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Fibroblast Growth Factor Differentially Modulates the Neurotransmitter Phenotype of Cultured Sympathetic Neurons

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Fibroblast growth factors (FGFs) are multifunctional growth factors that increase the proliferation of mesoderm- and neuroectoderm-derived cells and promote neuronal survival and neurite outgrowth in various regions of the brain, yet the physiological role(s) they may play in nervous system function and/or development is unclear. The present report demonstrates, using a well-characterized system, avian sympathetic neurons *in vitro*, that acidic and basic FGFs increase ChAT but decrease tyrosine hydroxylase (TH) activity in these cells, without affecting neuronal growth and survival. Heparin, which binds to FGFs with a high affinity, potentiates the activity of FGF on ChAT, but not TH. The time course of FGF action on the neurotransmitter phenotype is slow since effects start to appear after 1–2 d only. FGFs may thus modulate the activities of ChAT and TH by differentially regulating the expression of the genes coding for these enzymes. In conclusion, this report provides evidence supporting the hypothesis that FGFs may play a role in regulating neurotransmitter expression in sympathetic neurons during development independently of any effect on neuronal survival.

Neuronal survival, growth, and differentiation are influenced by various environmental signals during neuronal development. Modulation of neuronal differentiation, that is, changes in neurotransmitter expression, is of particular interest since neurotransmitters are the agents responsible for neuronal communication (for reviews, see Patterson, 1978; Black et al., 1987; Landis, 1990; Zurn, 1991). Several polypeptide growth factors have been shown to be capable of influencing neuronal survival and differentiation. NGF is the first and best-characterized factor. It promotes the survival, growth, and differentiation of several distinct populations of neurons in the PNS and CNS (for reviews, see Levi-Montalcini, 1987; Barde, 1989; Dreyfus, 1989). Two neurotrophic factors closely related to NGF, brain-derived neurotrophic factor and neurotrophin-3, have also recently been discovered and cloned (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990a). They differ from NGF both in tissue distribution of the messenger RNA, and in their neuronal specificity (Maisonpierre et al., 1990a,b; Hyman et al., 1991; Knüsel et al., 1991). Recently, it has been reported that fibroblast growth factors (FGFs), multifunctional and ubiquitous growth factors, may also act as neurotrophic factors, since they increase the survival and neurite outgrowth of neurons from multiple brain regions (Morrison et al., 1986; Unsicker et al., 1987; Walicke, 1988; Ferrari et al., 1989). They also promote cholinergic differentiation (and/or the survival of cholinergic neurons) in chick ciliary ganglion, rat spinal cord, and septal cholinergic neurons *in vitro* (Unsicker et al., 1987; Grothe et al., 1989; Knüsel et al., 1990). FGFs thus appear to act as multifunctional neurotrophic factors in the brain. FGF had originally been identified as an activity present in pituitary and brain that stimulates the proliferation of fibroblasts *in vitro* (reviewed by Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). The activity was later shown to be due to two proteins, one with an acidic pI (5.6), acidic FGF (aFGF), and a second, basic FGF (bFGF), with a basic pI (>9.0) (Esch et al., 1985). bFGF has been identified in many normal and malignant tissues, including the brain, whereas aFGF occurs predominantly in the brain, with a restricted distribution (Ernfors et al., 1990; Wilcox and Unnerstall, 1991).

The present report provides evidence that FGF can regulate the expression of the cholinergic and noradrenergic phenotype independently in sympathetic neurons *in vitro*, without affecting the survival of these cells. FGF may thus play a role in regulating neurotransmitter expression in the developing nervous system without having any effect on neuronal survival.

Materials and Methods

Cell cultures. Superior cervical ganglia (SCG) from 9-d-old chicken embryos were dissociated and cultured as described previously (Zurn and Mudry, 1986). Briefly, dissociated neurons were plated at a density of 100–120,000 cells per culture well (24-well dishes, Costar) in Eagle's minimal essential medium containing 1.5% (v/v) fetal calf serum, 1.5% horse serum, 10 ng/ml 2.5 S nerve growth factor (Sigma) (or as otherwise stated) and treated with 7×10^{-7} M AraA (9β-D-arabinofuranosyladenine) from culture day 1 to day 4 to remove non-neuronal cells (Zurn and Mudry, 1986). Basic FGF (bFGF; recombinant; Boehringer Mannheim) and acidic FGF (aFGF; bovine brain; Sigma and Boehringer Mannheim) were added to the culture medium starting day 3–6 and throughout the entire culture period. The media were changed every 2–3 d. After 8–12 d in culture, the neurons were counted (only phase-bright cells with one or more processes were counted), and the incorporation of ^{3}H-leucine into neuronal protein was measured as described previously (Zurn, 1987).

