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TGF-β superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP^+ toxicity

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The superfamily of transforming growth factors-β (TGF-β) comprises an expanding list of multifunctional proteins serving as regulators of cell proliferation and differentiation. Prominent members of this family include the TGF-βs 1–5, activins, bone morphogenetic proteins and a recently discovered glial cell line-derived neurotrophic factor (GDNF). In the present study we demonstrate and compare the survival promoting and neuroprotective effects of TGF-β1, -2 and -3, activin A and GDNF for midbrain dopaminergic neurons *in vitro*. All proteins increase the survival of tyrosine hydroxylase-immunoreactive dopaminergic neurons isolated from the embryonic day (E) 14 rat mesencephalon floor to varying extents (TGF-βs 2.5-fold, activin A and GDNF 1.6-fold). TGF-βs, activin A and GDNF did not augment numbers of very rarely observed astroglial cells visualized by using antibodies to glial fibrillary acidic protein and had no effect on cell proliferation monitored by incorporation of BrdU. TGF-β1 and activin A protected dopaminergic neurons against *N*-methyl-4-phenylpiridinium ion toxicity. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that TGF-β2 mRNA, but not GDNF mRNA, is expressed in the E14 rat midbrain floor and in mesencephalic cultures. We conclude that TGF-βs 1–3, activin A and GDNF share a neurotrophic capacity for developing dopaminergic neurons, which is not mediated by astroglial cells and not accompanied by an increase in cell proliferation.

Key words: activin A/GDNF/neuroprotection/nigrostriatal dopaminergic neurons/transforming growth factors-β

Introduction

Mesencephalic dopaminergic neurons in the substantia nigra play an important role in motor functions and degenerate in Parkinson's disease. Oxidative stress, increased iron, defects in enzymes with a capacity to cope with free radicals, neurotoxicity mediated by excitatory amino acids and lack of availability of growth factors to balance the toxic influences have been implicated in the etiology of the idiopathic disease (Shoffner *et al.*, 1991; Turski *et al.*, 1991; Przedborski *et al.*, 1992; Edwards, 1993; Gerlach *et al.*, 1994; Unsicker, 1994). The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite, *N*-methyl-4-phenylpyridinium ion (MPP^+) produce *in vivo* a syndrome with striking clinical and pathological resemblance to Parkinson's disease (Kopin and Markey, 1988; Forno *et al.*, 1993; Otto and Unsicker, 1990). The drugs are also toxic to cultured dopaminergic neurons of the substantia nigra, allowing the testing *in vitro* of putative beneficial agents that can sustain toxically impaired dopaminergic neurons (Ferrari *et al.*, 1989; Hyman *et al.*, 1991; Otto and Unsicker, 1993; Unsicker, 1994). Neurotrophic factors, i.e. molecules that are essential for neuron development, survival and maintenance and that act by retro- and anterograde axonal transport as well as auto- and paracrine mechanisms (Korsching, 1993; Unsicker *et al.*, 1992), are major candidates for protecting dopaminergic neurons from toxic damage and degeneration. Several of these factors occur in the developing and adult substantia nigra and striatum (Unsicker, 1994; Beck, 1995) and may have a physiological relevance for dopaminergic nigrostriatal neurons.

Transforming growth factors-β (TGF-β), activin A and a distant member of the TGF-β superfamily, a glial cell line-derived neurotrophic factor (GDNF), have a capacity to promote the survival of embryonic neurons *in vitro* (Martinou *et al.*, 1990; Schubert *et al.*, 1990; Chalazonitis *et al.*, 1992; Lin *et al.*, 1993; Prehn *et al.*, 1993b). Moreover, TGF-β has marked neuroprotective effects for ischemically lesioned neurons *in vivo* (Prehn *et al.*, 1993a). TGF-β2 and -3 immunoreactivities have been demonstrated in the striatum and substantia nigra of adult rat (Unsicker *et al.*, 1991), as well as in the developing murine mesencephalon floor (Flanders *et al.*, 1991). Activin A and GDNF mRNAs are widely expressed in the developing brain (for references, see Discussion). Localization of TGF-β in mitochondria in several tissues and cells (Heine *et al.*, 1991) has raised speculation concerning a putative role in the regulation of mitochondrial enzymes. In the present study we have demonstrated and compared the survival promoting and protective effects of TGF-β1–3, activin A and GDNF for unlesioned and toxically impaired mesencephalic dopaminergic neurons. We show that TGF-βs maintain a larger number of dopaminergic neurons than activin A and GDNF. The neurotrophic effects are not mediated by astroglial cells or any type of proliferating cell population.

