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Schwann cell-derived factors support serotonergic neuron survival and promote neurite outgrowth

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SUMMARY

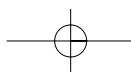
During embryogenesis and the postnatal period, neurons and glia interact in the development and differentiation of specific populations of nerve cells. Both in the peripheral (PNS) and in the central nervous system (CNS), glial cells have been shown in various experimental conditions to constitute a favorable substrate for neural adhesion, neural polarity, shape and axonal extension, while numerous soluble molecules secreted by neurons influence the survival and differentiation of the glial cells themselves. The aim of the present work was to investigate the influence of postnatal Schwann cells (SC) on embryonic serotonergic (5-HT) neurons of the raphe, in order to study the possible influence of the peripheral glia on the CNS neurons. Cultures of SC from sciatic nerve of postnatal rats and neurons from rat embryonic rhombencephalon were successfully established and cells were immunocytochemically characterized. The number of 5-HT neurons, and the number and length of their branches were quantified in the cultures of 5-HT neurons, in cultures added with Nerve Growth Factor (NGF) and Insulin-like Growth Factor I (IGF-I), in co-cultures with SC and in cultures added with conditioned medium obtained from SC cultures.

The results indicated that SC have the capacity to promote the survival and growth of 5-HT neurons in culture, and that this activity is mediated by soluble factors. Although the precise nature and mechanism of action of the growth factor or factors produced by SC in the presence of 5-HT neurons was not identified, our results add more data on the possible activity of the peripheral glia in promoting and enhancing the survival and outgrowth of the CNS neurons.

INTRODUCTION

During embryogenesis and the postnatal period neurons and glia in the central nervous system (CNS) and the peripheral nervous system (PNS) interact on the survival, maturation, migration and differentiation of specific neural and glia populations of cells. The possible mechanisms involved in the mutual relationship between neurons and glia have been extensively investigated. In the CNS, in fact, astrocytes have been shown to produce a wide variety of trophic factors affecting the development and survival of different neuronal cell types (Barde, 1989; Knusel *et al.*, 1990; Hyman *et al.*, 1991; Mayer *et al.*, 1993; Beck *et*

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al., 1993; Hyman *et al.*, 1994; Murphy *et al.*, 2000), the morphology of isolated neurons *in vitro* (Banker *et al.*, 1980; Muller and Seifert, 1982; Hatten *et al.*, 1988) and the function of neurons by regulating their size, synthesis of transmitter enzymes and neuritic outgrowth (Purves, 1988; Snider and Johnson, 1989; Franke *et al.*, 2000). Conversely, the reciprocal influence of neurons and glia was established by the demonstration that trophic factors produced by neurons significantly promote the proliferation, morphology, maturation and survival of astroglia and oligodendroglia cells (Hatten, 1985).

A close interaction between neuronal and glial cells also occurs in the PNS. Nerve growth factors support the survival of cultured sympathetic neurons (Barde *et al.*, 1982), and members of the neurotrophin gene family are expressed by developing sensory and motor neurons (Friedman *et al.*, 1991; Russel *et al.*, 2000). Schwann cells (SC) produce neurotrophic factors that activate specific cell surface receptors, which start a cascade of intracellular events modifying neuronal morphology, survival and/or functional capacity (Yuen *et al.*, 1996; Munson *et al.*, 1997).

The close interaction between nerve cells and glial cells is present during the adult lifetime (Mirsky *et al.*, 1999) and probably trophic substances are continuously produced acting as factors necessary for normal neuronal function and survival (Levi—Montalcini, 1987; Ruit and Snider, 1991; Longo *et al.*, 1992; Holtzman *et al.*, 1995; Friedman *et al.*, 1999). The amount of trophic factors are likely to increase during degenerative and especially regenerative processes of the nervous tissue. For instance, Nerve Growth Factor (NGF) mRNA and Brain-derived Neurotrophic Factor (BDNF) mRNA are upregulated after axotomy of the peripheral nerve at the lesion site and in the distal stump, at the same time when SC express on their surface a large amount of the p75 low affinity receptor for neurotrophins. Considering the reparative capacities of the PNS, and the great abundance of growth factors produced by SC (Frostick *et al.*, 1998), it has been suggested that SC might influence repair and regeneration also in the CNS (Xu *et al.*, 1995).