Analysis of the activities of ChAT and TH. Tyrosine hydroxylase (TH) activity was measured by following the formation of $^{14}CO_2$ from L-1-^{14}C-tyrosine (Amersham) in the presence of an excess hog kidney DOPA decarboxylase, as described by Swerts et al. (1983).

Choline acetyltransferase (ChAT) activity was determined by measuring the synthesis of ^{3}H-acetylcholine (AcCho) from ^{3}H-acetylcoenzyme A (AcCoA) (Amersham) according to the method of Fonnum (1975), with minor modifications (Erkman et al., 1989). Ninety-eight percent of the activity could be blocked in the presence of 10^{-4} M Br-AcCho, a specific inhibitor of ChAT. Results were usually calculated in cpm since nonsaturating concentrations of AcCoA were used.

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Table 1. Effect of FGF on neuronal growth, survival, and ChAT and TH activities

	% control	
	bFGF	aFGF
ChAT	539% ± 58 (15)	427% ± 32 (6)
TH	69% ± 6 (14)***	74% ± 13 (6)**
^{3}H-Leu/N	119% ± 2.4 (8)***	108% ± 7 (4)***
Survival	98% ± 5 (5)	102% ± 8 (4)

The data show ChAT and TH activities, incorporation of ^{3}H-leucine into neuronal protein (^{3}H-Leu/N), and number of neurons surviving (survival) in SCG neurons grown for 8 d in the presence of 10 ng/ml bFGF and 100 ng/ml aFGF, with no heparin present. The values are expressed as percentages of control cultures (100%) grown in the presence of NGF alone. The actual values after 8 d in culture in the presence of NGF alone are, for ChAT, 0.88 fmol of ^{3}H-AcCho/neuron/hr; TH, 231 fmol of $^{14}CO_2$/neuron/hr; ^{3}H-Leu/N, 3.8 fmol of ^{3}H-leucine/neuron/hr; survival, 49,700 neurons/well. Each value represents the mean of (n) independent experiments ± SEM.

*** $p < 0.001$.

** $p < 0.01$.

TH and ChAT activities were measured in the same culture wells, whereas the ^{3}H-leucine incorporation was analyzed in separate culture wells from the same experiment.

Graphs were drawn using a HARVARD GRAPHICS computer program. Statistical significance was evaluated by using the Student's t test.

Results

aFGF and bFGF increase the activity of ChAT, the enzyme responsible for the biosynthesis of AcCho from choline and AcCoA, four- to sixfold in cultured chick sympathetic neurons. In contrast, the activity of TH, the rate-limiting enzyme in the biosynthesis of catecholamines, is decreased to approximately 70% of the control values (Table 1). Similar results were obtained when FGF was added from culture day 3–10 or culture day 6–12. Sympathetic neurons grown for 10 d in the presence of NGF and 5 ng/ml bFGF produce 5.3 fmol of ^{3}H-AcCho/neuron/hr ($n = 3$) (specific activity measured by using saturating concentrations of AcCoA), and the specific activity of TH is of 162 fmol of $^{14}CO_2$/neuron/hr ($n = 3$). FGF has no effect on neuronal survival and only a small effect on neuronal growth (Table 1, Fig. 2*C*).

The concentration-dependent effects of aFGF and bFGF on the activities of ChAT and TH are shown in Figure 1. Half-maximal increases in ChAT activity are obtained at concentrations of 0.8 ng/ml bFGF (4–5 × 10^{-11} M) and 15 ng/ml aFGF (Sigma) (8–10 × 10^{-10} M). Note that the half-maximal concentration for an optimal increase in ChAT activity with aFGF from a different source (Boehringer) is higher (approximately 40 ng/ml; A. Zurn, unpublished observation; Fig. 3*C,D*). Because the effects of aFGF and bFGF are similar, some of the subsequent experiments were only done with bFGF.

bFGF also promotes cholinergic and depresses noradrenergic differentiation in sympathetic neurons grown in the absence of NGF (Fig. 2*A,B*). The effect on ChAT is larger in the absence (1378% increase) than in the presence (575% increase) of NGF, possibly because basal ChAT levels without NGF are 55% lower than with NGF (Fig. 2*A*). In contrast, there is a larger depression in the activity of TH by FGF with than without NGF. Note that basal TH levels in the absence of NGF correspond to 70% of the values with NGF (Fig. 2*B*). Neuronal survival with or without NGF and FGF is unchanged (Fig. 2*C*). Neuronal growth, however, is 62% lower in the absence of NGF, and FGF increases growth approximately threefold in neurons grown without NGF (Fig. 2*C*).