Results

Activin A, GDNF and TGF-βs promote in vitro survival of dopaminergic neurons

Immunocytochemical staining for tyrosine hydroxylase (TH), the rate limiting enzyme in the biosynthesis of

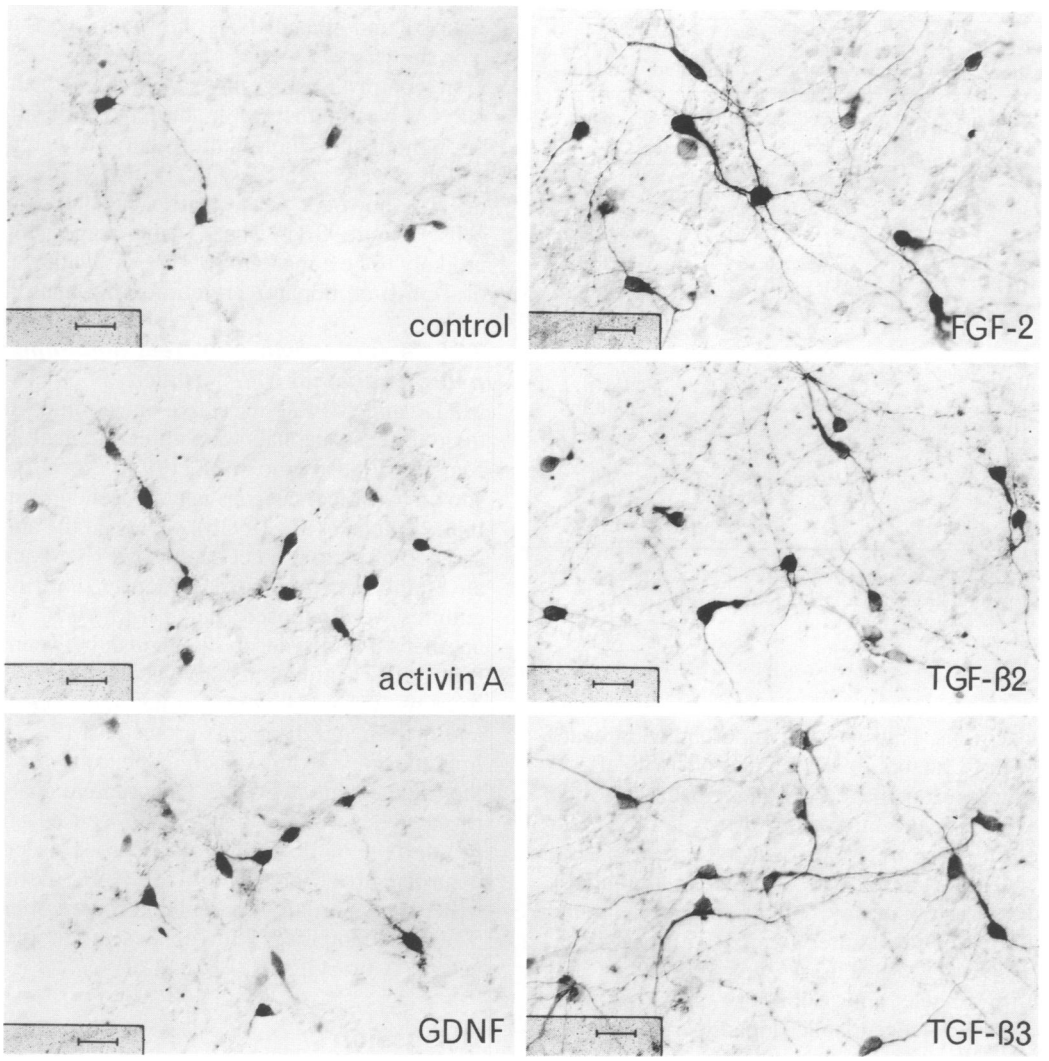

Fig. 1. Photomicrographs of dissociated cultures of rat E14 ventral mesencephalic cells after 8 days in culture stained with monoclonal antibodies against TH. Cultures shown are control cultures and cultures treated with activin A (20 ng/ml), GDNF (20 ng/ml), FGF-2 (10 ng/ml) and TGF-β2 and -β3 (2 ng/ml). Bar = 50 μm.

dopamine and other catecholamines, was employed to identify dopaminergic neurons. TH is a marker for dopaminergic neurons in these cultures, since the dissection procedure used (Shimoda *et al.*, 1992) excludes noradrenaline-containing cells, as for example the locus coeruleus, from these cultures.

Typically, our cell cultures contained ~98% neuronal cells after 1 day *in vitro* (DIV 1), using neurofilaments as a marker (cf. Hyman *et al.*, 1991; Shimoda *et al.*, 1992). About 8% of these neuronal cells were TH-immunoreactive. Glial fibrillary acidic protein (GFAP)-immunoreactive cells were not detectable (cf. Hyman *et al.*, 1991; Shimoda *et al.*, 1992). To evaluate possible survival promoting effects of activin A and TGF-βs on dopaminergic neurons and to compare these effects with the established neurotrophic effects of GDNF (Lin *et al.*, 1993), cultures were treated with each of these growth factors, starting treatment at DIV 1. Figure 1 shows that treatment with saturating doses of activin A, TGF-βs or GDNF, as compared with untreated controls, clearly maintained more TH^+ neurons after 8 days in culture. This effect was comparable with that elicited by basic fibroblast growth factor (FGF-2; Figure 1). Activin A, the TGF-β isoforms 1–3 and GDNF were tested for their survival promoting effect at various concentrations (0.5, 2, 5 and 10 ng/ml; Table I). Each of the three TGF-β isoforms increased survival of mesencephalic dopaminergic neurons 2.3- to 2.4-fold above controls. The IC_{50} was 0.5 ng/ml and concentrations of 2 ng/ml and higher were already saturating. Both activin A and GDNF were less potent than the TGF-βs, raising survival to about 160% of control values (100%) at 20 ng/ml.

