton X-100). Antibody incubations were carried out overnight at 4°C. Rabbit antiserum I (a gift from M. Sendtner) was diluted 1:200, mouse monoclonal antibody 4-65 (hybridoma supernatant, generously provided by G. Breitfels) was diluted 1:1 with PGT. Control stainings were carried out using monoclonal antibody 4-65, which had been absorbed with 400 μg/ml of recombinant rat CNTF during a 1 hour preincubation at room temperature. After the incubation with anti-CNTF antibodies, the sections were washed (3 times for 15 minutes) and then incubated for 2 hours with biotinylated goat anti-mouse antibody or biotinylated donkey anti-rabbit antibody (1:100, Amersham). After repeated washings the sections were incubated for 2 hours with Texas red streptavidin (1:100, Amersham), washed and mounted in PBS/glycerol (1/1).

Results

VIP-induction in cultured chick sympathetic neurons as specific assay for CNTF-like cholinergic differentiation factors in footpad extract

CNTF induces ChAT in cultures of rat sympathetic neurons and also produces a strong increase of VIP-immunoreactivity (VIP-IR) in cultures of chick sympathetic neurons (Ernsberger et al., 1989a). The proportion of VIP-positive neurons increases with

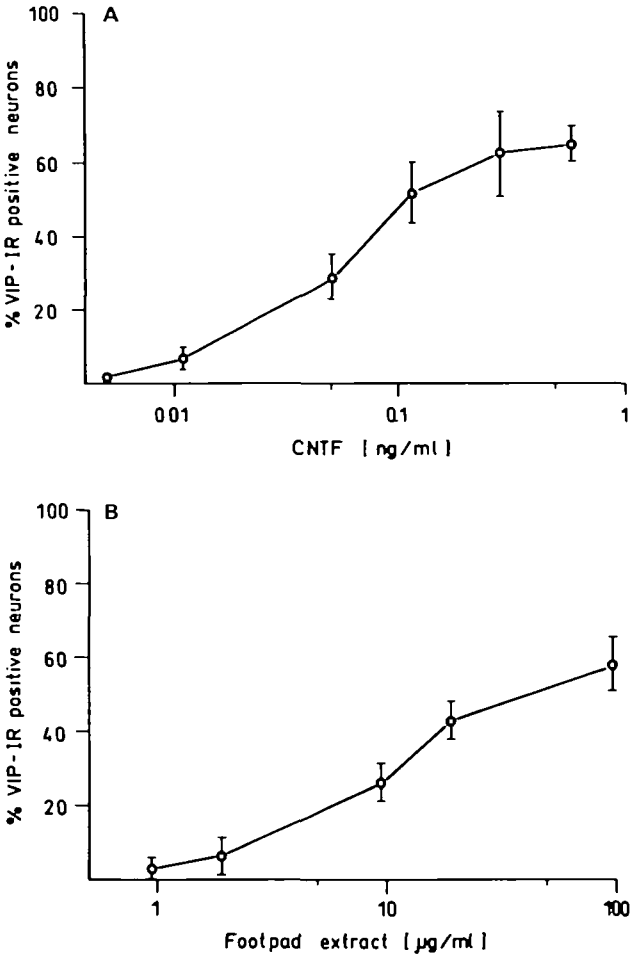

Fig. 1. (A) Induction of VIP-immunoreactivity in chick sympathetic neurons by CNTF. Increasing concentrations of rat CNTF were added to cultures of E7 chick sympathetic neurons. After 4 days the cells were stained for VIP-IR and the proportion of VIP-positive neurons was determined. Each point represents the mean ± s.e.m. of at least 3 independent experiments. (B) Induction of VIP-immunoreactivity in chick sympathetic neurons by footpad extracts. Increasing concentrations of P21 rat footpad extract were added to cultured E7 chick sympathetic neurons. The cultures were processed and analysed as in A. Each point represents the mean ± s.e.m. of 5 independent experiments.

increasing amount of CNTF and in the presence of saturating CNTF concentrations about 60% of E7 sympathetic neurons express VIP-immunoreactivity after 4 days in culture (Fig. 1A). Half-maximal effects were observed at a CNTF-concentration of 0.07 ng/ml.