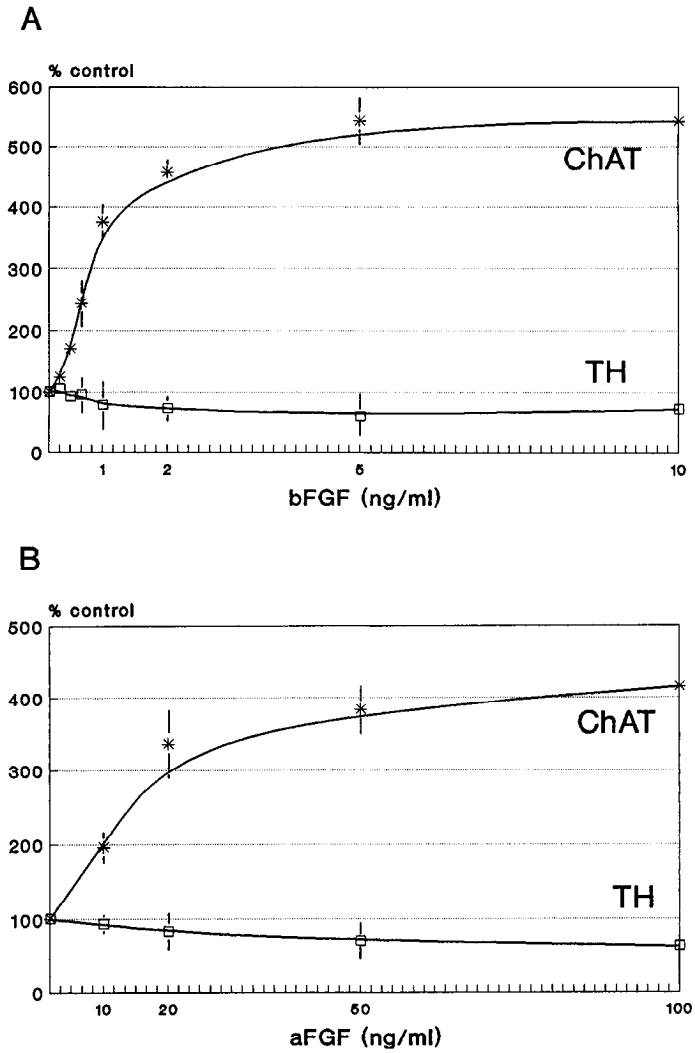

Figure 1. Dose-dependent effect of aFGF and bFGF on ChAT and TH activity. The SCG neurons were grown for 10 d in the presence of increasing concentrations of bFGF (*A*) and aFGF (*B*) (Sigma). FGF was added to the cultures starting on day 3. ChAT activity was determined by measuring the synthesis of ^{3}H-AcCho from ^{3}H-AcCoA. TH activity was measured by following the formation of $^{14}CO_2$ from L-1-^{14}C-tyrosine. The values are expressed as percentages of control cultures (100%) grown in the presence of NGF alone. The actual values for the ChAT and TH activities in sympathetic neurons grown for 10 d in the presence of NGF alone are 1.1 fmol of ^{3}H-AcCho/neuron/hr and 253 fmol of $^{14}CO_2$/neuron/hr, respectively. Each point represents the mean of four culture wells from two separate experiments, and the error bars indicate the SEM. TH activity: +10 ng/ml bFGF, $p < 0.001$; +100 ng/ml aFGF, $p < 0.001$.

Heparin (1 μg/ml) potentiates the effects of both bFGF (1.3–1.5-fold) and aFGF (1.5–2-fold) on the activities of ChAT, but not TH (Fig. 3). Heparin alone has little effect on ChAT activity, but it increases TH activity by approximately 150% (Fig. 3*A,B*). Concentrations higher than 1 μg/ml heparin could not be used since heparin decreases cell adhesion to the substratum.

bFGF increases ChAT and decreases TH activity to the same extent whether it is added to the cultures at the time of plating (day 0) or 4 d later (Fig. 4) (non-neuronal cells are present in these cultures up to day 3–4 only). Furthermore, addition of bFGF at the time of plating does not promote the incorporation

of ^{3}H-thymidine into cultured cells grown in the absence of AraA (Zurn, unpublished observation).