The trophic effects of activin A, GDNF and TGF-βs are not mediated by astroglia and proliferating cells

TGF-βs and activin A can act as mitogens and differentiation factors on various cell types (Roberts and Sporn, 1990; Sakurai *et al.*, 1994). For GDNF, which is widely expressed in most mammalian organs and tissues (Suter-Crazzolara and Unsicker, 1994), similar mitogenic functions might be expected. We therefore investigated whether the TGF-β superfamily members used in this study induced proliferation and/or differentiation of neural progenitor

Table I. Percentages of surviving TH-immunoreactive neurons of rat mesencephalic cultures (E14) at the end of the 8 day culture period

Treatment	Concentration (ng/ml)	Percent survival	*P* value
Control		100 ± 7.98	
bFGF	10	201.0 ± 18.17	<0.001
TGF-β1	0.5	161.5 ± 23.19	<0.001
	2	228.5 ± 6.36	<0.001
	10	231.3 ± 9.95	<0.001
TGF-β2	0.5	164.6 ± 3.77	<0.001
	2	239.9 ± 7.44	<0.001
	10	237.3 ± 12.32	<0.001
TGF-β3	0.5	172.6 ± 8.86	<0.001
	2	237.0 ± 28.10	<0.001
	10	241.2 ± 13.75	<0.001
Activin A	5	125.0 ±14.53	<0.05
	20	162.2 ± 22.80	<0.001
GDNF	5	152.3 ± 25.54	<0.01
	20	161.8 ± 14.93	<0.001

Controls were set as 100%. Results are mean ± SEM of triplicate determinations of two replicate experiments for each isoform and concentration.

cells, which subsequently would elicit trophic effects on dopaminergic neurons. This was also important with regard to the fact that most factors for which neurotrophic effects on dopaminergic neurons have been described exert their actions by expanding the number of astroglial cells (for references, see Discussion). We first tested for the presence of astroglial cells by staining with a monoclonal antibody against GFAP. Very few, if any $GFAP^+$ cells were found in control cultures or in cultures treated with activin A, GDNF or TGF-β at DIV 8 (Figure 2). FGF-2, which has been shown by Engele and Bohn (1991) to promote *in vitro* survival of mesencephalic dopaminergic neurons acting via glial cells, caused a massive increase in $GFAP^+$ astroglial cells (Figure 2). We next studied cell proliferation by applying bromodeoxyuridine (BrdU) to cultures at various time points for periods of 48 h and visualizing incorporated BrdU using monoclonal antibodies. Cultures treated with any of the TGF-βs, activin A or GDNF or maintained in the absence of factors showed very sparse, if any, BrdU incorporation at any time period during the entire culture period (Figure 2). In contrast, yet as expected, FGF-2-treated cultures showed large numbers of labeled nuclei (Figure 2). We therefore conclude that the survival promoting effects of activin A, GDNF and TGF-βs are not mediated through any mitogenic activity on any cell type, neural or non-neural, in this culture system. Moreover, the neurotrophic effects are apparently not mediated by astroglial cells.

TGF-β2 mRNA, but not GDNF mRNA, is expressed in the E14 rat midbrain floor in situ and in cultured cells

In order to ascertain that the observed neurotrophic effects of TGF-βs reflected potentially physiological rather than pharmacological actions, we performed reverse transcription-polymerase chain reaction (RT-PCR) on freshly dissected tissues of E14 midbrain floor and on cultured midbrain cells at various time points. As shown in Figure 3A, TGF-β2 mRNA was clearly detectable both in dissected tissues and in untreated and TGF-β-treated cultures. TGF-β1 and -β3 mRNAs, however, were not detectable (not shown).

In contrast, using the same cDNA samples, GDNF mRNA was undetectable in E14 midbrain tissues and throughout the whole culture period, whether or not the cultures were treated with TGF-β (Figure 3B). GDNF mRNA, however, was clearly detectable in B49 glioma cells (Figure 3B). These data suggest that GDNF is unlikely to be a mediator of the survival promoting effects of TGF-β on dopaminergic midbrain neurons.