Extracts of postnatal rat footpads induced a dose-dependent increase in the proportion of VIP-expressing sympathetic neurons (Fig. 1B) and the activity that caused half-maximal effects was arbitrarily defined as 1 VIP-inducing unit. This biological assay for VIP-inducing activity is very convenient because of its sensitivity, reproducibility, and since it tolerates most tissue extracts even at high protein concentrations. Neuronal cell number was unaffected by the added

extracts except for liver extracts which were slightly toxic at high concentrations. It should be pointed out here that sympathetic neurons from E7 embryos do not require any neuronal survival factor (Ernsberger et al., 1989b), excluding effects of the added factors via the selective survival of subclasses of sympathetic neurons. As cultures of E7 sympathetic neurons are virtually devoid of non-neuronal cells (Rohrer and Thoenen, 1987; Rodriguez-Tébar and Rohrer, 1991), the VIP-induction is due to a direct action of the added factor(s) on sympathetic neurons rather than to indirect effects via non-neuronal cells. The activity in footpad extracts not only induced VIP in chick sympathetic neurons, but also interfered with sympathetic neuronal proliferation and supported the survival of ciliary neurons (data not shown), as demonstrated previously for purified CNTF (Ernsberger et al., 1989a).

Expression of VIP in cultured rat sympathetic neurons is affected by several factors, like CDF/LIF, CNTF and other factors in conditioned media and extracts whose molecular nature is still unclear (Nawa and Patterson, 1990; Nawa and Sah, 1990). Thus it was important to examine to what extent CNTF contributes to the VIP-inducing activity in rat footpad extracts. We observed that chick sympathetic neurons, in contrast to rat sympathetic neurons, do not respond to murine CDF/LIF and thus CDF/LIF does not contribute to the signal obtained in the assay used. Also acidic and basic fibroblast growth factor (aFGF, bFGF), which can act in a CNTF-like manner as survival factor for chick ciliary neurons (Unsicker et al., 1987; Watters and Hendry, 1987, Eckenstein et al., 1990), did not induce VIP in chick sympathetic neurons. To determine the identity of the VIP-inducing activity, immuno-precipitation experiments were carried out, using a polyclonal antiserum raised against a synthetic peptide whose sequence corresponds to the C-terminal end of CNTF (Stöckli et al., 1991). Extracts of P21 rat footpad were incubated with protein A-sepharose-bound anti-CNTF antibodies and the activity in the supernatant was assayed. The activity is referred to control immuno-precipitations using unrelated antiserum. Whereas incubations with control antiserum did not reduce the VIP-inducing activity in footpad (data not shown), treatment with anti-CNTF eliminated 90% of the VIP-inducing activity of footpad extracts (Fig. 2). A similar proportion of VIP-inducing activity was eliminated from rat sciatic nerve extract which is a rich source of CNTF (Manthorpe et al., 1986). The antiserum seems not to recognize chick CNTF, as VIP-inducing activity from CNTF-rich tissues, like adult chick sciatic nerve (Eckenstein et al., 1990) or E15 chick eye (Barbin et al., 1984) was not affected by the antiserum in immuno-precipitation experiments (Fig. 2; eye extract and sciatic nerve extracts gave identical results).

Developmental expression of CNTF in rat footpad

The time course of the target-induced switch of sympathetic neurons from noradrenergic to cholinergic phenotype during sweat-gland innervation has been analysed in detail (see Landis, 1988, 1990 for review).

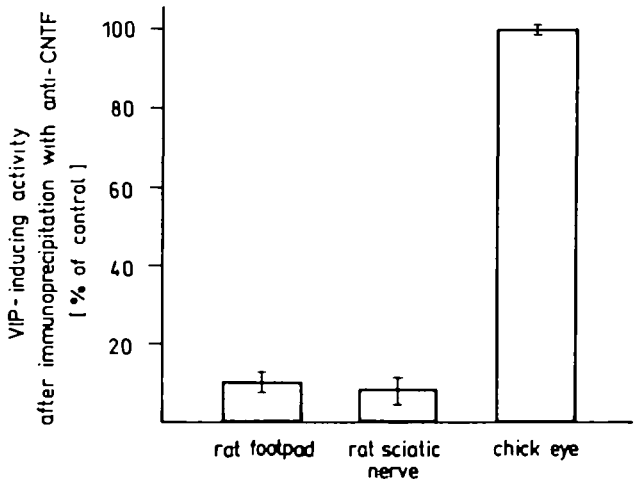

Fig. 2. Effect of immunoprecipitation with anti-CNTF antibodies on the VIP-inducing activity in extracts of rat footpad, rat sciatic nerve and chick eye. Tissue extracts were incubated with either anti-CNTF antibodies, or control antibodies, bound to protein A-sepharose beads. The beads were pelleted and the VIP-inducing activity in the supernatants was analysed using E7 chick neuron cultures. The activity remaining after immunoprecipitation with anti-CNTF-antibodies is compared to incubations with control antibodies and expressed as a percentage of control.