Vasoactive intestinal polypeptide (VIP) has recently been shown to promote ChAT activity in chick sympathetic neurons via a rise in the intracellular cAMP levels (Beretta and Zurn, 1991). FGF, however, does not alter the cAMP content of the sympathetic neurons (Zurn, unpublished observation). Yet, although the intracellular pathways involved in VIP and FGF action are distinct, their combined effects are additive at suboptimal (10^{-7} M and 0.5 ng/ml), but not at optimal (2×10^{-6} M and 5 ng/ml) chick VIP and FGF concentrations, respectively (Fig. 5).

The time course of the effect of bFGF is slow. Increases in the activity of ChAT start to be apparent 24–36 hr after its addition to the cultures (Fig. 6). The effect of bFGF on TH is more complex since an initial small and rapid increase in TH activity (1–8 hr) is followed by a decrease in activity starting 2 d later (Fig. 6).

Discussion

FGFs are multifunctional and ubiquitous growth factors that affect a wide variety of biological events including induction of mesoderm, angiogenesis, cell proliferation, neuronal survival, and neurite outgrowth (reviewed by Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). Using a well-characterized *in vitro* system, cultured avian sympathetic neurons (Zurn and Mudry, 1986), we demonstrate that aFGF and bFGF increase the activity of ChAT, the enzyme responsible for the biosynthesis of AcCho, and decrease the activity of TH, the rate-limiting enzyme in the biosynthesis of catecholamines, in these cells (Table 1, Fig. 1). Neuronal survival is unchanged under those conditions (Table 1, Fig. 2*C*). This is evidence that FGF can regulate the cholinergic and noradrenergic phenotype of chicken sympathetic neurons *in vitro* independently of any effect on neuronal survival. A specific increase in the activity of ChAT has also been observed with FGF in cultured chick ciliary ganglion neurons (Unsicker et al., 1987). However, increases in the cholinergic differentiation of cultured rat spinal cord and septal neurons probably reflect an action of FGF on neuronal survival rather than neuronal differentiation (Grothe et al., 1989; Knüsel et al., 1990).

The cholinergic properties of avian sympathetic neurons are also increased in the presence of the neuropeptide VIP (Beretta and Zurn, 1991). Note that, in contrast to FGF, VIP also increases the activity of TH in these cells. VIP acts via a rise in the intracellular cAMP levels, whereas FGF does not alter the cAMP content of the sympathetic neurons (Beretta and Zurn, 1991; Zurn, unpublished observation). Indeed, FGF action is probably mediated via distinct intracellular pathways, since FGF receptors belong to the class of protein-tyrosine kinase receptors (Coughlin et al., 1988; Presta et al., 1989; Ullrich and Schlessinger, 1990; Meldolesi and Magni, 1991). Nevertheless, the combined effects of optimal VIP and FGF concentrations on the activity of ChAT are not additive (Fig. 5). Additional control mechanisms such as product inhibition by AcCho may therefore additionally be involved in the regulation of ChAT activity in these cells (Salvaterra and Vaughn, 1989).

FGF action on the chick sympathetic neurons does not depend on the presence of NGF since basic FGF also promotes cholinergic and depresses noradrenergic differentiation in neurons cultured in the absence of NGF (Fig. 2). Note that the same number of neurons survive in the presence or the absence of NGF, as well as in the presence or the absence of FGF (Fig. 2*C*). This may be due to the fact that the sympathetic neurons are cultured on heart-conditioned medium–coated culture dishes, and in the presence of depolarizing medium (Ernsberger et al., 1989a; Bhave