TGF-βs and activin A protect dopaminergic neurons against MPP^+ toxicity

MPTP and MPP^+, its active metabolite, are selectively toxic to mesencephalic dopaminergic neurons (Kopin and Markey, 1988; Forno *et al.*, 1993). Since TGF-β has been shown to protect neurons against ischemic and excitotoxic injury (Prehn *et al.*, 1993b), we tested the ability of TGF-β and activin A to protect against MPP^+ toxicity. Following an established protocol (Hyman *et al.*, 1991), 4-day-old cultures were exposed for 48 h to MPP^+ and fixed after another 48 h in order to distinguish between slowly dying (but still TH-immunoreactive neurons) and surviving TH^+ neurons. Figure 4 demonstrates that TGF-β1 (2 ng/ml) and activin A (20 ng/ml) protected MPP^+-lesioned, TH-immunostained dopaminergic neurons to approximately the same extent as FGF-2. Quantitative analysis (Table II) revealed that treatment with MPP^+ caused a 67% reduction in survival of dopaminergic neurons as compared with controls. In the presence of TGF-β1, the toxic effect of MPP^+ was partially prevented, reducing neuron losses to 32%, as compared with 45% seen in the presence of activin A or FGF-2.

Discussion

Our results provide evidence that several members of the TGF-β superfamily, including TGF-βs 1–3, activin A and GDNF, promote survival of dopaminergic neurons cultured from the embryonic rat mesencephalon floor. The neurotrophic effects are seen on both unlesioned and toxically impaired neurons. The TGF-βs 1–3, activin A and GDNF, together with nerve growth factor (NGF) and platelet-derived growth factor, belong to a structural superfamily of growth factors that share a conserved structural motif of cystines referred to as the 'cystine knot motif' (McDonald and Hendrickson, 1993). The mammalian TGF-βs 1–3 have long been known to act as multifunctional cytokines, with prominent effects on extracellular matrix formation, cell adhesion and proliferation (Roberts and Sporn, 1990; Kingsley, 1994). Activin, a member of the activin/inhibin subfamily, has been isolated from many tissues and functionally characterized on the basis of its ability to modulate the release of follicle-stimulating hormone (FSH) from pituitary cells (Vale *et al.*, 1994). GDNF was first cloned from a glioma cell line (Lin *et al.*, 1993), but is widely expressed in all organs studied so far (Suter-Crazzolara and Unsicker, 1994). For TGF-βs and activin the signaling receptor complexes have been identified as consisting of heteromeric high affinity serine/threonine kinase receptors and a β-glycan low affinity receptor (Massague, 1992; Wrana *et al.*, 1992; Franzen *et al.*, 1993). Receptors for GDNF have not been identified

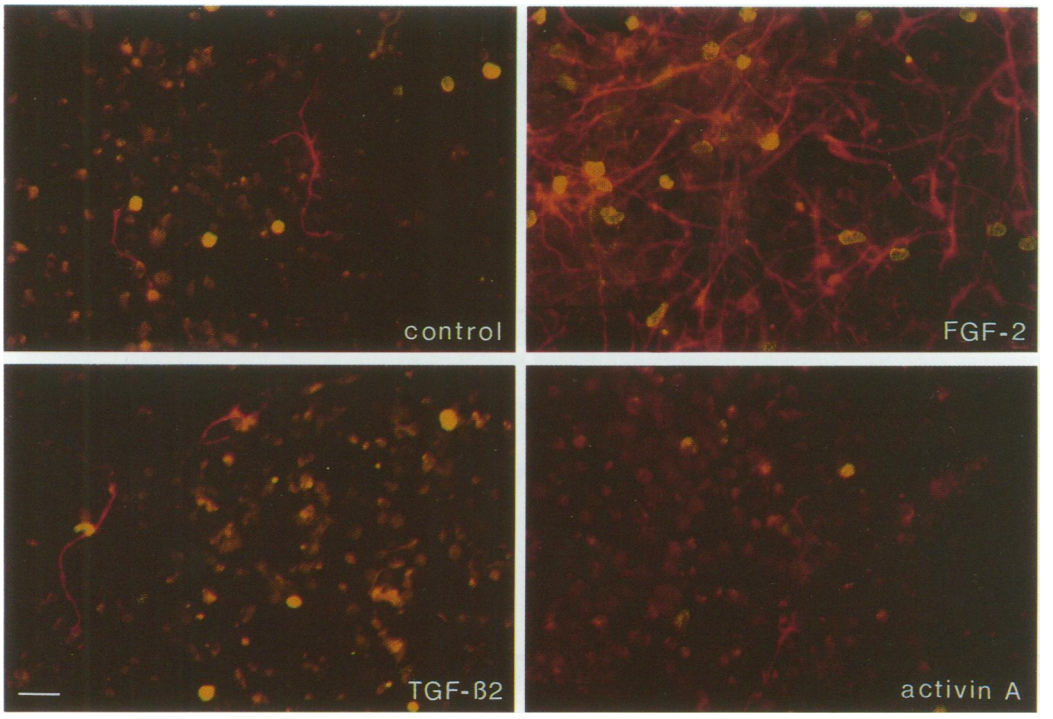

Fig. 2. Photomicrographs of dissociated cultures of rat E14 mesencephalon floor after 8 days in culture double stained for GFAP (red cells) and BrdU incorporation (yellow nuclei). Cultures shown are control cultures and cultures treated with TGF-β2 (2 ng/ml), activin A (20 ng/ml) and FGF-2 (10 ng/ml). Bar = 50 μm.