Studies by Collier *et al.* (1993, 1999) have shown that SC are able to secrete a diffusible molecule or a combination of molecules that influence the sur-

vival and axonal outgrowth of embryonic dopaminergic neurons. These data have induced us to verify if postnatal SC have the capacity to influence in the same way the survival and the neuronal branching of other central monoamine neurons. In CNS, monoamine neurons are distributed in different areas, such as the locus coeruleus (containing norepinephrine), the substantia nigra (containing dopamine) and the raphe nuclei (containing serotonin) (Dahlstrom and Fuxe, 1964).

In this study, we determined the relation between SC and serotonergic (5-HT) neurons in culture.

The results showed that SC significantly increased the number and the dendritic sprouting of 5-HT neurons, and that this effect was probably mediated by soluble factors.

MATERIALS AND METHODS

Neuronal Cultures

Cell suspensions were prepared from rat embryos (crown-rump length 10-11 mm). Pregnant female rats (Sprague Dawley, Nossan) were anaesthetized in ether. The embryos were removed by caesarian section and placed in cold Leibowitz L-15 medium (4°C) where all dissection processes were carried out. The rostral rhombencephalon, which contains developing 5-HT neurons from the raphe complex (Konig *et al.*, 1988), was dissected out and pieces of tissue were incubated for 6 min in 0.1% trypsin in a solution of 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 0.02% EDTA at 37°C. Then 0.001% deoxyribonuclease was added and the cells were centrifuged; the supernatant was removed and the cells were resuspended and triturated through a fire-polished Pasteur pipet in 1 ml of triturating solution [1% bovine serum albumine (BSA), 0.001% DNase, 0.1% soybean trypsin inhibitor in PBS].

Dissociated cells were then plated on 13 mm diameter poly-L-lysine (PLL; 10 µg/ml) coated glass coverslips at a final density of 2×10^4 cells/coverslip and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (50 µg/ml) and streptomycin (50 U/ml). Contaminating non-neuronal cells were reduced by treatment with the antimetabolic agent cytosine arabinoside (10^{-5} M) added 24 h after the dissection.

Cells were incubated in this medium for a week with two medium changes. Some wells containing neurons were treated with NGF 2.5 S (Boehringer Mannheim, 50 ng/ml) or IGF I (Insulin-like Growth Factor, somatomedin C, Boehringer Mannheim, 10 ng/ml).

Schwann cell cultures

SC were isolated from the sciatic nerves of 2-day old rat pups (P2) using the procedure described by Brockes *et al.* (1979). Briefly, sciatic nerves were removed and treated with 0.1% collagenase and 2.5% trypsin in DMEM, mechanically dissociated by trituration and filtration through a 150 μm nylon mesh. Cells were resuspended in fresh complete medium and plated at a density of 5×10^6 cells. Cytosine arabinoside (10^{-5} M) was added 24 h after initial plating to reduce the number of dividing fibroblasts. To eliminate fibroblasts that survived the antimetabolic agent, SC cultures were treated with monoclonal antibody Anti-thy 1.1 (Serotec) and a rabbit complement (Cedarlane Lab.). Fibroblast contamination was minimal after this stage (about 1.5%).

5-HT Neuron-SC co-cultures

Dissociated SC at a final density of 1.5×10^4 cells/cover slip were plated onto coverslips containing 5-HT neurons as previously described.

Both neuronal cultures and co-cultures were kept at 37°C with 5% CO₂ for a week and then were processed for immunocytochemical staining.

Preparation of conditioned medium

The conditioned medium (CM) was obtained from SC cultures grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, penicillin/streptomycin. The cultures were washed several times with fresh medium. After 3 days, the CM was removed from the cultures and immediately passed through a membrane filter (0.2 μm pore diameter) to remove cells and debris. The retained medium was resuspended in fresh medium and added to the serotonergic neuronal cultures.