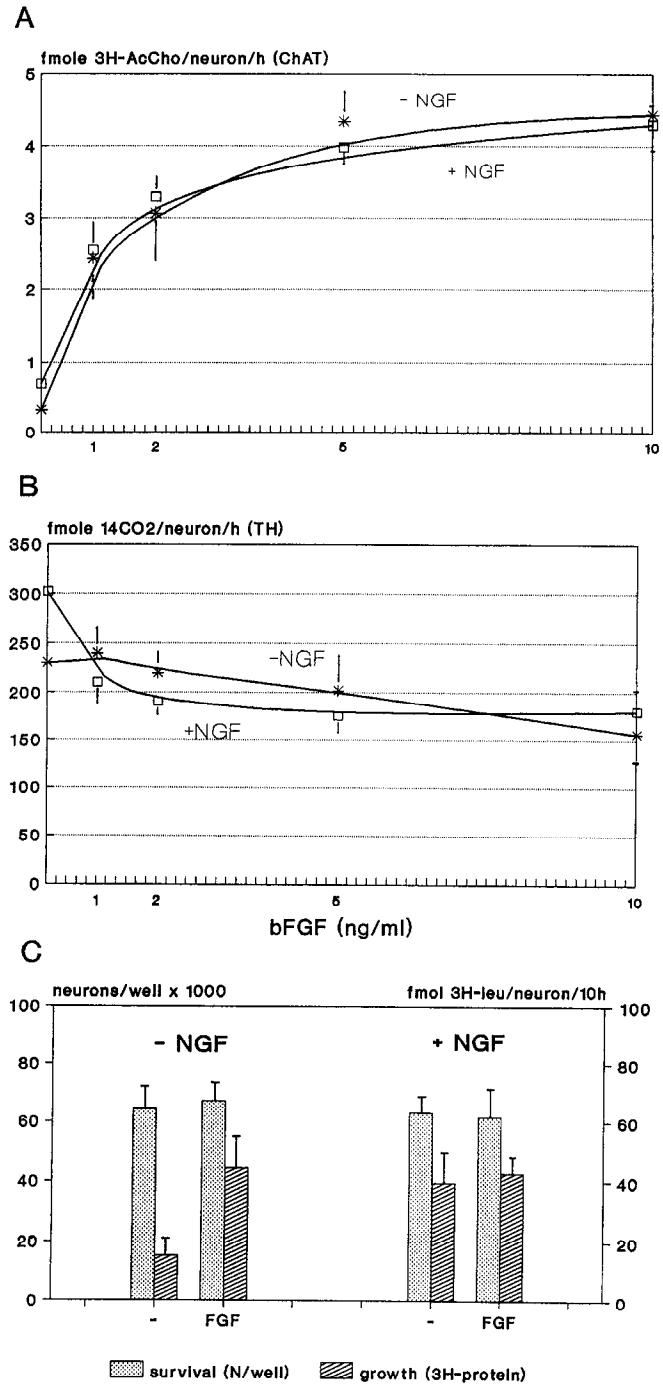

Figure 2. Dose-dependent effect of bFGF on the activities of ChAT (*A*), TH (*B*), and on neuronal growth and survival (*C*) in SCG neurons grown for 8 d in the presence (□) or the absence (*) of NGF. FGF is added to the cultures starting on day 0. The values are expressed as fmol of ^{3}H-AcCho/neuron/hr (ChAT, *A*), fmol of $^{14}CO_2$/neuron/hr (TH, *B*), number of neurons per well (survival, *C*), and fmol of ^{3}H-leucine incorporated per neuronal protein per 10 hr (growth, *C*). Each point represents the mean of four culture wells from two separate experiments, and the error bars indicate the SEM. TH activity, +10 ng/ml bFGF: +NGF, $p < 0.001$; −NGF, $p < 0.01$.