as yet. TGF-β2 and -β3 as well as activin messages and proteins are widely distributed in the embryonic and adult peripheral and central nervous system (Flanders *et al.*, 1991; Unsicker *et al.*, 1991, 1994; Jakowlew *et al.*, 1994; Vale *et al.*, 1994; Krieglstein *et al.*, 1995). TGF-β1 is constitutively expressed in meninges, but significantly induced upon lesioning in astro- and microglia as well as in neurons (Lindholm *et al.*, 1992; Logan *et al.*, 1992; Pasinetti *et al.*, 1993). Radial and astroglial cells seem to be predominant sites of TGF-β2 and -β3 synthesis, but both astroglial cells and neurons are TGF-β2- and -β3-immunoreactive (Flanders *et al.*, 1991; Unsicker *et al.*, 1991, 1994). Activin A has been reported to be localized in particular sets of neurons, e.g. in the medullary reticular formation, and there are extensive projections to the fore- and midbrain (Vale *et al.*, 1994). GDNF mRNA seems to be predominantly expressed in the late embryonic and early postnatal rat brain (Schaar *et al.*, 1993; Strömberg *et al.*, 1993; Springer *et al.*, 1994) and is up-regulated upon lesioning (Humpel *et al.*, 1994). Its cellular localization has not been unequivocally revealed. The distributional patterns of these TGF-β superfamily members suggest multiple target cells and functions in the brain, including the nigrostriatal system. Consistent with the above demonstration of TGF-β2 mRNA in the E14 midbrain floor by RT-PCR, TGF-β2 immunoreactivity has been shown to occur as early as E12.5 in the embryonic mouse mesencephalon floor (Flanders *et al.*, 1991). Moreover, both neurons and astroglia display TGF-β2- and -β3-immunoreactivities in the adult rat substantia nigra and striatum (Unsicker *et al.*, 1991). Activin can be immunocytochemically shown in nerve fibres projecting to the midbrain (Vale *et al.*, 1994) and GDNF mRNA has been detected in dopaminergic target areas beginning at E20, reaching

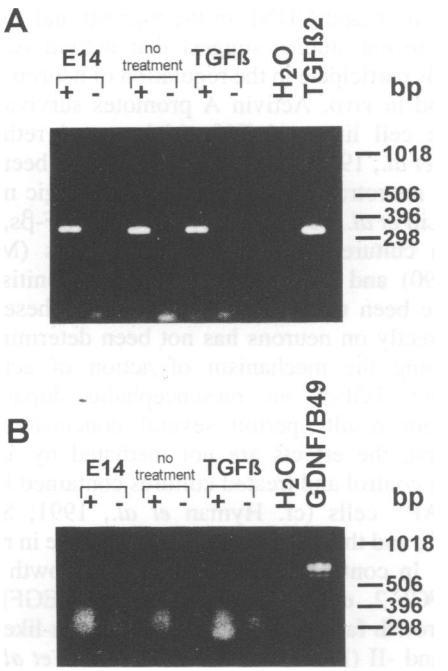

Fig. 3. Expression of TGF-β2 (**A**) and GDNF (**B**) in E14 rat mesencephalon floor and in mesencephalic cell cultures (DIV 4) detected by RT-PCR. − lanes represent controls, in which total RNA was not transcribed, + lanes represent cDNA. Lane marked H_2O shows amplification using H_2O instead of cDNA. (A) A positive signal for TGF-β2 (359 bp) is obtained from E14 mesencephalon floor, TGF-β1-treated (2 ng/ml) and untreated mesencephalic cultures. (B) A positive signal for GDNF (700 bp) is obtained from B49 glioma cells, but not from E14 mesencephalon floor and not from TGF-β1-treated (2 ng/ml) or untreated mesencephalic cultures, using the same cDNA samples as for the TGF-β2 PCR.

