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Mechanisms of Development 68 (1997) 115–126

# The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation

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Received 21 July 1997; revised version received 22 August 1997; accepted 25 August 1997

**Abstract**

Cholinergic properties in chick sympathetic neurons are detectable early during development of paravertebral ganglia and mature after target contact. The cholinergic marker choline acetylytransferase (ChAT) is first detectable at embryonic day 6 and its expression partly overlaps with that of the noradrenergic marker tyrosine hydroxylase (TH). At late embryonic stages, when sympathetic neurons have established target contact, ganglia consist of two major neuronal populations, TH-positive noradrenergic neurons and cholinergic neurons that at this stage express vasoactive intestinal peptide (VIP) in addition to ChAT. The maturation of sympathetic neurons is paralleled by changes in their response to the neurokine ciliary neurotrophic factor (CNTF). These findings suggest that expression of neurotransmitter properties is controlled differentially before and during target innervation. © 1997 Elsevier Science Ireland Ltd.

*Keywords:* Choline acetyltransferase; Vasoactive intestinal peptide; Sympathetic neuron; Chick; Tyrosine hydroxylase; Development

## 1. Introduction

Sympathetic ganglia are composed of neuronal subpopulations that differ in their neurotransmitter phenotype. Although the vast majority of mammalian sympathetic neurons uses noradrenaline as neurotransmitter, a smaller subpopulation uses acetylcholine (Elfvin et al., 1993). In addition, different neuropeptides are expressed and released as co-transmitters from distinct subpopulations (Lundberg and Hökfelt, 1986). The expression of neuropeptides and synthesis of classical neurotransmitters can be correlated with specific functions and innervation targets of the sympathetic neuron subpopulations (Lindh and Hökfelt, 1990; Gibbins, 1992).

Even though the development of sympathetic ganglia is well analyzed in chick embryos, noradrenergic but not cholinergic differentiation has been studied in detail (reviewed in Ernsberger and Rohrer, 1996; Heller et al., 1996). Catecholamine-producing cells are first detectable at embryonic day 2.5–3 (E2.5–3), stage 18 (Hamburger and Hamilton, 1951), of chick embryo development in primary sympathetic ganglia, near the dorsal aorta. These catecholaminergic cells migrate dorsolaterally to form secondary paravertebral ganglia and ventrally to the adrenal and to the sites of prevertebral ganglia. Neuron proliferation occurs both in primary and secondary sympathetic ganglia and proceeds at low levels until late embryonic stages. Although the first postmitotic cells are generated at E4, target innervation occurs much later and was first observed at E9 (Enemar et al., 1965; Kirby et al., 1978, 1980; Rush et al., 1986; Devay et al., 1994). Direct evidence for the presence of cholinergic sympathetic neurons was only obtained from choline acetylytransferase (ChAT) activity measurements and revealed significant levels of ChAT activity at E6 (Marchisio and Consolo, 1968), i.e. before target contact. These data on ChAT activity are, however, not entirely conclusive since a contribution from presynaptic terminals could not formally be excluded (Hruschak et al., 1982; Ross et al., 1990; Devay et al., 1994). In dissociated culture of sympathetic neurons from 12 day old chick embryos, ChAT activity could be directly attributed to ganglionic cells

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*PII* S0925-4773(97)00135-4

(Edgar et al., 1981). The expression of vasoactive intestinal peptide (VIP) in chick sympathetic neurons (Hayashi et al., 1983; New and Mudge, 1986) was taken as indirect evidence for the cholinergic phenotype as VIP-immunoreactivity (-IR) was found in neurons devoid of tyrosine hydroxylase (TH) (Hayashi et al., 1985) and since this neuropeptide is co-expressed with ChAT in mammalian cholinergic sympathetic neurons (Elfvin et al., 1993). VIP-IR was first observed at E10 in a small subpopulation of ganglion cells (New and Mudge, 1986). The presence of a strong plexus of VIP-IR fibers in blood vessels of skin and muscle (Zechbauer and Rohrer, in preparation) suggests that the vasculature is the major target of VIP-IR sympathetic neurons.

A recent analysis of the expression of ChAT and the vesicular acetylcholine transporter (VAChT) in rat paravertebral sympathetic ganglia concluded that the cholinergic phenotype is generated before target innervation (Schäfer et al., 1997). In addition, VAChT expression was observed in the initial sympathetic innervation of sweat glands in the rat footpad, the most thoroughly analyzed target tissue of cholinergic sympathetic fibers in mammals. Earlier studies analyzing the development of the cholinergic sympathetic sweat gland innervation came to the conclusion that cholinergic differentiation can be promoted by target tissues (Landis, 1990). During early postnatal development, the sweat glands receive noradrenergic sympathetic innervation. While the sweat glands and their innervation mature during the following 2 weeks, the axon terminals loose a number of noradrenergic characters (Landis and Keefe, 1983), whereas ChAT and VIP are upregulated (Leblanc and Landis, 1986; Stevens and Landis, 1987; Landis et al., 1988). These results in mammalian sympathetic ganglia suggest a complex regulation of the cholinergic phenotype that may involve both early expression of the cholinergic locus (ChAT, VAChT) and later target-dependent signaling.

In the present study, we have investigated the expression of ChAT and VIP mRNA by in situ hybridization to delineate the differentiation of cholinergic neurons in paravertebral chick sympathetic ganglia during development. Using cultured sympathetic neurons, we have addressed the question whether the expression of ChAT, VIP and TH are regulated coordinately. We also investigated whether sympathetic neurons derived from early and late stages of development respond in a different way to signals that in vitro are known to affect ChAT and VIP expression.

We provide evidence that ChAT mRNA, but not VIP mRNA, is expressed at E6, before target contact. At this stage, co-expression of ChAT and TH mRNA is detectable. Several days later, during the time of target encounter, VIP starts to be expressed in neurons that are ChAT-positive and negative for TH mRNA. The effect of the cholinergic differentiation factor ciliary neurotrophic factor (CNTF) on cultured sympathetic neurons indicates that the control of ChAT and TH expression is different in sympathetic neurons derived from ganglia before and after target innervation. The data suggest that ChAT and TH expression may be subject to CNTFR$\alpha$-mediated signaling during late but not early development. These data also indicate that in chick and rat both target-independent and target-dependent signals may be involved in the generation of the cholinergic as well as the noradrenergic sympathetic phenotype. Parts of this work have been published in abstract form (Ernsberger et al., 1996; Rohrer and Ernsberger, 1998).

## 2. Results

### *2.1. ChAT but not VIP mRNA can be detected before target contact of sympathetic fibers and may be co-expressed with TH*

To investigate the differentiation of cholinergic sympathetic neurons at the cellular level, ChAT mRNA expression in paravertebral sympathetic ganglia was studied by in situ hybridization and by enzyme activity determinations during embryonic development and compared with the expression of VIP and TH.

ChAT-positive cells were already detectable in the primary sympathetic chain and in cells migrating from the primary ganglia to the sites of the secondary sympathetic ganglia (Fig. 1). Areas of ChAT mRNA expression overlapped with areas of TH mRNA expression indicating that the cells are indeed migrating sympathetic precursors. Earliest detectable expression of ChAT mRNA was on E6 (stage 29). No expression of ChAT mRNA was detectable in sympathetic ganglia of the thoracic region at younger stages (data not shown).