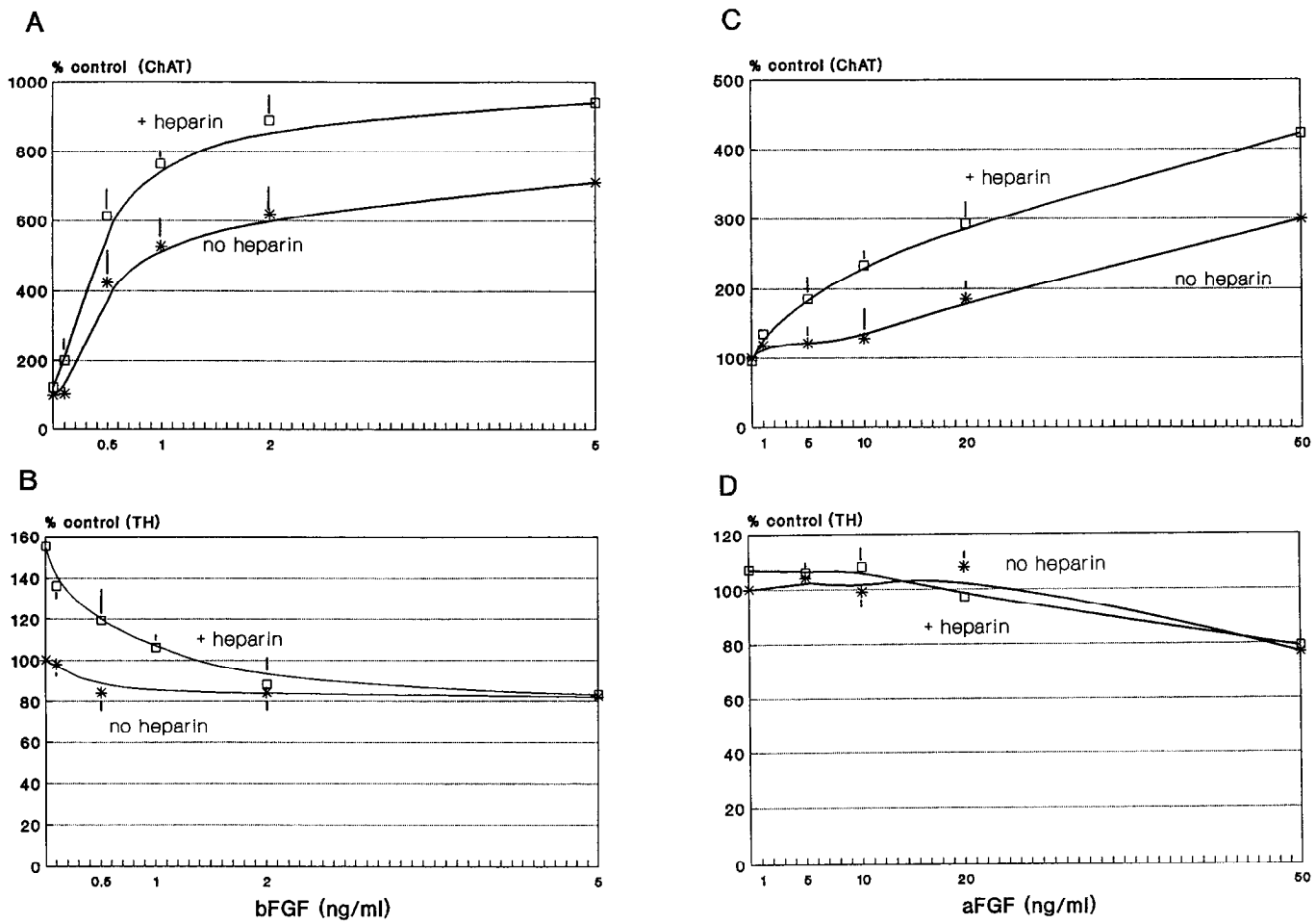

Figure 3. Dose-dependent effect of bFGF (*A, B*) (Sigma) and aFGF (*C, D*) (Boehringer) on the activities of ChAT (*A, C*) and TH (*B, D*) in SCG neurons grown for 10 d in the presence or the absence of heparin. □, +1 μg/ml heparin; *, no heparin. The values are expressed as percentages of control cultures (100%) grown in the presence of NGF alone. The actual values for the ChAT and TH activities in SCG neurons grown for 10 d in the presence of NGF alone and no heparin are 0.95 fmol of ^{3}H-AcCho/neuron/hr and 274 fmol of $^{14}CO_2$/neuron/hr, respectively. Each value represents the mean of three culture wells from the same experiment, and the error bars indicate the SEM. +2 ng/ml bFGF: ChAT activity, ±heparin, $p < 0.05$; TH activity, ±heparin, NS. +20 ng/ml aFGF: ChAT activity, ±heparin, $p < 0.05$; TH activity, ±heparin, $p < 0.05$. +50 ng/ml aFGF: TH activity, ±heparin, NS.

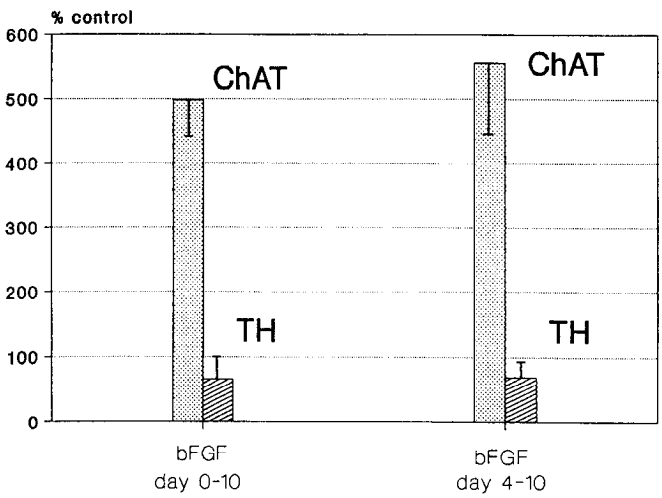

Figure 4. Effect of early and late addition of bFGF on the activities of ChAT and TH. The SCG neurons were grown in the presence of bFGF (5 ng/ml) starting culture day 0 (*left*) or day 4 (*right*). The values are expressed as indicated in Figure 3. The actual values for the ChAT and TH activities in SCG neurons grown for 11 d in the presence of NGF alone are 1.25 fmol of ^{3}H-AcCho/neuron/hr and 283 fmol of $^{14}CO_2$/neuron/hr, respectively. Each value represents the mean of six culture wells from two separate experiments, and the error bars indicate the SEM.