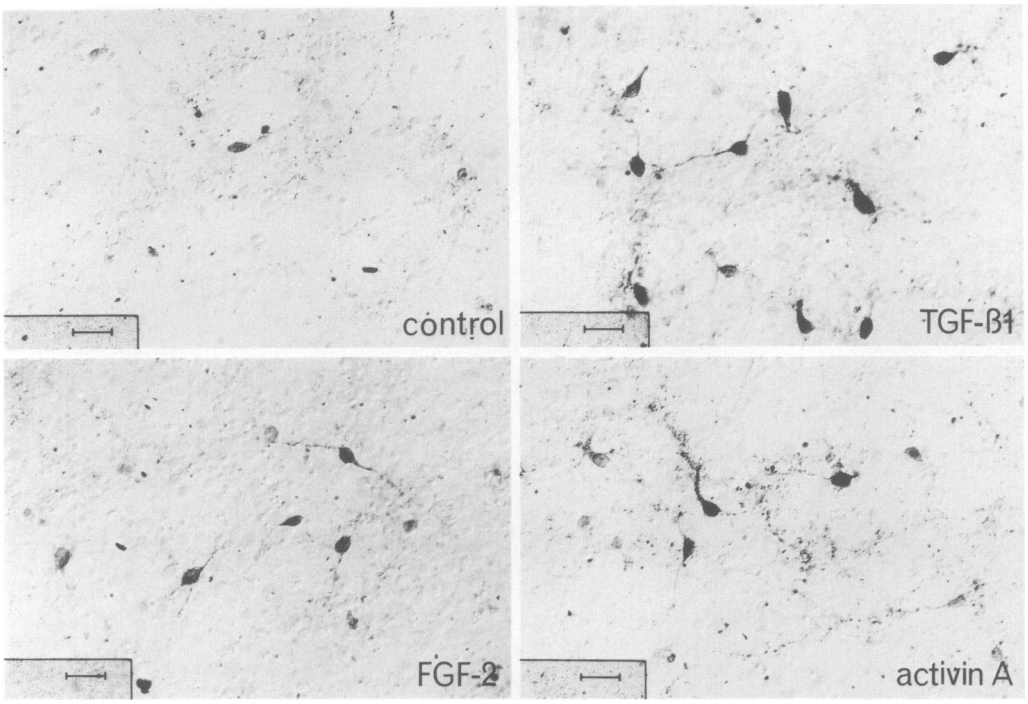

Fig. 4. TH-immunoreactive cells in mesencephalic cultures (E14) treated with MPP^+. MPP^+ (2 μM), TGF-β1 (2 ng/ml) plus MPP^+, activin A (20 ng/ml) plus MPP^+ and FGF-2 (10 ng/ml) plus MPP^+. Bar = 50 μm.

a peak at birth (Strömberg *et al.*, 1993). Taken together, these data suggest yet to be defined functional roles for TGF-βs, activin and GDNF in the nigrostriatal system.

Several recent studies suggest that activin A, GDNF and TGF-βs participate in the regulation of neuron survival *in vitro* and *in vivo*. Activin A promotes survival of the B50 nerve cell line and E10 chick neural retina cells (Schubert *et al.*, 1990). GDNF has previously been shown to act as a neurotrophic factor for dopaminergic midbrain neurons (Lin *et al.*, 1993). With regard to TGF-βs, trophic effects on cultured embryonic motoneurons (Martinou *et al.*, 1990) and sensory neurons (Chalazonitis *et al.*, 1992) have been reported. Whether any of these factors can act directly on neurons has not been determined.

Concerning the mechanism of action of activin A, GDNF and TGF-β on mesencephalic dopaminergic neurons, our results permit several conclusions to be drawn. First, the effects are not mediated by astroglial cells. Both control and treated cultures contained less than 0.2% $GFAP^+$ cells (cf. Hyman *et al.*, 1991; Shimoda *et al.*, 1992) and their numbers did not increase in response to TGF-β. In contrast, several mitogenic growth factors, such as FGF-2, epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and insulin-like growth factors-I and -II (IGF-I and IGF-II) (Knüsel *et al.*, 1990; Casper *et al.*, 1991; Engele and Bohn, 1991; Alexi and Hefti, 1993), have trophic effects on mesencephalic dopaminergic neurons which are accompanied by a drastic increase in cell numbers, including astroglial cells, and are, as shown for FGF-2 and EGF, abolished upon inhibition of cell proliferation (Knüsel *et al.*, 1990; Engele and Bohn, 1991). Next, we can exclude the possibility that the neurotrophic effects of activin A, GDNF and TGF-βs are mediated by a numerical expansion of non-astroglial cell populations, since cell proliferation was virtually absent from these cultures. Since >98% of the cells in our cultures kept under any condition (except with FGF-2) were neurofilament-immunoreactive (data not shown; cf. Hyman *et al.*, 1991; Shimoda *et al.*, 1992), the survival promoting effects of activin A, GDNF or TGF-βs, given that they are indirect ones, would be mediated by neuron- rather than by glial-derived factor(s). In fact, for none of the hitherto established neurotrophic factors for mesencephalic dopaminergic neurons have indirect, neuron-mediated effects been excluded. The trophic effect of TGF-βs observed is unlikely to be mediated by GDNF, since its mRNA was not induced to detectable levels in TGF-β-treated cultures. In contrast, TGF-β2 mRNA was detectable throughout the whole culture period, making it possible that TGF-βs might act in an autocrine fashion. Activin A, GDNF and TGF-βs might exert their neurotrophic effects by inducing neurotrophins. However, hitherto the only documented example of such a possibility is the induction of NGF mRNA by TGF-β1 in cultured rat astrocytes (Lindholm *et al.*, 1990). NGF, however, is

Table II. Percentages of surviving TH-immunoreactive neurons when treated with MPP^+ in combination with growth factors