In the secondary sympathetic chain of 7 day old embryos (stage 31), ChAT mRNA could be detected over large parts of the ganglion (Fig. 2). As the size of the neuronal somata is relatively small at this stage of development, staining patterns from adjacent sections even at 7–10 $\mu$m cannot prove expression of TH and ChAT in the same or distinct cells. Therefore in situ hybridization for ChAT mRNA has been combined with staining for the TH protein. Regardless of the detection methods used, cells that expressed either one or both of the molecules were readily observed (Fig. 3). The data show that the cholinergic character ChAT is expressed in sympathetic neurons before the cells contact their final targets. Moreover, ChAT and TH may be expressed in the same cells, indicating that cholinergic and noradrenergic lineages are not completely segregated at this early time.

In contrast to the early expression of ChAT mRNA, the first few and isolated cells expressing VIP mRNA could be detected at E10 (stage 36) (data not shown). This is in agreement with immunohistochemistry showing the first VIP-immunoreactive cells at day 10 in embryonic chick sympathetic ganglia (New and Mudge, 1986). It should be mentioned that in sections from early stages that lack VIP-

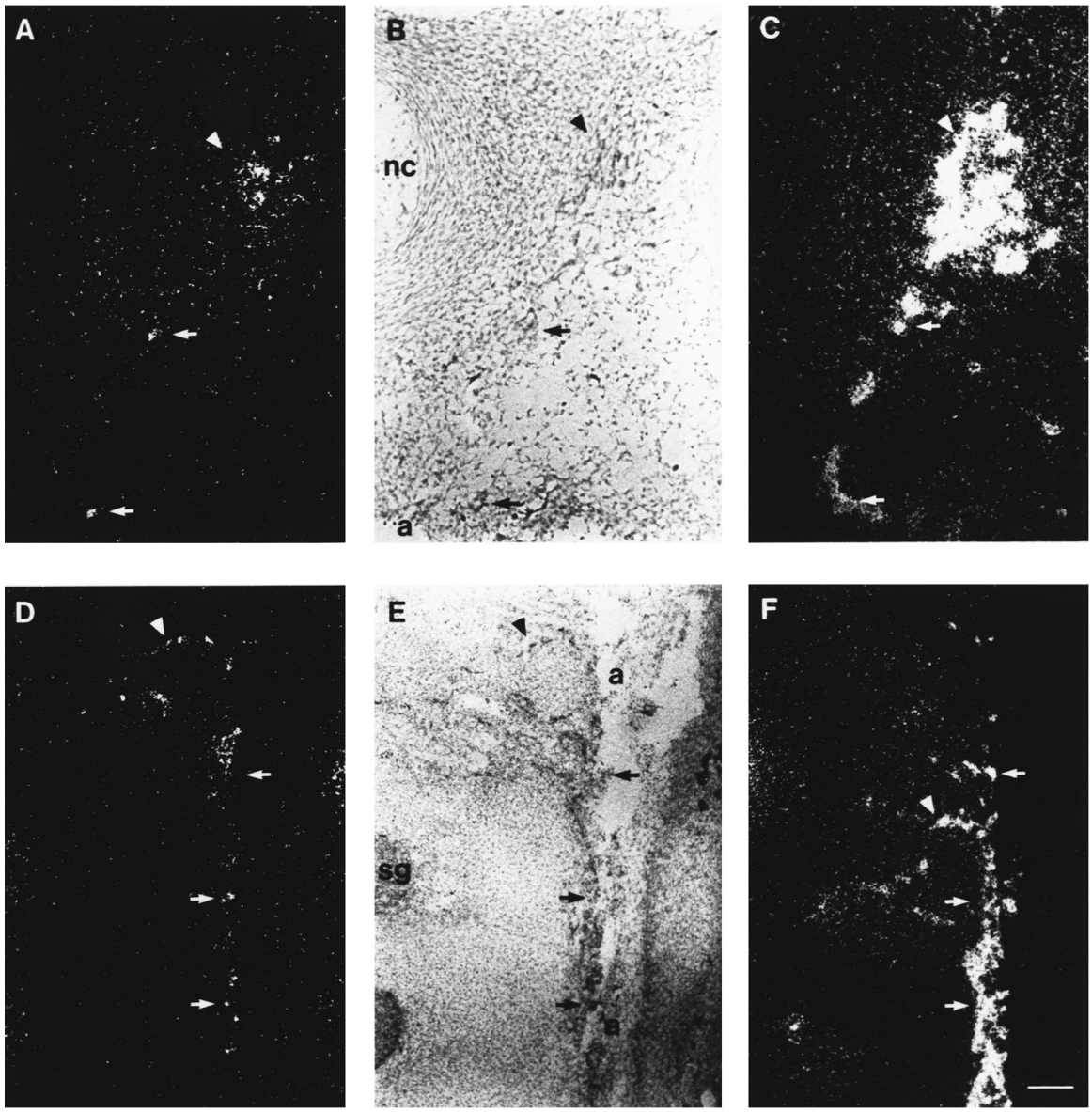


Fig. 1. ChAT mRNA signals are detectable in cells of the primary sympathetic strands and on the migration pathways to the secondary sympathetic ganglia in 6 day old (stage 29) chick embryos. (A–C) Consecutive cross sections two segments caudal to the wing bud are hybridized with radioactive probes to ChAT mRNA (A) and TH mRNA (C) (dark field optics). (B) is the phase contrast picture of (A). Arrows indicate regions ventrally to the developing secondary sympathetic ganglion where TH and ChAT mRNA positive cells are detected. In the forming ganglion, TH as well as ChAT signals are detected, TH being expressed in larger parts of the ganglion than ChAT. a, aorta; nc, notochord. (D–F) Parasagittal sections of the same region as shown in (A–C) from a different embryo of the same stage hybridized for ChAT (D) and TH (F) mRNA (dark field optics). (E) is the phase contrast picture of (D). The sections shown in (D) and (F) are 40 $\mu$m apart. The top is directed rostrally, the left dorsally. The primary sympathetic strand dorsal to the aorta contains cells positive for ChAT and for TH (arrows). Groups of positive cells dorsal to the primary sympathetic strands (arrowheads) likely represent migrating sympathetic cells on the way to the locations of secondary ganglia. a, aorta; sg, spinal ganglia. Scale bar, 50 $\mu$m.

expressing cells in sympathetic ganglia, VIP-positive cells were detected in the spinal cord and gut.

### *2.2. A subpopulation of sympathetic neurons expresses ChAT and VIP mRNA but not TH mRNA after target innervation*

At advanced developmental stages, sympathetic ganglia are composed of two cell populations that can be distinguished by the expression of the catecholaminergic marker enzyme TH and the neuropeptide VIP (Hayashi et al., 1985; New and Mudge, 1986). To examine the identity of ChAT-expressing sympathetic neurons, mRNA for ChAT, TH, VIP and for the general neuronal marker SCG 10 was visualized by in situ hybridization in alternate sections from E18 paravertebral sympathetic ganglia, using DIG-labeled cRNA probes. As the cell body size (15–20 $\mu$m) is large compared to the thickness of the sections (7–10 $\mu$m), cellular patterns from adjacent sections are comparable but must not be identical. Comparison of SCG 10 (Fig. 4A,E) and TH expression