Immunocytochemistry

Individual populations of cells were identified by immunocytochemical procedures. The cells were fixed by exposure to 4% paraformaldehyde in 0.1 M PBS for 30 minutes. After washing in PBS, the cell

membranes were permeabilized with 5% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 (PBS-Triton) at room temperature for 15 min. This step was followed by incubation overnight at 4°C in the primary antibody, a polyclonal anti-serotonin (Incstar) used at the concentration 1:100. Co-cultures were incubated overnight both with anti-serotonin and with monoclonal anti-Nerve Growth Factor receptor (anti-NGFr, Boehringer Mannheim) at the concentration 1:10 which reacts with the low affinity NGF-receptor. All primary antibodies were diluted in PBS-Triton containing 1% NGS. After washing in PBS, coverslips were incubated in secondary antibody, [dichlorotriazinyl amino (DTAF) labelled anti-rabbit antibody (Jackson Immuno Res. Lab.) at the concentration 1:50 to visualize the polyclonal primary antibody (anti-serotonin) and tetramethyl rhodamine isothiocyanate (TRITC) labelled anti-mouse antibody (Jackson Immuno Res. Lab.) at the concentration 1:50 to visualize monoclonal antibody (NGFr)] in PBS-Triton containing 1% NGS, at 4°C for 30 min. Coverslips were washed and mounted in PBS/glycerol (50:50) and placed on glass microscope slides.

In all instances negative controls without primary antibody were performed.

Morphometric study

Coverslips were analysed on a Leitz fluorescent microscope and 5-HT positive neurons were counted over the entire coverslips. Moreover, for each 5-HT positive neuron, the number of neuronal branches and total length of fibers were determined using image analysis. For each culture condition, a minimum of 10 experiments were carried out, the means were subsequently obtained and data were assessed by statistical analysis: fiber length data were tested by the Friedman Test and the number of neurons was tested by the Wilcoxon's Test.

RESULTS

Effects of growth factors on survival and branching of serotonergic neurons

Surviving 5-HT neurons kept for 7 days under previously described culture conditions, increased after treatment with NGF or IGF-I. Figure 1 shows the clear difference between controls and trophic