et al., 1990). In the absence of NGF and FGF, the values for ChAT and TH activity, and for ^{3}H-leucine incorporation, are 55%, 30%, and 62% lower than with NGF, respectively (Fig. 2). This probably explains why the effects of FGF on ChAT are larger (in percentage increase) without than with NGF (see Results).

bFGF modulates the ChAT and TH activity levels to the same extent whether it is added to the cultures at the time of plating or 4 d later (Fig. 5). We conclude that FGF does not rescue a possible subpopulation of cholinergic neuroblasts for survival, and that it acts directly on the sympathetic neurons (hardly any non-neuronal cells are left on culture day 4; see also Walicke and Baird, 1988). FGF does not induce cell proliferation since ^{3}H-thymidine incorporation is not increased by FGF in cultures grown in the absence of AraA (Zurn, unpublished

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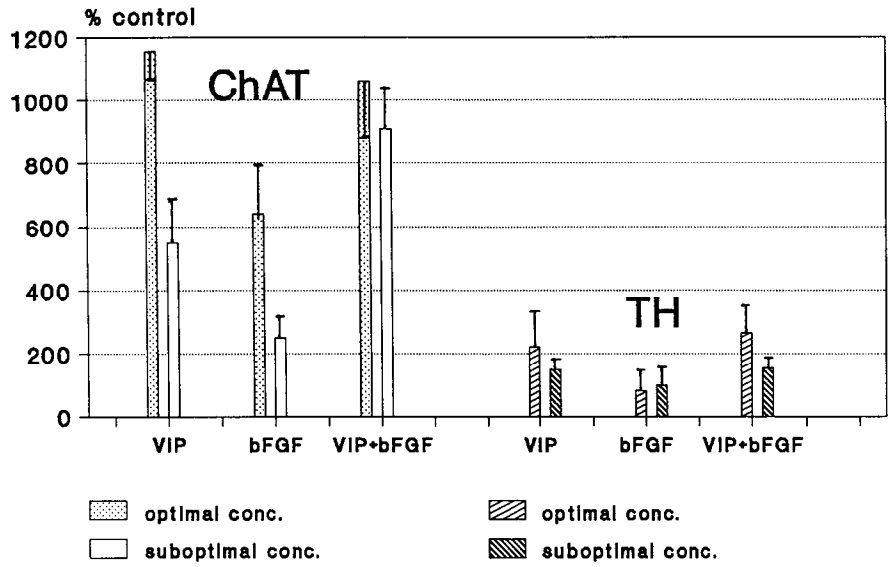

Figure 5. Combined effects of chicken VIP and bFGF on the activities of ChAT and TH. The SCG neurons were grown for 11 d in the presence or the absence of suboptimal concentrations of VIP (10^{-7} M) and bFGF (0.5 ng/ml), and optimal concentrations of VIP (2×10^{-6} M) and bFGF (10 ng/ml). The actual values for the ChAT and TH activities in SCG neurons grown for 11 d in the presence of NGF alone are 0.78 fmol of ^{3}H-AcCho/neuron/hr and 217 fmol of $^{14}CO_2$/neuron/hr, respectively. Each value represents the mean of six culture wells from three separate experiments, and the error bars indicate the SEM. Differences between VIP and VIP + FGF at optimal concentrations are not statistically significant.

observation). Similarly, FGF does not promote cell division in cultured avian sympathetic neuroblasts (Ernsberger et al., 1989b).

aFGF and bFGF appear to bind to high-affinity FGF receptors present on the sympathetic neurons since half-maximal increases in ChAT activity are obtained at concentrations of 0.5–1 ng/ml bFGF (4–5 $\times$ 10^{-11} M) and 15–30 ng/ml aFGF (6–9 $\times$ 10^{-10} M) (Fig. 1). High-affinity FGF receptors, as well as FGF receptor transcripts, have indeed been shown to be present in neuronal cells (Walicke et al., 1989; Heuer et al., 1990).