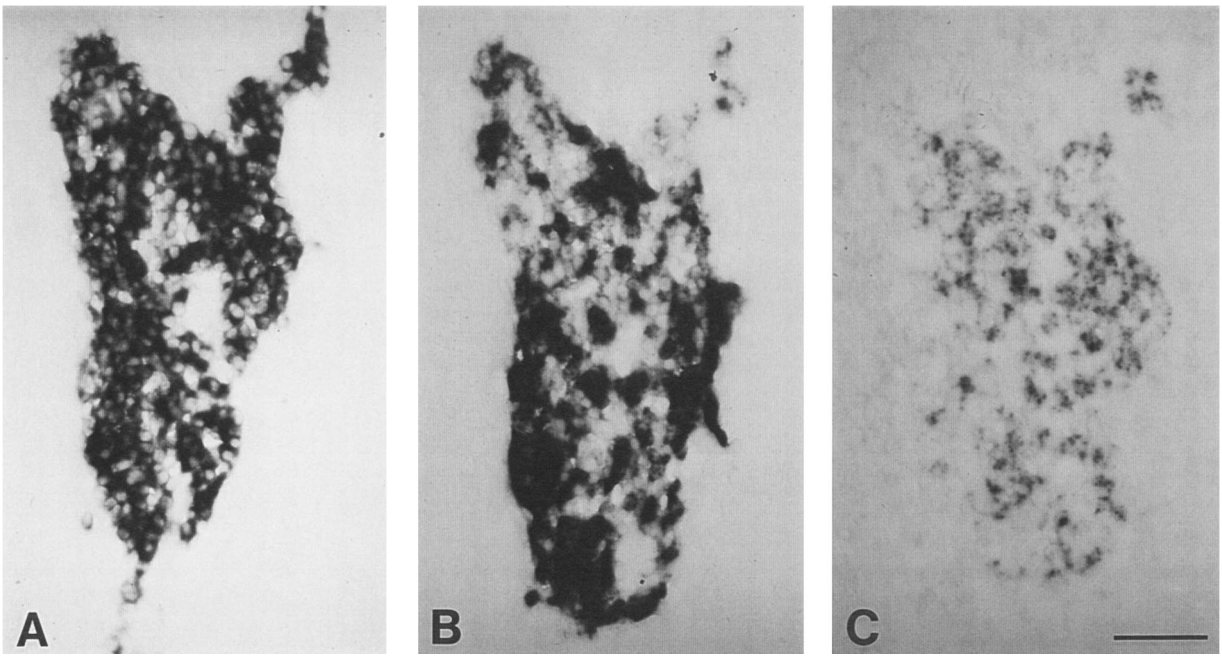


Fig. 2. ChAT is expressed in paravertebral sympathetic ganglia of 7 day old (stage 31) chick embryos. Non-radioactive in situ hybridization has been performed on tissue sections from the thoracic region of chick embryos to demonstrate mRNA for SCG 10 (A), TH (B), and ChAT (C). Hybridizations were performed on adjacent sections. Strong TH signals are seen in large parts of the ganglion. Weak signals indicating ChAT mRNA expression are observed throughout the ganglion. Scale bar, 50 $\mu$m.

(Fig. 4B,F), demonstrates the expression of TH in the majority but not all neurons of the ganglion. Clusters of cells that express the neuronal marker SCG 10 but not the adrenergic marker TH can be recognized. The TH-negative clusters of cells are observed to express ChAT mRNA (Fig. 4C,G). In the same region of the ganglion, VIP mRNA is detected (Fig. 4D,H). The results demonstrate the presence of a cholinergic, VIP-positive and TH-negative neuronal subpopulation in sympathetic ganglia during late development when target innervation is firmly established (Enemar et al., 1965; Kirby et al., 1978, 1980; Rush et al., 1986; Devay et al., 1994). The present results cannot exclude the existence of a minority of cells that expresses VIP but not ChAT or ChAT but not VIP. However, previous studies, using immunohistological procedures, have shown that TH and VIP are not co-expressed in E14 sympathetic ganglia (Hayashi et al., 1985; H. Rohrer, unpublished results).

### *2.3. Regulation of ChAT, VIP and TH expression in cultured embryonic sympathetic neurons*

The different timing of ChAT and VIP expression during sympathetic neuron development suggests that these two genes are differently regulated during early development. As sympathetic neurons from E7 chick sympathetic neurons express VIP in response to CNTF in culture (Ernsberger et al., 1989b), we investigated whether ChAT is also induced by CNTF and whether sympathetic neurons from E7 and E12 ganglia display different responses with respect to the expression of ChAT, VIP and TH. For this purpose, ChAT mRNA expression (Figs. 5 and 6), ChAT activity (Table 1), TH mRNA expression and VIP-IR (Fig. 6) were compared in cultures of sympathetic neurons kept with or without CNTF.

To reduce the effects of CNTF on neuron proliferation and survival (Ernsberger et al., 1989b) that would complicate the interpretations of the culture experiments, cells were kept under conditions that reduce proliferation as well as cell death (35 mM $K^+$; see Section 4). When E7 sympathetic neurons were grown in the presence or absence of CNTF, no significant difference in cell number was observed between CNTF-treated and untreated cultures after a 4 day culture period (Fig. 6D). In addition, no significant difference in the percentage of ChAT-positive neurons (Fig. 6A) was observed when CNTF was added to the culture medium. Also specific ChAT activity was not increased in the presence of CNTF (Table 1). Interestingly, however, specific ChAT activity was increased after 4 days in culture as compared to freshly isolated ganglia under both culture conditions (Table 1). Even though the percentage of TH mRNA positive neurons was reduced in CNTF-treated cultures, the effect was not significant (Fig. 6B). The induction of VIP-IR in more than 75% of the cells (Fig. 6C) demonstrates that the vast majority of E7 sympathetic neurons is able to respond to CNTF in culture.

Different from E7 sympathetic neurons, ChAT was affected by CNTF in E12 sympathetic neuron cultures (Fig. 6A). The number of ChAT mRNA positive neurons was significantly higher after 4 days in CNTF-treated cultures (10113 ± 2186 ChAT mRNA positive neurons; mean ± SD; $n = 4$) than in control cultures (4086 ± 1735 ChAT mRNA positive neurons; mean ± SD; $n = 4$) (Fig.