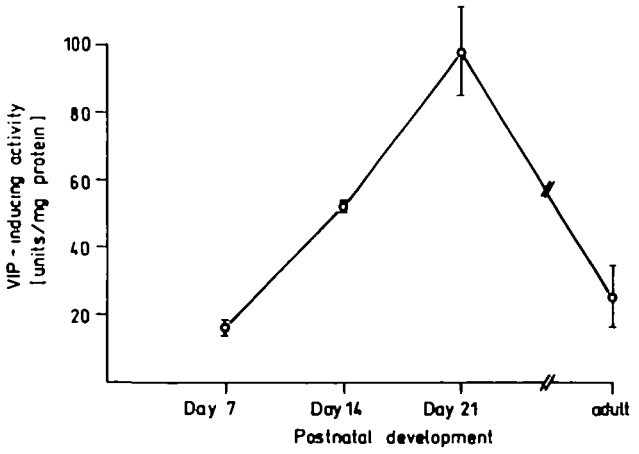

Fig. 3. Developmental increase of CNTF-like activity in rat footpad. Footpads were dissected at different developmental time points and extracts were analysed for VIP-inducing activity.

During the first postnatal week the sympathetic fibers exhibit noradrenergic properties like TH and dopamine β-hydroxylase. Cholinergic markers are absent during early development but appear during the second and third postnatal weeks. Both ChAT activity and VIP-immunorectivity become detectable around postnatal day 11. ChAT activity then increases about 4-fold up to P21.

We observed an 6-fold increase in the specific activity of the VIP-inducing factor in footpad extracts between P7 and P21 (Fig. 3). In adult tissue CNTF is maintained

at reduced levels. As CNTF-dependent cholinergic differentiation of rat sympathetic neurons is a slow process (Saadat et al., 1989), CNTF expression in vivo should also precede cholinergic neuron differentiation. In agreement with this notion, we found that the factor is expressed in footpad before the cholinergic differentiation of sympathetic neurons is detectable. Since the specific activity was highest at P21, footpad extracts were made routinely from this age unless indicated otherwise.

Table 1. *VIP-inducing activity in tissues of 21-day-old rats*

Tissue	VIP-inducing activity (units/mg protein*)
Footpad	98±13
Hairy skin	20±5
Kidney	53±17
Skeletal muscle	9±1.5
Heart	<5
Liver	<5
Sciatic nerve	7500±1100

*Mean ± s.e.m. of 3-11 determinations.

Tissue distribution and cellular localisation of the CNTF

The acquisition of cholinergic properties by noradrenergic sympathetic neurons is controlled by interactions with specific target tissues. In the skin, for instance, only sympathetic fibers innervating sweat glands in the glabrous skin of rat foot pads become cholinergic, but not sympathetic fibers in hairy skin, innervating vascular smooth muscle and piloerectors (Landis, 1990). Thus it was of interest to investigate the tissue distribution of the VIP-inducing activity. Compared with rat footpad, other tissues like hairy skin, muscle and kidney contain less, but still measurable levels of VIP-inducing activity (Table 1). Heart and liver extracts also induced VIP immunoreactivity but at levels that were too low to be quantified. The highest VIP-inducing activity was found in sciatic nerve, as expected from the high CNTF levels present in this tissue. Similarly, in chick, CNTF-rich tissues, like sciatic nerve (Eckenstein et al. 1990) and E15 eye (Barbin et al., 1984), showed elevated levels of VIP-inducing activity (710±173 and 182 ± 43 units/mg protein, respectively).

The high concentrations of VIP-inducing activity in extracts of sciatic nerve raised the possibility that the activity in footpad extracts may be derived from CNTF localized in the Schwann cells of the nerve plexus within the footpad tissue. To address this question, the cellular localization of CNTF in footpad tissue was determined immunohistologically. Frozen sections from P21 rats were stained with either rabbit antiserum or a monoclonal anti-CNTF antibody (Stöckli et al., 1991). Both antibodies detected strong CNTF-IR in nerve Schwann cells. Schwann cell staining could be completely abolished by preabsorption of the antibodies with CNTF or peptide. In contrast, the diffuse staining of sweat glands was maintained also with absorbed antibodies (see Fig. 4 and Fig. 5 for staining with monoclonal or polyclonal antibodies, respectively).