FGFs have a strong affinity for heparin, and several biological effects of FGF are potentiated by heparin (Neufeld et al., 1987; Ruoshlati and Yamaguchi, 1991). Yayon and colleagues have recently shown that heparin-like molecules are required for binding of bFGF to its high-affinity receptor (Yayon et al., 1991). Our results show that heparin also potentiates the effects of FGF on the activity of ChAT (Fig. 3), yet the potentiation by externally added heparin is relatively small. This may be due to the presence of proteoglycans on the neuronal cell surface and/or in the substrate-attached material (Matthew et al., 1985; Yayon et al., 1991). The decrease in the activity of TH by FGF, however, is not potentiated by heparin. The significance of this discrepancy remains to be elucidated. Note that heparin alone increases the activity of TH (Fig. 3*B*). This may be explained by the fact that heparin decreases cell adhesion to the substratum and therefore induces neurite fasciculation (Zurn, unpublished observation). We have indeed shown previously that cell–cell contact increases the activity of TH, but not ChAT, in these cells (Zurn and Mudry, 1986).

The time course of FGF action on the activity of ChAT is slow (Fig. 6). This speaks in favor of a regulation of ChAT gene expression. FGF action on the activity of TH, however, is more complex since an initial small increase is followed by a decrease in TH activity 2–3 d later. FGF may thus regulate TH by rapidly increasing its activity via phosphorylation, and by decreasing it subsequently via effects on TH mRNA levels (see also Vyas et al., 1990). Further experiments are required to clarify this point.

FGF has been shown to be localized to sympathetic neurons of the developing avian embryo *in vivo* and to neurons of the dorsal root ganglion *in vitro* (Kalcheim and Neufeld, 1990). FGF, as well as FGF receptor transcripts, increases from embryonic day 3.5 (E3.5) to E8, progressively decrease until hatching, and are very low in adult chicken sympathetic ganglia (Heuer et al., 1990; Kalcheim and Neufeld, 1990). The presence of both FGF and its receptor in developing neurons suggests that FGF may have an autocrine or paracrine effect during development. Alternatively, it is also possible that FGF is released via naturally occurring cell death. It has, for instance, been proposed that cholinergic neuroblasts present in avian sympathetic ganglia early during development progressively decrease until hatching (Hruschak et al., 1982). Taken together, these observations and our results suggest that FGF released via normally occurring cell death may be one of the factors responsible for inducing or maintaining cholinergic properties in sympathetic neuroblasts early during development. At the end of the period of neuronal cell death, however, sympathetic neurons may not be exposed to FGF any longer and, in the absence of any other

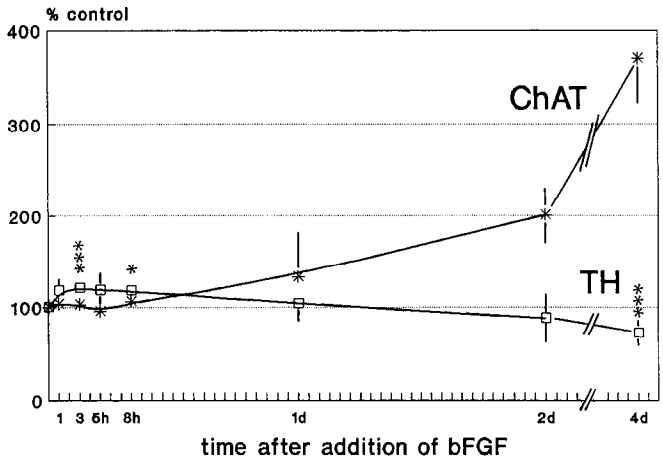

Figure 6. Time course of the effect of bFGF on the activities of ChAT and TH. The SCG neurons were grown in the presence or the absence of bFGF for 10 d. FGF was added to the cultures starting day 6. The values are expressed as percentages of control cultures (100%) grown in the presence of NGF alone. The actual values for the ChAT and TH activities in SCG neurons grown for 10 d in the presence of NGF alone are 1.4 fmol of ^{3}H-AcCho/neuron/hr and 298 fmol of $^{14}CO_2$/neuron/hr, respectively. Each point represents the mean of three culture wells from two separate experiments, and the error bars indicate the SEM. ***, $p < 0.001$; *, $p < 0.05$.

cholinergic differentiation factors, may therefore only produce catecholamines. In contrast, FGF receptors may be derepressed *in vitro* and externally added FGF may thus still be capable of increasing cholinergic properties in cultured sympathetic neurons.

The present report provides evidence, using a well-characterized *in vitro* system, that FGF is capable of specifically regulating neurotransmitter expression in developing sympathetic neurons *in vitro*, without affecting neuronal growth and survival. In addition to playing a role as a neuronal survival and proliferation factor in the nervous system, FGF may thus also participate in the modulation and/or induction of neurotransmitter expression during development.

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