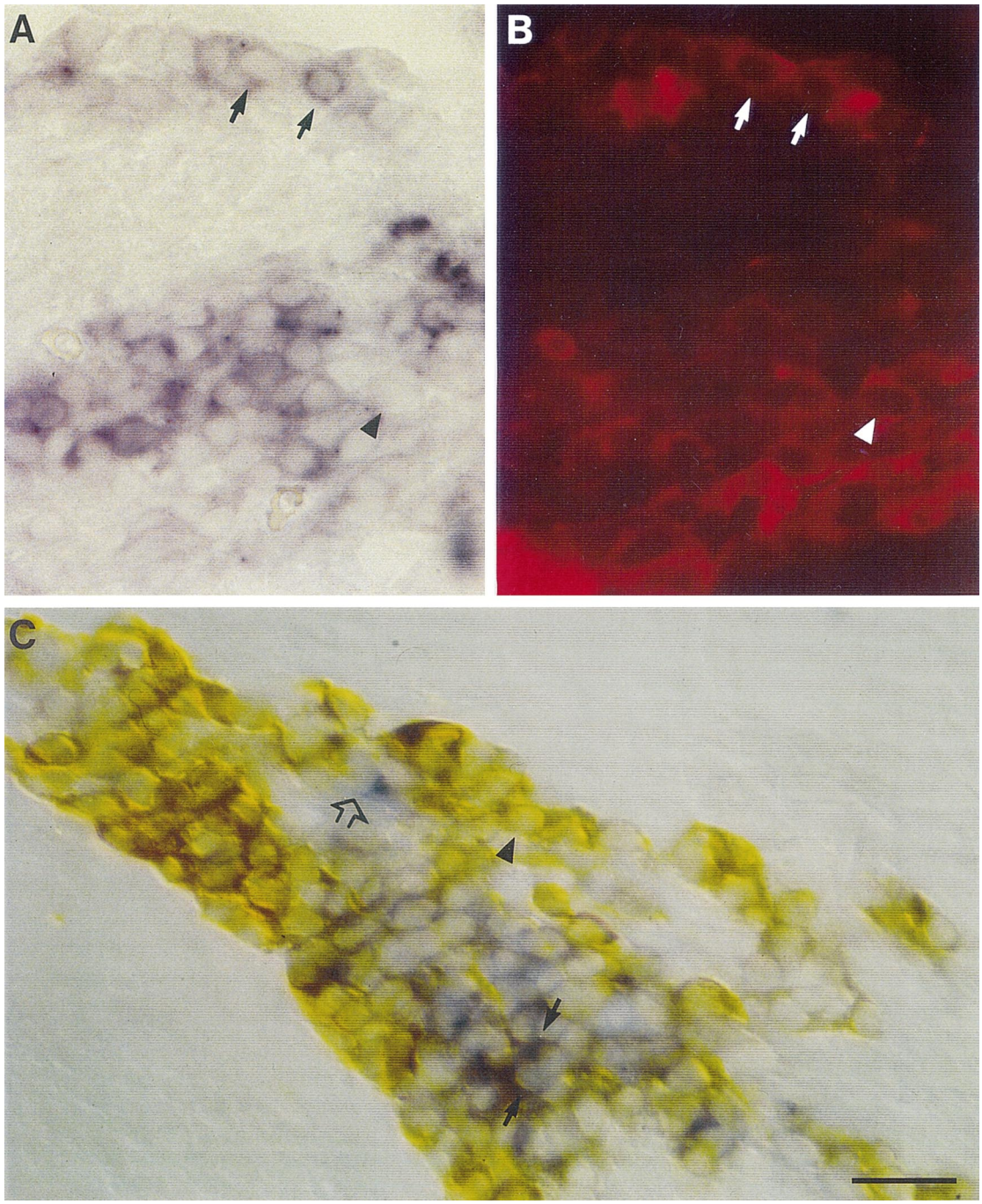


Fig. 3. ChAT and TH are co-expressed in some neurons in sympathetic ganglia of 6.5 day old (stage 29/30) embryos. (A,B) In situ hybridization for ChAT mRNA with alkaline phosphatase and NBT/BCIP substrate yielding blue signal (A). Immunohistochemistry with CY3-labeled streptavidin for TH protein yielding red fluorescence (B). Arrows indicate cells co-expressing ChAT and TH. Cells expressing only TH are indicated by triangles. The area shown was selected for a high proportion of double-positive cells. (C) In situ hybridization for ChAT mRNA with alkaline phosphatase and NBT/BCIP substrate yielding blue signal. Immunohistochemistry for TH with peroxidase and DAB substrate yielding brown signal. Blue precipitate (open arrow) indicates cells expressing preferentially or only ChAT, whereas brown precipitate (triangle) indicates cells expressing TH. Mixed precipitates (arrows) indicate cells expressing ChAT and TH. Scale bar, (A,B) 15 $\mu$m; (C) 20 $\mu$m.

6A). Very similar to the increase in the percentage of ChAT mRNA expressing neurons to a 2.3-fold level of that in control cultures, specific ChAT activity was increased by CNTF to a 2.4-fold level of that in control cultures (Table 1). It should be noted that specific ChAT activity after 4 days in control culture conditions is similar to that in freshly isolated ganglia (Table 1) which differs from the results with E7 neurons. Whereas ChAT did increase in response to CNTF, the percentage of TH mRNA positive neurons was significantly reduced by CNTF (Fig. 6B) from 7047 ± 1847 to 3974 ± 1738 TH mRNA positive neurons (mean ± SD; $n = 4$). In E12 sympathetic neuron cultures the CNTF-

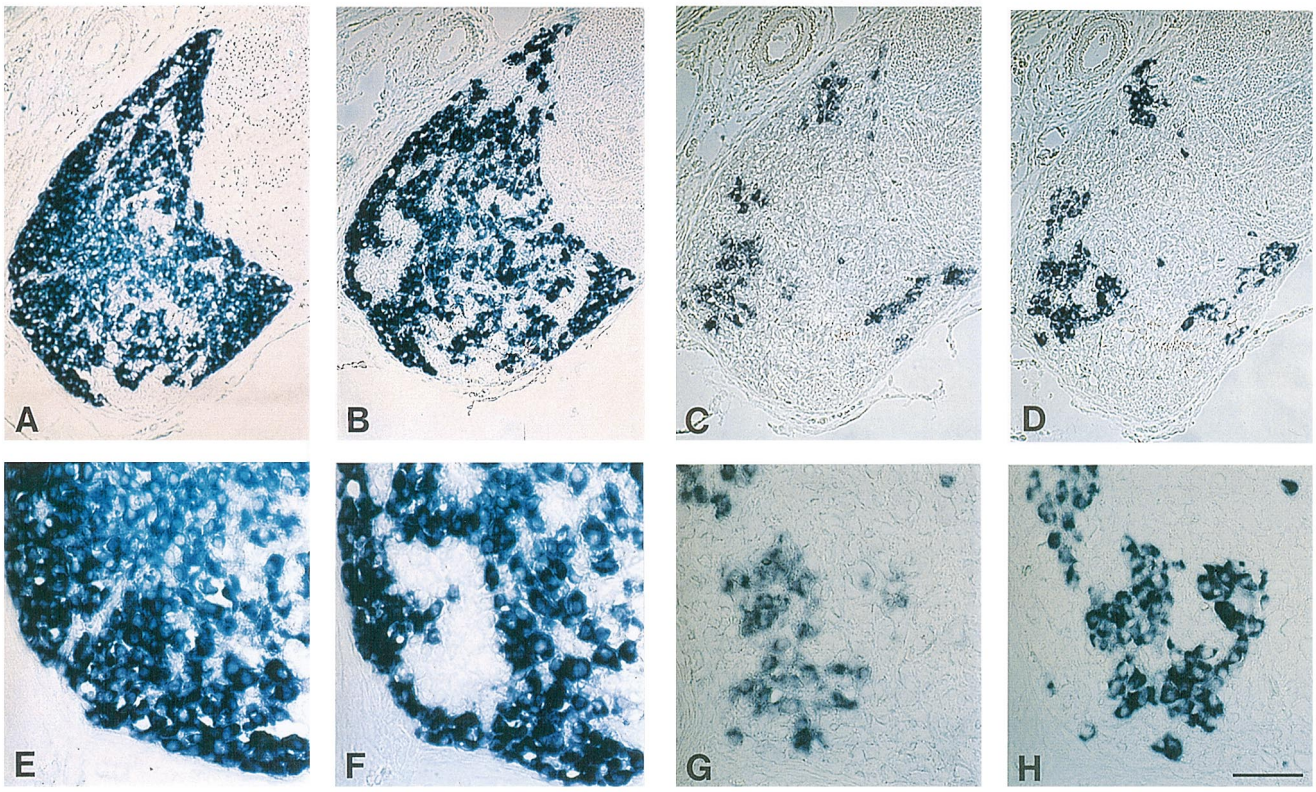


Fig. 4. A population of neurons in paravertebral sympathetic ganglia of 18 day old chick embryos expresses ChAT and VIP and is distinct from TH expressing cells. Non-radioactive in situ hybridization was performed on tissue sections from the thoracic region of stage 44 chick embryos to demonstrate mRNA for SCG 10 (A,E), TH (B,F), ChAT (C,G) and VIP (D,H). Hybridizations were performed on adjacent sections ((A–D) showing consecutive sections in this sequence) such that staining patterns are comparable. Pictures are taken from the region of the sympathetic ganglion with the segmental artery on top of the ganglion and part of the spinal nerve to the upper right. Enlargements of (A–D) are shown in (E–H), respectively. They show a region of the left part of the ganglion with a group of SCG 10-positive, TH-negative cells that express ChAT and VIP mRNA. Scale bar, (A–D) 100 $\mu$m; (E–H) 50 $\mu$m.