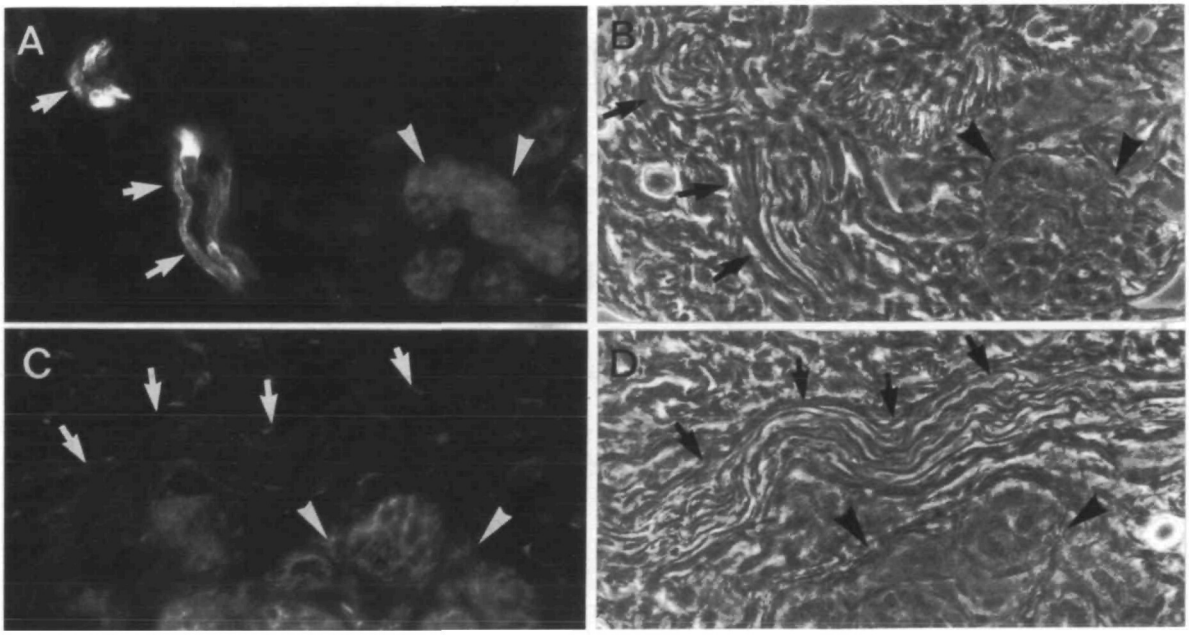

Fig. 4. Immunohistological localization of CNTF-like factor in footpad. Frozen sections of P21 rat footpad were stained with anti-CNTF antibody 4-65, followed by biotinylated anti-mouse antibodies and fluorescently labelled streptavidin (A, B). (A) Immunofluorescence; (B) phase contrast. Note the strong immunoreactivity in nerve Schwann cells (arrows) and weak immunoreactivity in sweat glands (arrowheads). (C,D) Preabsorption of the antibody with CNTF completely abolished the staining of Schwann cells (arrows), whereas the sweat gland staining was maintained (arrowheads). (C) immunofluorescence; (D) phase contrast.