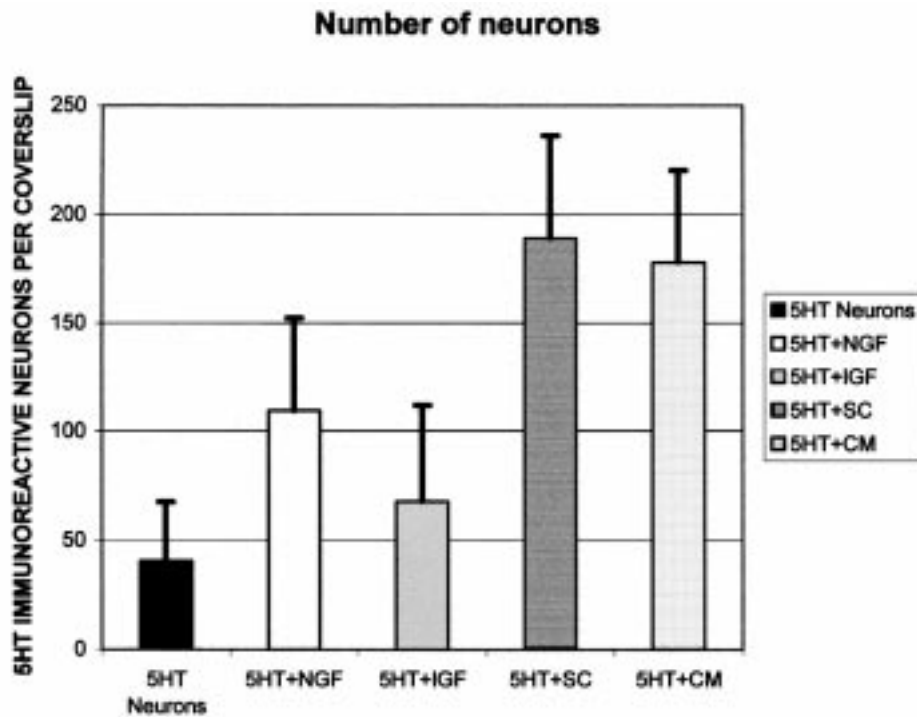


Fig. 1 - The influence of trophic factors and SC on the survival of serotonergic neurons after 7 days *in vitro*. The neuron counts are expressed as absolute numbers. Histograms show the increase in number of neurons when treated with NGF ($P < 0.05$), IGF-I (not significant) when co-cultured with SC ($P < 0.05$) or incubated with CM ($P < 0.05$).

factor-treated cultures. In NGF-treated cultures, we obtained a higher number of 5-HT neurons as compared to control (109 vs 40 per coverslip, $P < 0.05$) while in IGF-I-treated neurons the increase (68 per coverslip) was not significant. These results

demonstrate that survival of the serotonergic neurons is enhanced in the presence of specific growth factors, although the increase in number of neurons reached statistical significance only when NGF was added to the culture.

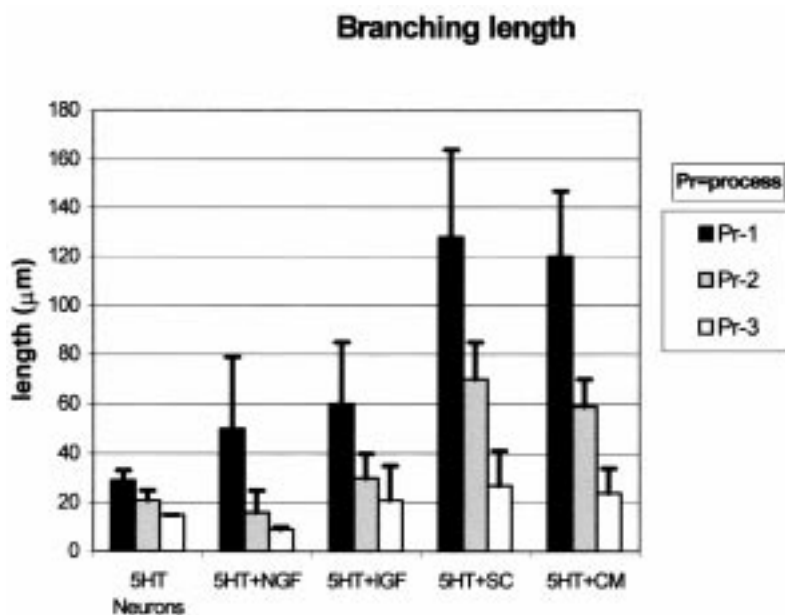


Fig. 2 - Histograms show the length of processes (Pr) exhibited by serotonergic neurons under each culture condition. Control cultures presented short processes by contrast to long processes exhibited by serotonergic neurons in the presence of NGF, IGF-I, SC ($P < 0.05$) or incubated with CM ($P < 0.05$).

Computerized measurements of immunocytochemically stained 5-HT neurons indicated that these cells exposed to trophic factors, such as NGF or IGF I, exhibited a greater complexity and degree of neuritic and dendritic outgrowth than in controls.

As shown in Figures 2, 3A and 4A, in control cultures serotonergic neurons exhibited short processes ($Pr_1 = 29 \mu\text{m}$) whereas in the presence of NGF (Fig. 2, 3B and 4B) or IGF-I (Fig. 2, 3C and 4C), the 5-HT neurons presented an increase in dendritic length, branching and number of primary dendrites with an average length of $50 \mu\text{m}$ if treated with NGF and about $60 \mu\text{m}$ if treated with IGF-I.

These findings demonstrate the capability of NGF and IGF-I to induce greater axonal and dendritic branching showing more branch points than control cultures.

Schwann cell-derived factors stimulate serotonergic neuron survival and branching

We found a clear increase in the number of surviving 5-HT neurons when embryonic rhombencephalic neurons were co-cultured with primary SC for 7 days.