induced increase in VIP-IR positive neurons was very similar to the results obtained with E7 neurons (Fig. 6C).

The experiments show that CNTF induces ChAT mRNA expression as well as ChAT activity and reduces TH mRNA expression in E12, but not E7 sympathetic neurons. The higher number of total E12 neurons in CNTF-treated cultures as compared to control cultures cannot account for the increase in ChAT mRNA positive cells, as the changes in transmitter phenotype markers are significantly greater than the change in total cell number (6027 ± 1117 additional ChAT-positive cells as compared to 3504 ± 375 more total cells; results are mean ± SD; $n = 4$; $t$-test; $P < 0.02$). As effects on ChAT activity are paralleled by the effects on the proportion of ChAT mRNA positive cells and cannot be explained by changes in cell number, we conclude that the CNTF-induced increase in ChAT activity is mediated at the transcript level. The results clearly demonstrate that ChAT expression is differentially regulated in cells derived from sympathetic ganglia of different developmental stages. Further evidence for differences in ChAT regulation during sympathetic neuron development comes from the comparison of ChAT activity in cultured neurons and freshly isolated ganglia. A striking increase in ChAT activity relative to levels in freshly isolated ganglia is observed in dissociated cultures of E7 but not E12 sympathetic neurons when cells are grown with elevated potassium concentrations in the absence of CNTF (Table 1).

Table 1
Effect of CNTF on specific ChAT activity in E7 and E12 neuron cultures

| Embryonic age | Culture conditions | Specific ChAT activity (fmol ACh/min per mg protein) | Specific activity levels in CNTF-treated relative to control cultures |
|---|---|---|---|
| E7 | NGF | 834 ± 354 ($n = 3$) | |
| | NGF + CNTF | 929 ± 380 ($n = 3$) | 1.1 ± 0.07 |
| E12 | NGF | 127 ± 31 ($n = 3$) | |
| | NGF + CNTF | 304 ± 126 ($n = 3$) | 2.4 ± 0.38* |

Cultures were maintained in the presence of elevated potassium and the factors indicated for 4 days and then analyzed for ChAT activity and protein content. CNTF-treated and control cultures were compared with the paired, two-tailed $t$-test. The asterisk indicates a significant difference (*$P < 0.05$). Specific activity levels were also determined in freshly isolated paravertebral sympathetic ganglia from different embryonic stages: 158 ± 15 fmol ACh/min per mg protein (mean ± SEM; $n = 5$) at E7–8 and 182 ± 54 fmol ACh/min per mg protein (mean ± SEM; $n = 4$) at E10–12.

## 3. Discussion

Here we document the development of a cholinergic neu-

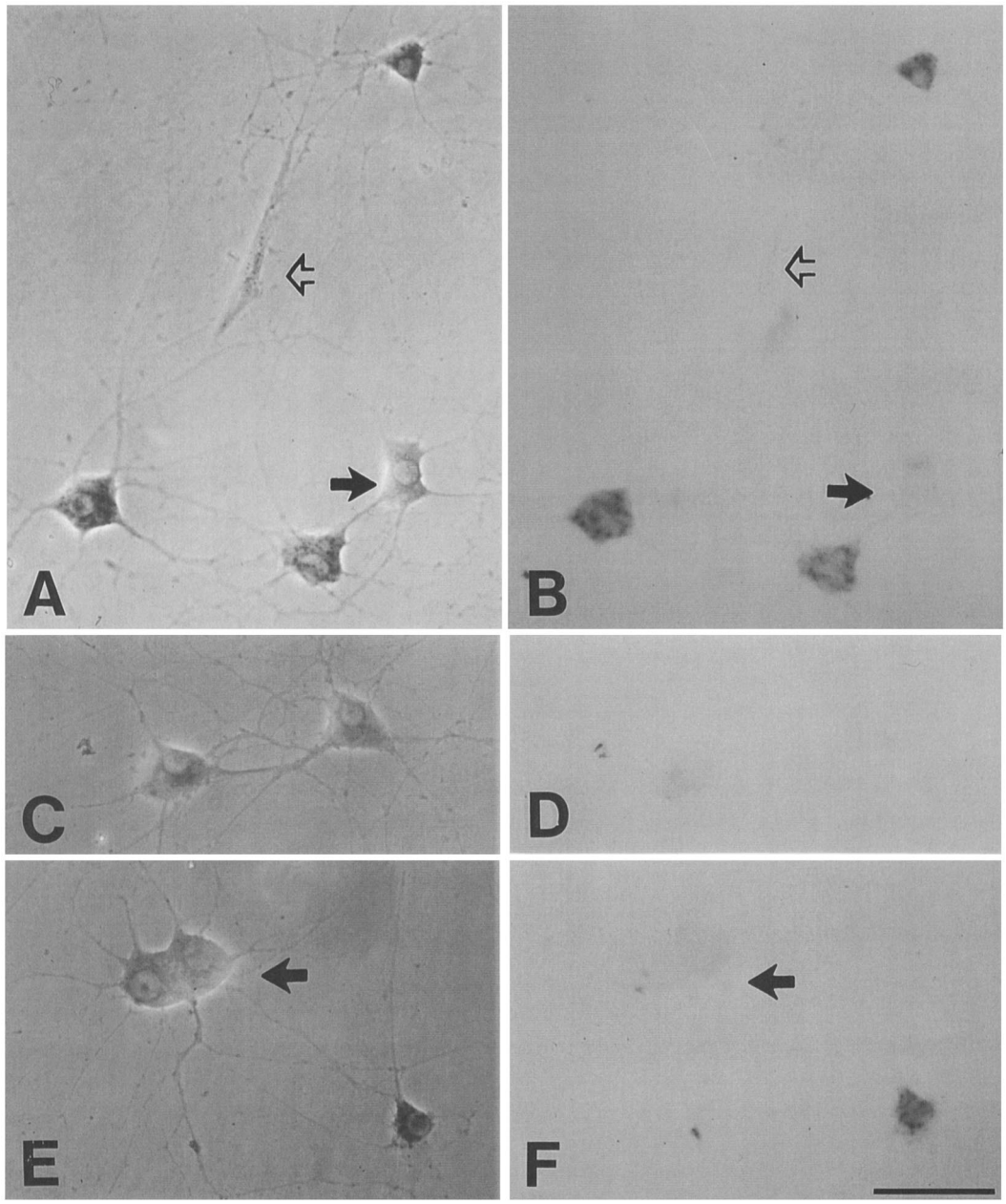


Fig. 5. CNTF induces expression of ChAT mRNA in sympathetic neurons from 12 day old chick embryos in culture. ChAT mRNA is detected by non-radioactive in situ hybridization in sympathetic neurons cultured 4 days in NGF-supplemented cultures with increased potassium concentrations in the presence (A,B) or absence (E,F) of CNTF. Cells are shown with phase contrast optics (A,E) and ChAT mRNA is detected by blue reaction product in bright field illumination (B,F). No signal is detected with a sense riboprobe for ChAT (C,D). Neurons negative for ChAT mRNA are indicated by filled arrows, a negative non-neuronal cell is indicated by an open arrow. Scale bar, 45 $\mu$m.