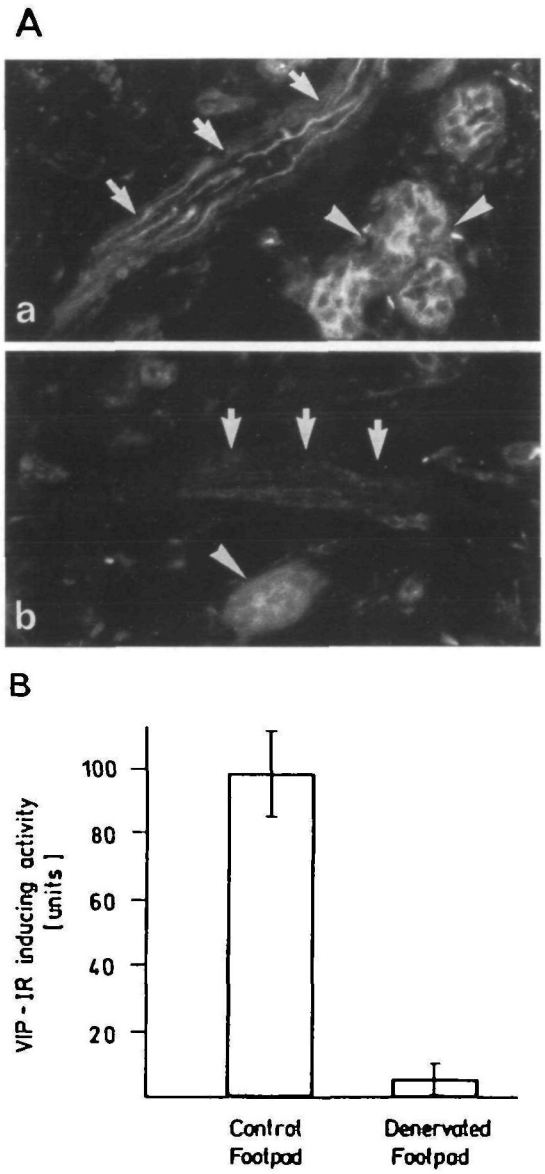

Fig. 5. Effect of nerve transection on VIP-inducing activity and CNTF-like immunoreactivity in rat footpad. After sectioning both sciatic and saphenous nerves unilaterally at P14, footpads were analysed for CNTF-like immunoreactivity (A) and VIP-inducing activity (B) at P21. (A) Frozen sections of unoperated control (a) and nerve sectioned animals (b) were stained for CNTF-like immunoreactivity using a rabbit polyclonal anti-CNTF antiserum. Nerve staining is indicated by arrows, sweat glands by arrowheads. (B) VIP-inducing activity in extracts of operated footpad is compared to the unoperated control. Please note that both CNTF-like immunoreactivity and VIP-inducing activity have disappeared virtually completely 7 days after sectioning the nerve.

To support the finding that the VIP-inducing, CNTF-like activity in rat footpad extracts is localized in, and derived from, Schwann cells, an attempt was made to eliminate the Schwann cell-derived CNTF from footpad tissue (Millaruelo et al., 1986). Previous studies have demonstrated that nerve transection results in a dramatic decrease of CNTF mRNA in the nerve distal to the lesion (Sendtner, personal communication). Therefore we investigated how much of the VIP-inducing activity could be removed from P21 footpads by eliminating the innervation of rat footpad. To obtain complete denervation of the footpad (Mills et al., 1989) both the sciatic and the saphenous nerves were sectioned unilaterally at P14 and one week later at P21 the footpads were analysed for CNTF immunoreactivity and VIP-inducing activity. The contralateral side was used as control. We found that the CNTF-IR in the nerve plexus was strongly decreased whereas the sweat gland staining was not affected. In addition, the VIP-inducing activity in footpad extracts of operated legs was drastically reduced. Taken together, these data suggest that the majority of the VIP-inducing, CNTF-like activity in rat footpad homogenates is localized in Schwann cells.

CNTF accounts only for part of the cholinergic activity in rat footpad

Having established the presence of CNTF as cholinergic differentiation factor in rat footpad during the period of target-induced switch of transmitter phenotype, it was of interest to determine if CNTF accounts for all, or only part, of cholinergic activity in the extracts.

To try to detect all cholinergic activities present in rat footpad homogenates we used a homologous system, i.e. cultures of rat sympathetic neurons and analysed the effect of footpad extracts on the ChAT levels expressed by the cells. Serum-free culture conditions were used to exclude possible interactions with ChAT-inducing factors in serum (Iacovitti et al., 1982; Wolinski and Patterson, 1985a,b).

Footpad homogenates induced ChAT in a dose-dependent way in cultures of newborn rat sympathetic neurons. After 7-11 days in culture in the presence of 10 VIP-inducing units, the specific ChAT activity was increased about 20-fold as compared with control cultures in the presence of NGF alone. ChAT induction in cultures of rat sympathetic neurons required higher concentrations of footpad extract (Fig. 6,7) as compared with VIP induction in chick sympathetic neuron cultures (Fig. 1). This may be either due to differences in the stability of CNTF in chick and rat sympathetic neuron cultures, because of different receptors/receptor occupancies required, or due to negative or potentiative interactions with other factors present in the tissue extracts. Previous experiments, using purified CNTF, demonstrated a similar, but smaller difference between chick and rat neuron cultures with respect to the CNTF concentration required for their specific differentiation effects (Ernsberger et al., 1989a; Saadat et al., 1989).