Treatment	Concentration (ng/ml)	Percent survival	*P* value
Control		100 ± 4.84	
MPP^+/control		32.9 ± 6.83	
MPP^+/bFGF	10	54.7 ± 3.60	<0.01
MPP^+/TGF-β1	2	66.5 ± 5.71	<0.001
MPP^+/TGF-β1	10	68.9 ± 5.34	<0.001
MPP^+/activin A	20	55.5 ± 4.67	<0.01

Controls were set as 100%. Results are mean ± SEM of triplicate experiments, of two replicate experiments, for each isoform and concentration.

not active on midbrain dopaminergic neurons, nor did our cultures contain significant numbers of astrocytes. Preliminary data using K252b, a molecule known to inhibit the action of brain-derived neurotrophic factor (BDNF; Knüsel and Hefti, 1992), failed to inhibit the neurotrophic action of TGF-β and activin A (K.Krieglstein, unpublished). This, together with the fact that the trophic action of BDNF on dopaminergic midbrain neurons requires a 50-fold higher dosage (Hyman *et al.*, 1991, 1994) than does TGF-β, makes it unlikely that BDNF is a mediator of the trophic actions of TGF-βs and activin in our cultures.

A number of growth factors, traditionally not viewed as mitogens, have previously been shown to have survival promoting effects for mesencephalic dopaminergic neurons (Unsicker, 1994). These include BDNF (Hyman *et al.*, 1991), neurotrophin-3 (NT-3) (Hyman *et al.*, 1994) and neurotrophin-4/5 (NT-4/5; Hyman *et al.*, 1994; Hynes *et al.*, 1994). However, even for BDNF, NT-3 and NT-4/5, the notion of a direct influence on dopaminergic neurons comes from circumstantial evidence, i.e. the documented presence of the p75 low-affinity neurotrophin receptor on a dopaminergic neuron subpopulation (Hyman *et al.*, 1991) and the presence of *trkB* and *trkC* mRNA in the adult rat substantia nigra (Hyman *et al.*, 1994). Given the diversity of putative neuronal and non-neuronal target cells for, for example, BDNF and NT-4/5 identified by expression of *trkB* (Frisen *et al.*, 1993; Zhou *et al.*, 1993), it is obvious that the precise mechanisms for the effects of neurotrophins on dopaminergic neuron survival remain to be clarified.

Irrespective of possible mechanisms of action of activin A, GDNF and TGF-βs, their marked neurotrophic effects for midbrain dopaminergic neurons raise hopes for a rational therapeutic approach to Parkinson's disease.

Materials and methods

Growth factors

Human recombinant TGF-β1 and -β2 and chicken recombinant TGF-β3 were kindly provided by Drs Sporn and Flanders (NIH, Bethesda, MD). TGF-βs were reconstituted in 4 mM HCl, 1 mg/ml BSA and stored at −80°C before being used. Bovine recombinant FGF-2 was from Boehringer (Mannheim, Germany).

Activin A was produced in *Trichoplusia ni* cells (BTI-TN-5B 1-4, High Five Cells; Invitrogen, San Diego, CA) transfected with recombinant baculovirus as described (Hüsken-Hindi *et al.*, 1993, 1994). The recombinant protein was isolated by immunoaffinity chromatography followed by reversed phase HPLC. Characterization by mass spectrometry, FSH release bioassay and receptor binding assay (Hüsken-Hindi *et al.*, 1994) showed that the recombinant protein was identical to authentic activin A.

In order to generate recombinant GDNF, mature rat GDNF was amplified by PCR using the primers (5′-AGGGATCCTCAGATACATCCACACCGTTT-3′) and (5′-AAGACTGCATATGTCACCAGATAAACAAGCGGCG-3′). The PCR product was cloned into the *Bam*HI and *Nde*I sites of pET14b (pET expression system; Novagen). The correct nature of one construct was determined by sequence analysis. Upon introduction into *Escherichia coli*, this plasmid allows the production of a histidine-tagged GDNF protein. Bacterial cultures were induced with IPTG and the protein was purified under denaturing conditions with the help of nickel chelation chromatography (according to the manufacturer's guidelines). Recombinant, His-tagged GDNF was allowed to refold in 5 mM $NaPO_4$ (pH 6.9) and 0.01% Triton X-100 at 4°C for 3 days. Denaturing protein gel electrophoresis revealed the presence of a 16 kDa protein, which corresponds to the expected size of the recombinant GDNF protein. The amount of contaminating, *E.coli*-specific proteins was less than 0.1%.