ronal subpopulation in paravertebral sympathetic ganglia of chick embryos. ChAT mRNA is first detectable in sympathetic ganglia of E6 embryos and at this stage some ChAT mRNA positive cells are also expressing TH. Using anterograde labeling with fluorescent dyes, sympathetic neurites have been observed to reach parts of the abdominal aorta at E8 (Devay et al., 1994). By labeling with fluorescent dyes or formaldehyde-induced catecholamine histofluorescence, the sympathetic innervation of a number of target tissues in the chick embryo has been shown to start from E9 onwards (Enemar et al., 1965; Pappano, 1977; Kirby et al., 1978; Rush et al., 1986; Devay et al., 1994; U. Zechbauer and H. Rohrer, unpublished results). Thus, our data indicate that the cholinergic marker enzyme ChAT is expressed several days before the bulk of sympathetic neurons establishes contact with their target tissues. VIP mRNA and protein are first detectable at E10 when some sympathetic neurons have established contact with their target tissues. In older embryos, the cholinergic cells are characterized by the expression of ChAT and VIP, whereas TH is expressed in a separate subpopulation of noradrenergic sympathetic neurons. Thus, during a second differentiation step that occurs

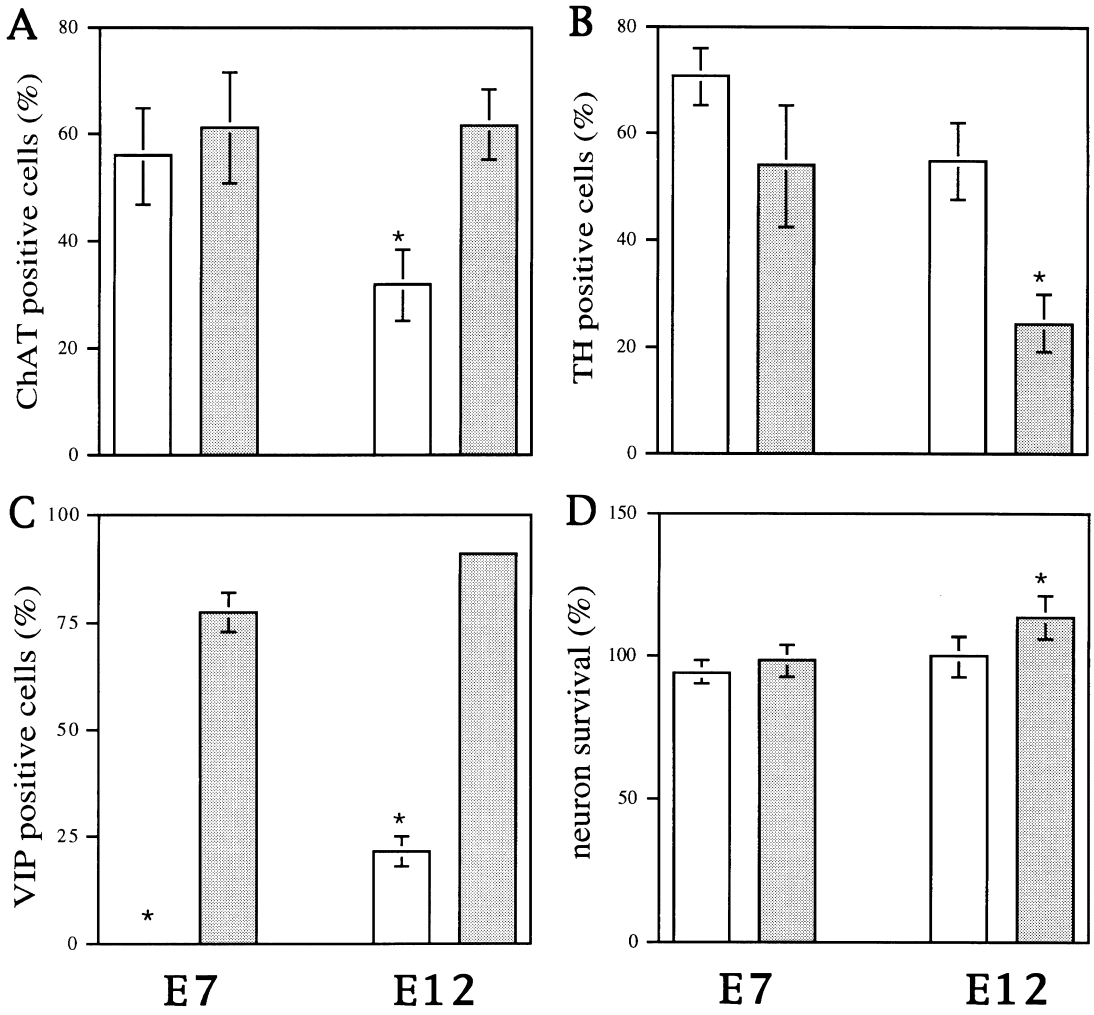


Fig. 6. CNTF affects the proportion of ChAT and TH mRNA expressing sympathetic neurons at E12 but not E7. Sympathetic neurons were cultured from lumbosacral ganglia of 7 and 12 day old chick embryos in the absence (white bars) or presence (grey bars) of CNTF. After 4 days in culture, in situ hybridization for ChAT (A) and TH (B) mRNA and immunohistochemistry for VIP (C) was performed and numbers of positive cells were determined. Results are expressed as percent positive neurons (means from three or more independent experiments, ± SEM). At E7 in the absence of CNTF no VIP-IR cells were detected (C). Error bars for E12 neurons in the presence of CNTF were so small that they do not show up in (C). In addition, neuron numbers were determined after 4 days and expressed in percent of the number of cells on the first day (D) to show that selective survival does not account for observed changes. Statistical differences were calculated with the paired, two-tailed *t*-test. The asterisk indicates significant differences ($*P < 0.05$). Data represent the mean ± SEM of at least three independent experiments.

during the time period when sympathetic neurons establish target contact, VIP expression is induced. The stage-dependent differentiation of cholinergic neurons is also reflected in culture where sympathetic neurons from different developmental stages display different regulation of ChAT and TH expression in response to CNTF. We also observed that during early development, ChAT and VIP are not co-ordinately expressed in response to CNTF. In E7 sympathetic neurons, VIP expression is induced whereas ChAT mRNA expression and enzyme activity are not affected.