To determine if the induction of ChAT is due to the CNTF in the footpad, CNTF was removed by immunoprecipitation. Interestingly only about 55% of the ChAT-inducing activity of footpad extracts was eliminated, whereas virtually all ChAT-inducing activity in sciatic nerve homogenates was precipitated (Fig. 6A,B). When immunoprecipitation supernatants of

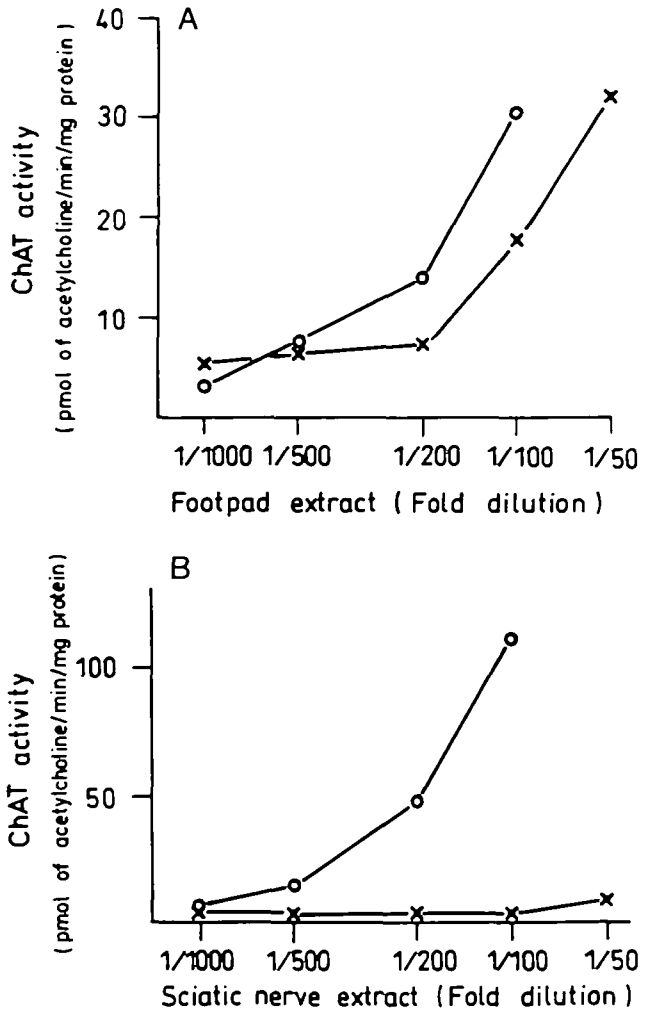

Fig. 6. Induction of ChAT in cultures of rat sympathetic neurons by footpad and sciatic nerve extracts: Effect of anti- CNTF antibodies. Tissue extracts were incubated with protein A- sepharose-bound anti-CNTF antiserum (×) or control immune serum (○). After centrifugation, the CNTF-depleted extracts were assayed for ChAT-inducing activity. Effect of anti-CNTF antibodies on ChAT-inducing activity in footpad (A) and sciatic nerve (B). Please note that only part of the ChAT-inducing activity in rat footpad, but virtually all ChAT-inducing activity in sciatic nerve is eliminated by anti-CNTF antibodies.

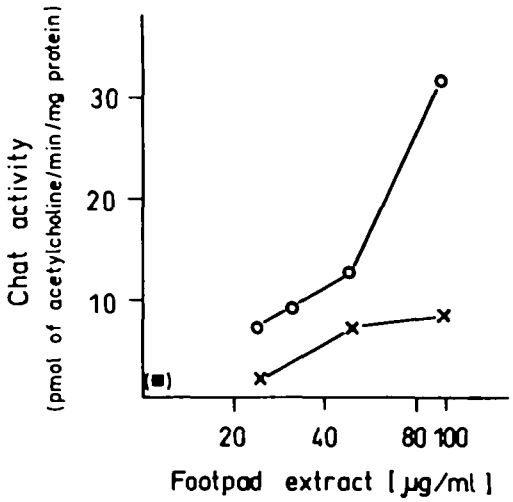

Fig. 7. Denervation results in a loss of ChAT-inducing activity in rat footpad tissue. After unilateral sectioning of both sciatic and saphenous nerves at P14, footpads were analysed for ChAT-inducing activity. Increasing concentrations of operated (×) and unoperated control (○) footpad extracts were added to rat sympathetic neuron cultures. (■) Cultures without added extract. ChAT activity was assayed after 11 days in culture. The data shown are from a representative experiment. In two additional experiments similar results were obtained.

footpad extracts were tested in parallel experiments for VIP and ChAT-inducing activity using cultures of chick and rat sympathetic neurons, respectively, 90% of the VIP-inducing activity, but only 55±8% of the ChAT-inducing activity was eliminated. These results suggest that at least two cholinergic factors are present in rat footpad homogenates, CNTF and a second factor(s) which is not recognized by antibodies against CNTF.