The number of neurons grown in the presence of SC was more than four times as high as controls (189 vs 40 per coverslip, $P < 0.05$). The co-cultures with SC had a higher number of neurons than the cultures treated with NGF or IGF-I but this increase did not reach statistical significance. These data suggest that, in our experimental conditions, SC contribute to promote the survival of 5-HT neurons. In addition, these experiments have shown that not only the number of surviving neurons increased but also a significant increase in cell body size was observed (Fig. 3A,D) using image analysis.

As described above, in co-cultures, SC exerted a positive effect on serotonergic neuron survival, accompanied by a significant increase in the length and branching processes ($Pr_1 = 128 \mu\text{m}$).

Figures 2, 3D and 4F show that, in the presence of SC, the length of neurites was four times as long as in controls and two times as long as those cultured with growth factors. Moreover, their length was also increased with more branch points compared to those 5-HT neuron cultures without SC (Figure 3A-E). Therefore, the results indicate that SC promote an increase in axonal and dendritic extension.

Effect of SC conditioned medium (CM) on serotonergic neuron survival and morphology

To determine whether SC supported serotonergic neuron survival through a release of soluble factors, CM was tested for a neurotrophic effect on serotonergic neurons. Figure 1 shows that these cultures presented an increase in the number of 5-HT neurons (178 vs 40 per coverslip $P < 0.05$), accompanied by an evident and dense axonal and dendritic outgrowth (Fig. 3E and 4D).

In all the different experimental conditions, the 5-HT neurons in culture did not express the low affinity NGFr, while SC were always NGFr positive.

DISCUSSION

In the present work we demonstrate that postnatal SC have the capacity to promote the survival and growth of embryonic cultured rhombencephalic serotonergic neurons. This property is probably mediated via soluble trophic factors, considering that the same results were obtained when the cultures of 5-HT neurons were co-cultured with SC or incubated with a conditioned medium derived from SC cultures. SC and their trophic factors have a remarkable effect not only on the survival of the serotonergic neurons but also on their axonal and dendritic outgrowth, increasing their number and enhancing their length. Often the axonal branching was clearly directed toward the surrounding SC, perhaps indicating a particular tropism of the growing neuritic arborization in the direction of the cells that produce the trophic factors.

We obtained similar results incubating the 5-HT neurons cultures with NGF or with IGF-I. NGF, besides its well known activity on the sympathetic ganglion cells (Levi-Montalcini and Angeletti, 1968; Thoenen *et al.*, 1971; Snider *et al.*, 1988) has a relevant trophic effect also on basal forebrain cholinergic neurons and on sensory neurons, increasing the number of surviving neurons, the total length of neurites and the number of branching points (Gnahn *et al.*, 1983; Hefti *et al.*, 1985; Hagg *et al.*, 1989; Holtzman *et al.*, 1995; Gavazzi *et al.*, 1999). IGF-I and IGF-II stimulate the differentiation of dopaminergic neurons in culture (Knusel *et al.*, 1990), regulating the growth of 5-HT and tyrosine hydroxylase neurons (Liu and Lauder, 1992),