Mesencephalic cell culture

The rostral mesencephalon floor (1 mm^3 containing 90% of all midbrain dopaminergic neurons) was dissected from E14 rat (Wistar) fetuses of two to four litters according to the procedure described by Shimoda *et al.* (1992). Tissues were enzymatically dissociated in 0.25% trypsin (ICN) in calcium−magnesium-free Hanks' balanced salt solution for 15 min at 37°C. An equal volume of ice-cold horse serum cells and 1 mg/ml DNase (Boehringer Mannheim) were added prior to trituration with wide- and narrow-bored fire-polished Pasteur pipettes. Cells were then washed with Dulbecco's modified Eagle's medium (DMEM)−F12 medium (1:1; Bio-Whittaker). Single cell suspensions were diluted in complete medium, DMEM−F12 containing the N1 supplements (Bottenstein *et al.*, 1980), 0.25% bovine serum albumin (BSA; Serva), 2 mM L-glutamine, 100 units/ml penicillin, 33 mM glucose, and seeded at a density of $2.0\times10^5/cm^2$ on polyornithine (0.1 mg/ml in 15 mM borate buffer, pH 8.4; Sigma)−laminin (5 μg/ml; Sigma)-coated glass coverslips placed in 24-well plates (Falcon). Cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C. After 24 h, and subsequently every 3 days, two-thirds (500 μl) of the medium was replaced and growth factors were added at the concentrations given below. After 8 days the cultures were processed for immunocytochemistry or extraction of RNA.

Immunocytochemistry

For visualization of dopaminergic neurons, cultures were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with acetone at −20°C (10 min) and washed in phosphate-buffered saline (PBS). After blocking with 1% H_2O_2 in PBS, washing and blocking with horse serum (Vector), coverslips were stained with a mouse monoclonal antibody to rat TH (1:200, 1 h at 37°C; Boehringer Mannheim) followed by Vectastain ABC kit (Vecto Labs) detection. GFAP was immunocytochemically demonstrated after fixation and permeabilizing cells with acetone at 20°C for 20 min. Cells were then washed with PBS and incubated with mouse monoclonal anti-GFAP (1:200; Sigma), followed by incubation with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse-IgG (1:100; Sigma). The 68 and 200 kDa neurofilament subunits were detected using monoclonal mouse antibodies (1:50; Boehringer) and TRITC-conjugated secondary antibodies. For monitoring cell proliferation, BrdU (Boehringer) was added to the culture medium at a final concentration of 10 μM for 48 h at various time points during the culture period. Cells were fixed following exposure to BrdU with 70% ethanol (in 50 mM glycine buffer, pH 2.0) for 20 min at −20°C, washed extensively and incubated with anti-BrdU mouse monoclonal antibody (1:10; Boehringer), which was then visualized with fluorescein-conjugated anti-mouse-IgG.

RT-PCR of TGF-β2 and GDNF

Total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Total RNA was first reverse transcribed into cDNAs, which in turn were subjected to PCR amplification using specific primers described below. cDNA was synthesized in a final volume of 20 μl with the following components: 2.5 μg total RNA, 50 mM Tris−HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM dNTPs, 25 mM oligo(dT) primer (Gibco), 20 units of RNase inhibitor (Boehringer) and 200 units SuperScript RNase H^- reverse transcriptase (Gibco). The mixture was incubated at 37°C for 60 min.

PCR was performed in a total volume of 25 μl containing cDNA (made from 625 ng total RNA), 10 mM Tris−HCl, pH 8.8, 10 mM KCl, 2.5 mM $MgCl_2$, 0.002% Tween 20, 0.2 mM dNTPs, 1 μM 5′ primers, 1 μM 3′ primers and 1.5 units UITma DNA polymerase (Perkin Elmer) using the hot start technique in a Perkin Elmer GeneAmp PCR system 9600. Buffer, primers, $MgCl_2$ and dNTPs were placed in a thin walled 0.2 ml tube, a piece of wax was added (Perkin Elmer) and the tube was heated to 80°C for 10 min in order to melt the wax and seal the mixture. cDNA, buffer and UITma DNA polymerase were added on top of the wax, separated from the primers. During the first melting step the wax layer rises to the surface, combining primers, cDNA, dNTPs, buffer and polymerase. The amplification steps involved denaturation at 94°C for 1 min, annealing for 2 min at 60°C with GDNF primers or 58°C with TGF-β2 primers and extention at 72°C for 3 min. Samples (5 μl) of the PCR mixtures were analyzed by electrophoresis in 2% agarose gels in the presence of ethidium bromide. The GDNF primers were used as described by Schaar *et al.* (1993) and the expected size of the PCR product was 700 base pairs. RNA isolated from B49 cells, the glial cell line GDNF was originally isolated from (Lin *et al.*, 1993), was used as a positive control for GDNF amplification. The TGF-β2 primers used were 5′-TCCTACAGACTGGAGTCACAACAG-3′ and 5′-ATCATATTGGAAAGCTGTTCGATC-3′ (S.Bieger, personal communication) and the expected size of the PCR product was 359 base pairs.

Treatment of cultures with MPP+

MPP^+ was added at day 4 to cultures at a final concentration of 2 μM for a period of 48 h. The toxin was then replaced by fresh culture medium for a final 48 h period. Treatment with FGF-2 or TGF-βs started 24 h prior to the addition of MPP^+ and lasted, with one more change of medium and factors (at day 6), until the end of the experiment (day 8).

Statistics

The data were analysed by a one way ANOVA and the significance of intergroup differences was determined by applying Student's *t*-test. Differences were considered significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

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