As CNTF is localized in Schwann cells and is reduced upon nerve transection, it was of interest to investigate to what extent transection would affect the ChAT-inducing activity. Since CNTF accounts for about half of the ChAT-inducing activity in rat footpad homogenates, transection was assumed to reduce the levels of ChAT-inducing activity by 50%. Surprisingly, the ChAT-inducing activity in rat footpad homogenates was drastically reduced after nerve transection, which indicates that all ChAT-inducing activities detectable in rat footpad homogenates are dependent on the presence of intact innervation. Since aFGF is present in sciatic nerve and rapidly disappears in the nerve distal to a lesion (Eckenstein et al., 1991), we investigated whether FGF is able to induce ChAT in cultures of rat sympathetic neurons. Neither bFGF nor aFGF had any significant effect on ChAT levels (100% and 112% of control levels, respectively), excluding FGF as a candidate for the non-CNTF-like cholinergic factor in rat footpad.

Discussion

After our previous demonstration that CNTF specifically affects the cholinergic differentiation of cultured chick and rat sympathetic neurons (Ernsberger et al., 1989a; Saadat et al., 1989), the present paper provides evidence that a CNTF-like activity is indeed present in vivo at the time of cholinergic differentiation. The biological and immunological characteristics of the VIP-inducing activity detected in footpad homogenates strongly suggest that this activity is CNTF or a related molecule. The cholinergic activity not only induced VIP in cultured chick sympathetic neurons but also interfered with sympathetic neuronal proliferation and supported E8 ciliary neuron survival. Other factors like CDF/LIF (Yamamori et al., 1989), aFGF, bFGF (Watters and Hendry, 1987; Unsicker et al., 1987; Eckenstein et al., 1990) which in other biological assays

produce CNTF-like effects, did not induce VIP in our chick sympathetic neuron assay and thus did not contribute to the signal obtained in footpad extracts. Virtually all of the VIP-inducing activity detected by our assay can be attributed to CNTF as 90% of the activity is recognized by the anti-CNTF antiserum. In a previous study, a cholinergic activity in rat footpad homogenates was described which displayed similar biological properties and developmental expression as the CNTF-like factor, but was not recognized by anti-CNTF antibodies (Rao and Landis, 1990). Although the negative finding may simply be due to a lower affinity of the antibodies used, it may be taken as indication that the CNTF-like activity in footpad extracts is not identical to CNTF and thus is not recognized by all antibodies that recognize CNTF.

The amount of VIP-inducing activity present in rat footpad is considerably higher than the activity in hairy skin, skeletal muscle, heart and liver. Elevated levels were also observed in kidney homogenates. Due to the small and only partly quantifiable amounts of activity, no attempt was made to identify the nature of the VIP-inducing activity in those tissues by immunoprecipitation. In previous studies, no CNTF mRNA was detected in these tissues (Stöckli et al., 1989). VIP-inducing activities are produced by primary cultures of different tissues analysed, i.e. heart, gut and skin, and the conditioned medium of heart muscle cells contains several VIP-inducing activities with prominent contributions by factors with an apparent relative molecular mass of 85×10^3 and 45×10^3 (Nawa and Patterson, 1990; Nawa and Sah, 1990). Thus it is unclear whether the low level expression is due to non-CNTF VIP-inducing factors or to CNTF, produced either by tissue cells or by Schwann cells.

In rat footpad, the majority of the CNTF-like VIP-inducing activity extract is derived from Schwann cells. This conclusion is supported by the specific Schwann cell staining using monoclonal and polyclonal antibodies against CNTF. In addition, VIP-inducing activity and Schwann cell staining both disappear after transection of sciatic and saphenous nerves and the developmental increase in the levels of VIP-inducing activity in rat footpad during the first 3 postnatal weeks is paralleled by the increase in CNTF mRNA (Stöckli et al., 1989) in the sciatic nerve. Although the existence of additional sites of low level CNTF production cannot be excluded, the available evidence suggests a specific glial origin of CNTF in footpad.