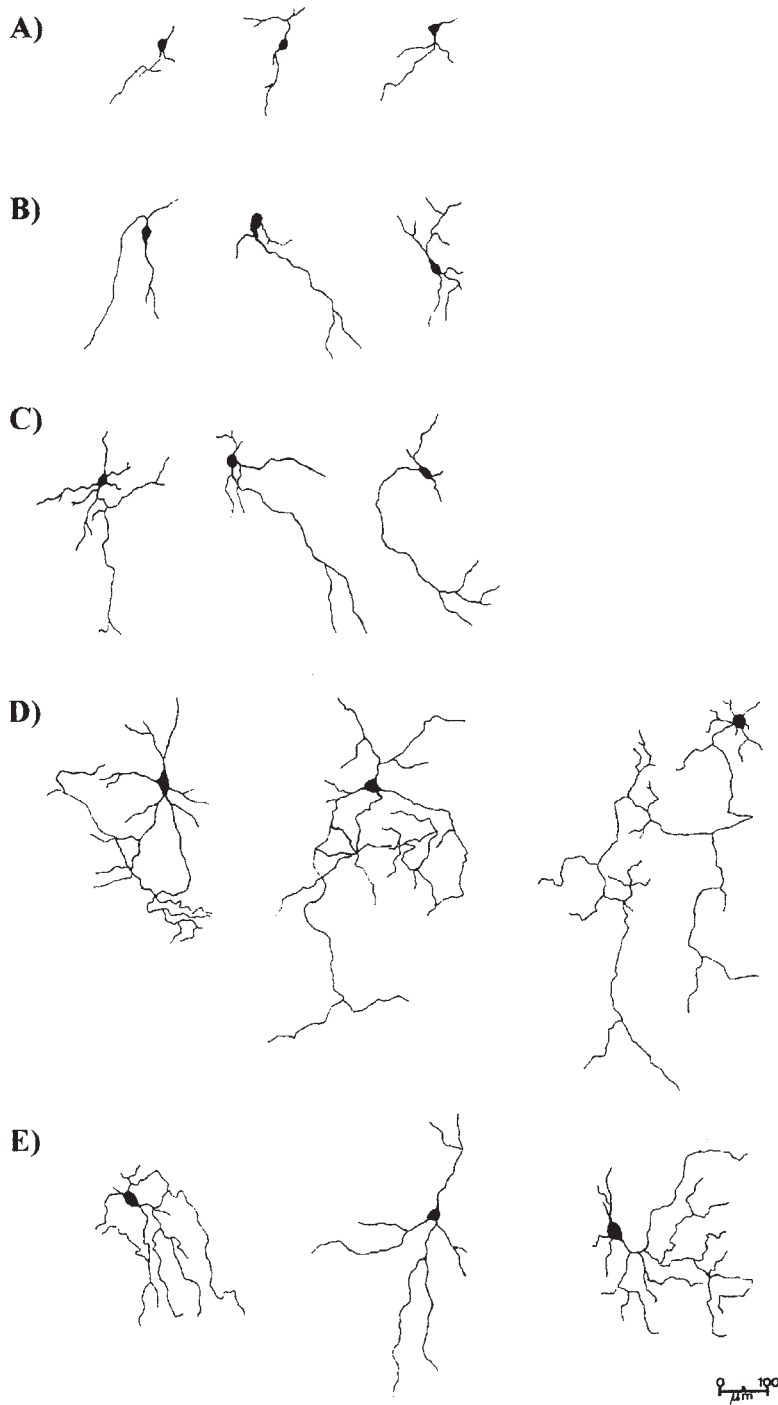


Fig. 3 - Camera lucida drawings of representative serotonergic neurons in different conditions. Control cultures of serotonergic neurons (A) showed a short neuritic and dendritic arborization. In the presence of NGF (B) and IGF-I (C), the cultures of serotonergic neurons exhibited a greater complexity of neurite and dendritic outgrowth than in controls. In co-cultures with SC (D), serotonergic neurons showed a significant increase in the length and branching of processes and in the cell body size (data not shown). In the presence of CM (E), serotonergic neurons showed an increase in neuritic and dendritic outgrowth with a high degree of complexity and an increase in the cell body size, in analogy to that observed in co-cultures with SC.

and neurite outgrowth in cultured motor, sensory, sympathetic, cortical neurons, and neuronal differentiation of stem cells (Brooker *et al.*, 2000). Moreover, IGF-I rescues SC from apoptosis (Delaney *et*

al., 1999) and enhances the formation of regenerated axons in SC implants if it is combined with Platelet-Derived Growth Factor (Oudega *et al.*, 1997). The results of our study demonstrate that