ChAT activity has previously been observed in E6 chick sympathetic ganglia (Marchisio and Consolo, 1968). Although a preganglionic origin of the activity could not formally be excluded, the results argued for ChAT expression in postganglionic sympathetic neurons (Hruschak et al., 1982). The present data confirm and extend these findings by demonstrating that ChAT mRNA is present from E6 onwards in chick sympathetic ganglia. The data obtained in the chick embryo show interesting similarities to results from the analysis of mammalian cholinergic sympathetic differentiation. In the mouse embryo, ChAT activity is detectable in the superior cervical ganglia at E13 (Coughlin et al., 1978), shortly after ganglion formation (Coughlin et al., 1977; Morin et al., 1997) when, by analogy with the rat embryo, sympathetic fibers are not expected to have innervated peripheral targets (Ernsberger and Rohrer, 1994). In the rat embryo, low levels of ChAT mRNA are present in a sympathoadrenal precursor cell isolated from E14.5 adrenal glands (Vandenbergh et al., 1991). In the rat stellate ganglion, which contains the neuron population innervating the sweat glands of the forepaw, both genes of the cholinergic locus, ChAT and VAChT were recently found to be

expressed before the onset of target innervation (Schäfer et al., 1997).

VIP is a neuropeptide which in the mammalian sympathetic nervous system is associated with cholinergic neurotransmitter phenotype (Lindh and Hökfelt, 1990). Here, we demonstrate that in the sympathetic nervous system of chick a similar situation exists but that the timing of ChAT and VIP expression during development differs between chick and rat. VIP mRNA was first detected at E10 in chick, several days after the onset of ChAT expression in the ganglion. The same result has been obtained by immunohistochemistry (New and Mudge, 1986; U. Zechbauer and H. Rohrer, unpublished results) and radioimmunoassay (Hayashi et al., 1983). Since at E10 sympathetic fibers are found in the target tissues, these results are consistent with the interpretation that VIP expression depends on target innervation by sympathetic fibers. In contrast, in the rat stellate ganglion both VIP mRNA and protein are expressed before target innervation (Tyrrell and Landis, 1994). However, the analysis of VIP-IR in the developing sympathetic axon terminals of rat sweat glands also implied an important role of the target in the expression of VIP (Landis et al., 1988).

During development of the sympathetic innervation of the rat footpad, sweat glands first receive catecholaminergic sympathetic innervation which under the influence of the target tissue changes such that cholinergic biochemical and functional properties are expressed (Landis, 1990). During this shift in neurotransmitter properties, ChAT activity, ChAT-IR, VIP-IR and acetylcholine esterase activity are induced at a similar time point after the sympathetic nerve fibers have established contact with the developing gland tissue in vivo (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis et al., 1988). From these results it was concluded that cholinergic characters including ChAT and VIP are only induced upon target contact of this sympathetic neuron subpopulation. The demonstration of ChAT and VAChT expression in rat sympathetic ganglia and sweat glands before target contact (Schäfer et al., 1997), taken together with the previously described embryonic expression of VIP in rat sympathetic ganglia (Tyrrell and Landis, 1994) and the present data on ChAT expression in chick sympathetic ganglia, suggest a more complex scheme of cholinergic differentiation, involving target-independent onset of expression of cholinergic properties that may be modulated by target contact.

The analysis of the in vivo expression of ChAT and VIP suggests two differentiation steps in the development of cholinergic sympathetic neurons, an early one occurring during formation of the paravertebral ganglia, and a later one after target contact, that may be controlled by two different extrinsic signals. In the chick there is evidence that the early step involves the expression of ChAT, whereas VIP expression is induced during the second differentiation stage. An alternative to the two-step differentiation model would be that ChAT and VIP expression represent distinct stages of cholinergic differentiation that proceeds cell autonomously after its initiation during early development. Importantly, ChAT as well as TH mRNA expression in cultures of sympathetic neurons did exhibit differential neurokine-mediated regulation, depending on the developmental stage of the neurons. In E12, but not E7 sympathetic neuron cultures, the cholinergic differentiation factor CNTF increased the proportion of neurons expressing ChAT mRNA and decreased the proportion of TH mRNA expressing cells. Similar results were obtained when ChAT enzyme activity was determined. Although it is not clear to which extent the differences in the control of ChAT and TH mRNA expression between E7 and E12 neurons in vitro reflect the in vivo situation it can be concluded that the cells isolated from E7 and E12 embryos display different properties with respect to neurokine sensitivity and regulation of transmitter synthesizing enzymes. The absence of CNTF effects on ChAT and TH expression in E7 neurons is not due to the absence of CNTF receptor signaling as VIP mRNA and protein are strongly upregulated in response to CNTF in these cells. The lack of VIP expression in sympathetic ganglia before E10, although the cells respond to CNTF at E7 by VIP expression in vitro (Ernsberger et al., 1989b; the present study) and in vivo (Heller, unpublished results), may be taken as an indication that E7 sympathetic neurons in vivo do not encounter significant amounts of CNTFR$\alpha$ ligand. The notion that early cholinergic differentiation does not involve CNTFR$\alpha$ signaling is also supported by the lack of CNTF effects on ChAT expression at E7. Interestingly, we observe a differential response of VIP and ChAT genes to cytokine signaling in E7 chick sympathetic neurons. Previous studies using cultures of postnatal rat sympathetic neurons observed a coordinate induction of both genes (Nawa and Patterson, 1990; Rao et al., 1992) that we observe with more mature neurons (E12).

The data obtained from sympathetic neurons in the chick embryo indicate that the neuron population also in relatively young sympathetic ganglia may not be homogeneous with respect to neurotransmitter phenotype. In situ hybridization in combination with protein immunohistochemistry showed co-expression of ChAT and TH in some cells as well as expression of either ChAT or TH in others. It should be mentioned in this context that previous in vitro studies also suggested the presence of subpopulations with different transmitter phenotype in chick sympathetic ganglia (Edgar et al., 1981; Rohrer et al., 1983). This heterogeneity may reflect cells in distinct stages of neuronal differentiation, proceeding over a large period of embryonic development. Alternatively it may reflect different cell populations. The latter interpretation provokes the question of whether ChAT expressing neurons in early ganglia are biased to innervate targets that receive functional cholinergic input in the mature animal. The findings that sweat gland innervation, but not sympathetic fibers to blood vessels in rat sweat gland displays a distinct expression pattern of neurotransmitter transporters (Schäfer et al., 1997) would support this possibility. On the other hand, most if not all rat and chick

sympathetic neurons have the potential for cholinergic differentiation as demonstrated by the effects of cholinergic differentiation factors on VIP and ChAT expression (Patterson, 1978; Ernsberger et al., 1989b; the present study). Furthermore a change of sympathetic innervation from noradrenergic to cholinergic has been described in vivo. Transplantation of sweat gland-containing footpad skin in neonatal rats to areas of hairy skin, normally innervated by noradrenergic sympathetic fibers, resulted in innervation of the food pad graft by catecholaminergic fibers starting after 2 weeks (Schotzinger and Landis, 1988). After the third week, catecholamine histofluorescence started to decrease while ChAT activity and acetylcholinesterase (AChE) staining strongly increased in the transplanted footpad tissue. These results demonstrate that catecholaminergic sympathetic neurons have the potential for cholinergic differentiation. In addition, they allow for the possibility that a population of young neurons which is homogeneous with respect to the transmitter properties yields distinct neuronal populations differing in their transmitter phenotype due to the inductive action of certain target tissues. Thus, evidence exists for the early existence of biased sympathetic subpopulations as well as for the plasticity of sympathetic neurons that allows for the respecification of the neurotransmitter phenotype. The degree to which target-independent differentiation, target-induced (re-) specification of transmitter pheoptype and selective survival contribute to the establishment of the final cell populations in sympathetic ganglia in vivo has still to be determined.