The postnatal increase in the amount of CNTF correlates with the increase in expression of cholinergic properties in sympathetic fibers innervating the sweat glands in the footpad (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis et al., 1988). As cholinergic sympathetic differentiation in vitro proceeds with a considerable delay (Saadat et al., 1989), it is expected that cholinergic factors must be present several days before the detection of cholinergic properties. However, CNTF is a cytosolic protein as indicated by the absence of a hydrophobic leader sequence and its non-release from transfected Hela or COS-cells (Stöckli et al., 1989; Lin et al., 1989). A physiological role of CNTF in vivo thus either involves a release during cell death or a novel release mechanism as described for IL-1 or for plasminogen activator inhibitor (Rubartelli et al., 1990; Belin et al., 1989). An interesting characteristic of these unconventional release mechanisms is the possibility of release under certain conditions i.e. by inducing cellular differentiation (Belin et al., 1989) or under stress conditions (Rubartelli et al., 1990). There are an increasing number of proteins without signal sequence but with known extracellular functions and cell surface receptors for these factors, such as bFGF (Abraham et al., 1986) and ADF (Tagaya et al., 1989). If the CNTF-like factor expressed by Schwann cells is involved in cholinergic sympathetic neuron differentiation, the availability of this factor must be restricted to those fibers that acquire cholinergic properties. There is considerable evidence to indicate that the cholinergic differentiation of sympathetic fibers in the rat footpad is due to interaction with its target, the sweat glands (Landis, 1988, 1990). The clearest evidence came from transplantation experiments where sweat glands were transplanted into areas of hairy skin. Cholinergic sympathetic fibers are not present in hairy skin; however, in the transplant situation, sweat gland innervating fibers became cholinergic (Schotzinger and Landis, 1988). As the innervating fibers are separated from the sweat glands by a basal lamina (Landis and Keefe, 1983), cholinergic sympathetic differentiation implicates a diffusible cholinergic differentiation factor produced by sweat glands. The virtually complete and parallel reduction of VIP-inducing activity and CNTF-immunoreactivity upon nerve transection suggests that the majority of the activity is localized in Schwann cells. Thus, the CNTF-like activity in footpad extracts seems to be a less likely candidate for the sweat gland cholinergic differentiation factor. However, a role for Schwann cell-derived CNTF in cholinergic differentiation, implicating a diffusible signal from sweat glands which would cause a local and specific release of CNTF, is not excluded.

The analysis of the effects of footpad extracts on ChAT-induction in rat sympathetic neurons provided evidence for a second, non-CNTF-like cholinergic activity. The inability to eliminate all ChAT-inducing activity from rat footpad homogenates is not due to limitations of the immmunoprecipitation conditions: increasing the amount of anti-CNTF antibody did not precipitate more ChAT-inducing activity. In addition, in sciatic nerve homogenates much higher concentrations of ChAT-inducing activity than those present in footpad homogenates were completely precipitated under identical conditions. One candidate for such a non-CNTF cholinergic factor is CDF/LIF (Yamamori et al., 1989; Yamamori, 1991). However, carefully controlled immunoprecipitation experiments with anti-CDF/LIF have shown that the ChAT-inducing activity in rat footpad homogenates is not immunologically related to CDF/LIF (Rao and Landis, 1990).

It is known that ciliary survival activity and CNTF mRNA in peripheral nerves of adult mammals is

decreased distally to a lesion site within a time period of a few days (Millaruelo et al., 1986; Sendtner, unpublished results). Therefore we investigated whether non-CNTF ChAT-inducing activity could be identified in footpads after lesion of the sciatic and saphenous nerves. Surprisingly, the ChAT-inducing activity in footpad extracts was drastically reduced, suggesting that both CNTF and non-CNTF-like factors are lost upon nerve transection. This may indicate an innervation-dependent synthesis of cholinergic factors in the target or a CNTF-like expression and localization of the second factor. Acidic FGF is present in sciatic nerve and transection results in dramatic decrease of aFGF content distal to the lesion (Eckenstein et al., 1990). As neither aFGF nor bFGF had any effect on VIP- or ChAT-induction, a contribution of FGF to non-CNTF-like cholinergic activity in footpad can be excluded.

The existence of a multitude of different cholinergic factors, detected by in vitro studies, may be explained by the assumption that different factors are responsible for the induction of specific neuronal characteristics in different cholinergic neurons. In this context it should be noted that the transcription of the VIP gene is regulated by two different signal transduction pathways (Fink et al., 1991). Although some factors affect only a single neuronal property (Nawa and Patterson, 1990), most of the factors described, like CDF/LIF and CNTF, are able to simultaneously influence a wide variety of neuronal characteristics. Cholinergic differentiation factors may be necessary not only for the induction, but also for the maintenance of the cholinergic phenotype, and it is not known whether induction and maintenance of specific neuronal traits are due to the same or different factors in vivo. Thus induction and/or maintenance of a specific neuronal phenotype may depend on the combinatorial interaction of different factors whose contribution to the final phenotype needs to be analysed by experiments where the function of individual factors is interfered with, i.e by the application of activity-blocking antibodies or by altered CNTF expression in transgenic mice.

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