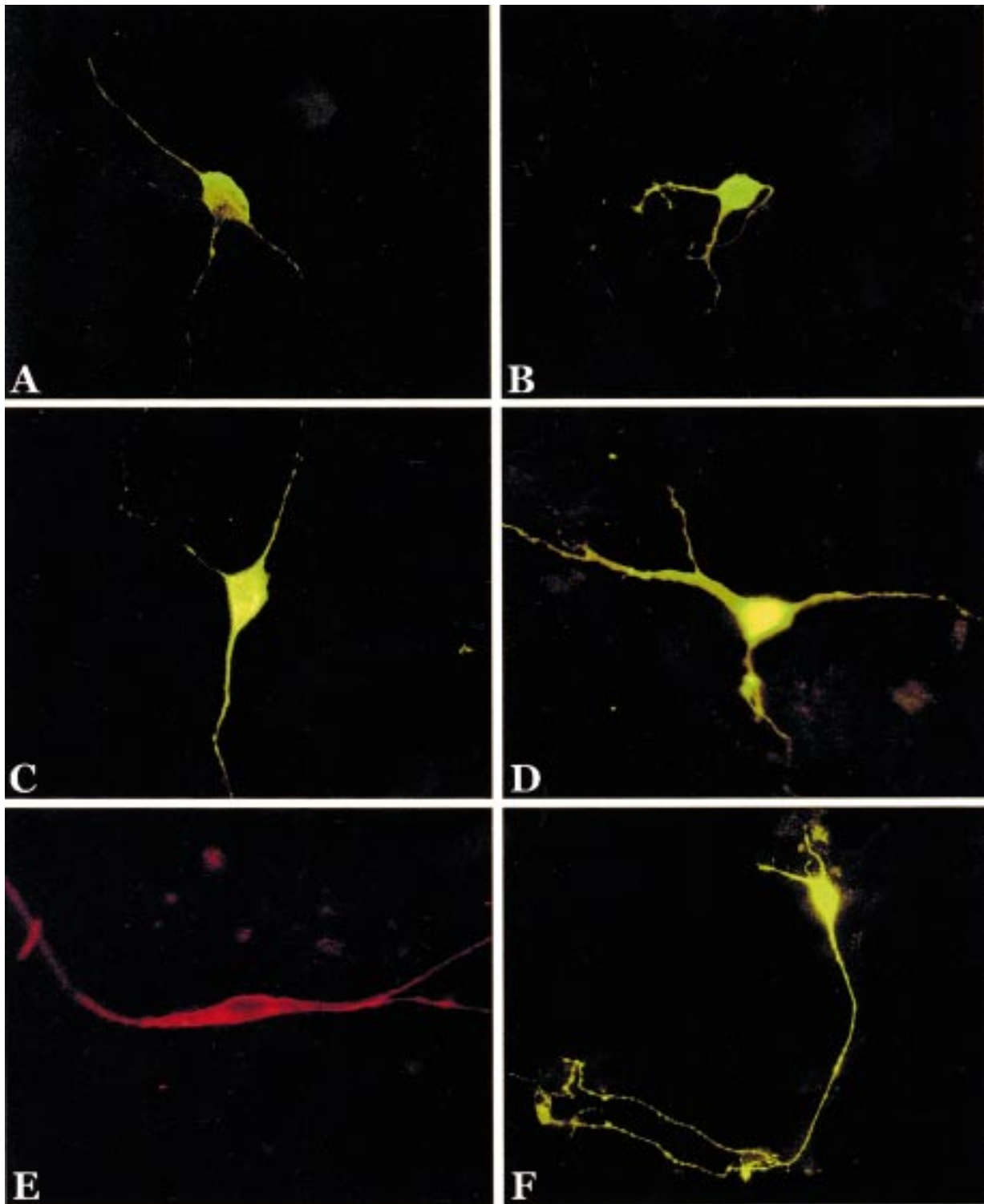


Fig. 4 - Fluorescence photomicrograph showing anti-serotonin immunocytochemical staining of neurons in different culture conditions. A) 5-HT positive neurons observed in control culture. B) 5-HT positive neurons grown in the presence of NGF (50 ng/ml). C) 5-HT positive neurons grown in the presence of IGF-I (10 ng/ml). D) 5-HT neurons grown in the presence of CM from SC. E) anti-NGFr positive SC and 5-HT neurons (F) both grown in the same co-culture.