The demonstration of changes in responsiveness of sympathetic neurons to cholinergic differentiation factors with development, together with the sequential expression of ChAT and VIP, supports the notion that cholinergic differentiation may be controlled by distinct signals that lead to an early, target-independent ChAT expression and a later, target-dependent VIP induction. The present study implicates CNTFR$\alpha$-mediated signaling during late, but not early sympathetic neuron development. Work in progress is directed to delineate the physiological role of neuropoietic cytokines in cholinergic differentiation by interfering with cytokine receptor signaling.

## 4. Materials and methods

Staging, preparation and sectioning of embryos was performed as described (Ernsberger et al., 1995). Embryonic stages are given according to Hamburger and Hamilton (1951).

### *4.1. In situ hybridization on tissue sections*

Radioactive in situ hybridization was performed as described previously (Ernsberger et al., 1995). Non-radioactive in situ hybridisations were performed according to a modification of a protocol by D. Henrique (IRFDBU, Oxford, UK). Cryosections were brought to room temperature and hybridization buffer with DIG-labeled cRNA treated for 10 min at 70°C was added. Hybridization was performed overnight at 65°C in a buffer containing 50% formamide (v/v), 10% dextrane sulfate (w/v), 1 mg/ml yeast RNA, 1× Denhardts, 5 mM EDTA, 12.6 mM Tris, pH 7.5, 185 mM NaCl, 5 mM $Na_2HPO_4$, 0.5 mM $NaH_2PO_4$. On the following day, slides were washed 2 × 30 min at 65°C in 1× SSC, 50% formamid, 0.1% Tween 20, and 2 × 30 min at room temperature in 100 mM maleic acid, 150 mM NaCl at pH 7.5, supplemented with 0.1% Tween 20 (MABT). Sections were blocked with MABT supplemented with 20% heat-inactivated (10 min, 70°C) chick serum (MABT/ChS) before incubating in anti-DIG alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) in MABT/ChS (1:500–1:3000) at room temperature overnight. On the following day, slides were washed 3 × 20 min in MABT and 2 × 10 min in alkaline phosphatase buffer containing 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.24 mg/ml levamisole, 0.1% Tween 20. The color reaction was started by adding NBT + BCIP (DIG-detection kit) (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. The reaction was performed in the dark under coverslips at room temperature for several hours to days and stopped by washing in phosphate-buffered saline (PBS). After washing, sections were mounted in Kaiser's Glyceringelatine (Merck, Darmstadt, Germany).

Riboprobes were synthesized from linearised plasmids with the DIG RNA labeling kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. cRNA probes were separated from unincorporated nucleotides with G50 quick spin columns (Boehringer, Mannheim, Germany). Chick tyrosine hydroxylase was available as full length cDNA (Ernsberger et al., 1995), a full length cDNA clone of chick ChAT was provided by Dr. Tom Jessell (Columbia University, New York, NY, USA) (Yamada et al., 1993), a prepro VIP cDNA from chick was provided by Dr. Peter Sharp (Roslin Institute, Midlothian, UK) (Talbot et al., 1995), a chick SCG 10 cDNA clone was provided by Dr. Peter Jeffrey (Children's Medical Research Institute, Wentworthville, Australia). Plasmid DNA was linearised at the cloning site and riboprobes were synthesized with the respective RNA polymerase. For radioactive in situ hybridization, cRNA probes were hydrolyzed to an average length of 0.25 kb. No hydrolysis was performed for DIG-labeled riboprobes since this led to decreased signal intensities. For control, sense probes were used and gave no hybridization signal.

### *4.2. Double-labeling protocol for mRNA in situ hybridization and protein immunohistochemistry*

Non-radioactive in situ hybridization using a DIG-labeled probe for ChAT was performed as outlined above. The color reaction was stopped by washing with PBS and

immunohistochemistry was performed on the same slides as described previously (Ernsberger et al., 1989b, 1995). It should be pointed out that both double-staining protocols used have technical limitations that restrict their use to qualitative rather than quantitative analysis: (1) in the combination of horseradish peroxidase (HRP) immunohistochemistry and in situ hybridization, double-labeled cells may only be detected when the amount of precipitate from the HRP reaction is low to moderate and does not obscure the reaction product from the in situ hybridization; (2) when in situ hybridization is combined with immunofluorescence, double-labeled cells may display weak immunoreactivity as the in situ reaction product quenches the fluorescence signal.

### 4.3. In situ hybridization in cell culture

After fixation of cultures in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 15 min, cultures were washed in PBS, permeabilized in PBS containing 0.1% Tween 20 for 15 min, and washed with PBS. DIG-labeled riboprobe was applied in hybridization buffer as described above. The remaining steps were as described for in situ hybridization on tissue sections.

### 4.4. ChAT assay

ChAT enzyme activity was assayed as described in detail previously (Saadat et al., 1989). Frozen cells were suspended in 100 $\mu$l 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 min at 10 000 $\times$ $g$). Fifteen to 25 $\mu$l of the supernatant was used for ChAT enzyme activity assays (Fonnum, 1969; Raynaud et al., 1987) and protein determination (Bradford, 1976) using ovalbumin as standard. The sensitivity of the enzyme assay was increased using subsaturating concentrations of acetyl-CoA as described previously (Raynaud et al., 1987; Saadat et al., 1989).

### 4.5. Cell culture

Chick sympathetic neurons were isolated from lumbosacral sympathetic chain ganglia of E7 and E12 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 essentially as described previously (Ernsberger et al., 1989a,b). For survival counts and in situ hybridization, dissociated cells from E12 ganglia were preplated on petri dishes (10 cm diameter; Falcon) in F14 supplemented with 10% horse serum to enrich for neurons. After 2–3 h non-adherent cells were harvested, pelleted by centrifugation, redissociated and plated in culture dishes (35 mm diameter; Greiner) on polyornithin-laminin. Cells were cultivated in the presence of nerve growth factor (NGF; 10 ng/ml) or CNTF (1 ng/ml) or both as indicated. To reduce cell proliferation at E7 and to support neuron survival, the potassium concentration was elevated to 35 mM by the addition of 3 M KCl. In E7 cultures, the proportion of [$^3$H]thymidine-labeled cells was reduced from 57 ± 8% (mean ± SEM; $n$ = 3) during the first day in regular $K^+$ concentration (5 mM) to 16 ± 1% (mean ± SEM; $n$ = 6) in medium with an increased $K^+$ concentration of 35 mM and from 32 ± 12% (mean ± SEM; $n$ = 3) during the second day in regular $K^+$ concentration (5 mM) to 5 ± 3% (mean ± SEM; $n$ = 6) in medium with an increased $K^+$ concentration of 35 mM.

Modifications for cultures used for ChAT assays include the use of larger culture dishes (6 cm diameter; NUNC), coating with a lower laminin-concentration overnight (4 $\mu$g/ml; 4 ml per dish). Cells were cultivated for 4 days with a change of the medium after 2 days. Cells were harvested in PBS after repeated washings of the cultures with PBS.

Tritiated thymidin incorporation was performed as described (Ernsberger et al., 1989b).

## Acknowledgements

We thank C. Thum and C. Krieger for excellent technical assistance and V. O'Connor for critical reading of the manuscript. We also thank T. Jessell, P. Sharp, and P. Jeffrey for ChAT, VIP and SCG 10 cDNA probes. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 269) and the Fonds der Chemischen Industrie.

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