NGF has a significant effect on the survival of serotonergic neurons while the effect of IGF-I was less pronounced and not statistically significant. Conversely, both trophic factors had the same positive effect on neuritic and dendritic arborization, resulting in a higher degree of complexity of dendritic branching. Although effective, the influence of NGF or IGF-I on survival and neurite outgrowth of 5-HT neurons was less pronounced than that obtained with SC or CM obtained from SC cultures. All these data seem to suggest that the positive effect of SC cultures on 5-HT cultured neurons is mediated through trophic factors, such as NGF or IGF-I: the more efficacious activity of SC cultures might be due to a larger production of a single trophic factor or, more probably, to the secretion of numerous trophic substances which could have a synergic and additive effect on the target cell. It is possible that the factors released by SC cultures act as substrate-bound agents promoting neurite outgrowth. It is known that the RN22 Schwannoma secretes agents which bind to tissue culture substrates to promote neurite outgrowth. According to this, SC could release a substrate-bound factor increasing 5-HT neuron attachment rather than survival. SC have been in fact demonstrated to have the capacity to produce not only NGF (Henderson *et al.*, 1993) and IGF-I (LeRoith, 1993), but also Epidermal Growth Factor (EGF) (Morrison *et al.*, 1987, 1988), BDNF (Koliatsos *et al.*, 1993), acidic and basic Fibroblast Growth Factor (aFGF and bFGF) (Morrison, 1986, 1988; Walicke *et al.*, 1986; Murphy *et al.*, 1990) and Ciliary Neurotrophic Factor (CNTF) (Sendtner *et al.*, 1994). It remains to be shown which trophic factors are produced by SC in the experimental conditions that we set up, considering that the type and amount of trophic substances secreted change in relation to the particular environment and, in particular, in relation to the target.

Neuron-glia interactions appear to be of main relevance not only during embryogenesis and the postnatal period but also during the process of degeneration and regeneration of the nervous system. Astrocytes more than fibroblasts have the capacity to facilitate neurite outgrowth of serotonergic neurons (Lieth *et al.*, 1990), and in coculture systems, where astrocytes and neurons grow together in the same environment but not in direct contact, it has been demonstrated that the regulation of axonal sprouting is mediated via a diffusible factor produced by astrocytes (Qian *et*

al., 1992). The expectation behind the studies on the neuron-glia relationship is that, by improving our knowledge of the mechanisms of the trophic effect on neuronal survival and growth, it could be, at least theoretically, possible to enhance regenerative and possibly to decrease degenerative processes of the nervous system (Terenghi, 1999). The use of neurotrophic factors has been suggested in neurological disorders such as Alzheimer's disease and Parkinson's disease and, in fact, the data of this study support their use in degenerative diseases of the CNS involving serotonergic neurons of the raphe, such as progressive supranuclear palsy (Holtzman *et al.*, 1994).

Given the remarkable capacities of regeneration and recovery of the PNS, and considering that the process is at least partially mediated by the activity of SC, grafts of SC cultures have been made to mammalian brain or spinal cord in order to promote axonal regeneration (Kromer and Cornbrooks, 1985; Paino and Bunge, 1991; Bunge, 1991, 1994; Xu *et al.*, 1994). Similarly, Aguayo *et al.* (1985) have shown that SC are essential elements in trophic support of central axon regrowth in peripheral nerve grafts. It appears, therefore, reasonable to utilize SC as a source of neurotrophic factors, taking into account that it is now possible to obtain a large number of SC in cultures (Rutkowski *et al.*, 1992) and that SC can be transplanted in nervous tissue (Tuszynski *et al.*, 1998). Our study adds further data on the capacity of postnatal SC to promote survival and growth of CNS neurons, particularly serotonergic neurons, but more effort is needed to precisely clarify the nature of the trophic factors produced by SC and their mechanism of action on the target cells of the CNS.

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