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# ADDITIVE EFFECT OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR AND NEUROTROPHIN-4/5 ON RAT FETAL NIGRAL EXPLANT CULTURES

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**Abstract**—Transplantation of embryonic dopaminergic neurons is an experimental therapy for Parkinson's disease, but limited tissue availability and suboptimal survival of grafted dopaminergic neurons impede more widespread clinical application. Glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-4/5 (NT-4/5) exert neurotrophic effects on dopaminergic neurons via different receptor systems. In this study, we investigated possible additive or synergistic effects of combined GDNF and NT-4/5 treatment on rat embryonic (embryonic day 14) nigral explant cultures grown for 8 days.

Contrary to cultures treated with GDNF alone, cultures exposed to NT-4/5 and GDNF+NT-4/5 were significantly larger than controls (1.6- and 2.0-fold, respectively) and contained significantly more protein (1.6-fold). Treatment with GDNF, NT-4/5 and GDNF+NT-4/5 significantly increased dopamine levels in the culture medium by 1.5-, 2.5- and 4.7-fold, respectively, compared to control levels, and the numbers of surviving tyrosine hydroxylase-immunoreactive neurons increased by 1.7-, 2.1-, and 3.4-fold, respectively. Tyrosine hydroxylase enzyme activity was moderately increased in all treatment groups compared to controls. Counts of nigral neurons containing the calcium-binding protein, calbindin-D28k, revealed a marked increase in these cells by combined GDNF and NT-4/5 treatment. Western blots for neuron-specific enolase suggested an enhanced neuronal content in cultures after combination treatment, whereas the expression of glial markers was unaffected. The release of lactate dehydrogenase into the culture medium was significantly reduced for GDNF+NT-4/5-treated cultures only.

These results indicate that combined treatment with GDNF and NT4/5 may be beneficial for embryonic nigral donor tissue either prior to, or in conjunction with, intrastriatal transplantation in Parkinson's disease. 

*Key words:* tissue culture, dopamine, Parkinson's disease, calbindin.

Pretreatment of fresh or cultured nigral donor tissue with neurotrophic factors or co-grafting of genetically engineered cell lines as well as intracerebral infusions of neurotrophic factors may improve survival of grafted dopaminergic neurons (Haque et al., 1996; Espejo et al., 2000; Mendez et al., 2000). Glial cell line-derived neurotrophic factor (GDNF) belongs to a distant branch of the transforming growth factor-β superfamily (Lin et al., 1993, 1994), which also includes neurturin (Kotzbauer et al., 1996), persephin (Milbrandt et al., 1998) and the recently described artemin/enovin/neublastin (Baloh et al., 1998; Masure et al., 1999; Rosenblad et al., 2000).

The mechanism of the GDNF action is not completely understood, but recent work demonstrates that the physiological response of GDNF requires glycosyl-phosphatidylinositol (GPI)-linked proteins designated GFR-α1 and GFR-α2 which are expressed on GDNF responsive cells and bind GDNF with high and moderate affinity, respectively (Jing et al., 1997; Glazner et al., 1998; Horger et al., 1998). GDNF has been found to form a complex with GFR-α1 or GFR-α2 and the receptor tyrosine kinase Ret to induce receptor tyrosine autophosphorylation and activation (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996).

In cultures of fetal midbrain, GDNF stimulates survival and differentiation of dopaminergic neurons and increases high-affinity dopamine uptake (Lin et al.,

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*Abbreviations:* BDNF, brain-derived neurotrophic factor; CB, calbindin; DAB, 3,3′-diaminobenzidine; E, embryonic day; FFRT, free-floating roller-tube; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HBSS, Hanks' balanced salt solution; HEPES, *N*-(2-hydroxy ethyl) piperazine-*N*′-(2-ethane sulfanic acid); HPLC, high-performance liquid chromatography; IR, immunoreactive; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; NSE, neuron-specific enolase; NT-4/5, neurotrophin-4/5; PBS, phosphate-buffered saline; PD, Parkinson's disease; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase.

1993, 1994; Schatz et al., 1999; Meyer et al., 2000; Widmer et al., 2000). GDNF has also been found to reduce apoptosis in dopaminergic neurons (Clarkson et al., 1995, 1997; Burke et al., 1998; Zawada et al., 1998) and to promote dopaminergic cell survival and fiber growth in fetal nigral grafts (Johansson et al., 1995; Rosenblad et al., 1996; Sinclair et al., 1996; Granholm et al., 1997; Yurek, 1998).

Neurotrophin-4/5 (NT-4/5; Berkemeier et al., 1991; Ip et al., 1992) is a member of the neurotrophin family, which also includes nerve growth factor (NGF; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3; Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990), and neurotrophin-6 (NT-6; Götz et al., 1994). Three different types of high-affinity neurotrophin receptors have been identified (TrkA, TrkB, TrkC), where NGF acts on TrkA, BDNF and NT-4/5 on TrkB, and NT-3 on the TrkC receptor with some binding to TrkB as well (Berkemeier et al., 1991; Glass and Yancopoulos, 1993; Ip et al., 1992). Binding of neurotrophins to Trk receptor tyrosine kinases initiates signaling cascades that involve stimulation of phosphatidylinositol hydrolysis and rapid phosphorylation of phospholipase-C γl (Widmer et al., 1992, 1993a,b) and promote cell survival and differentiation through Ras-MAP kinase and phosphatidylinositol 3-kinase activities (Greene and Kaplan, 1995). The non-tyrosine kinase low-affinity NGF receptor (p75NTR) has been classified as a member of the tumor necrosis (TNF) receptor superfamily (Chao, 1994). Also BDNF, NT-4/5, and NT-3 bind to p75NTR, but the functional significance is still not completely understood (for review see Kaplan and Miller, 2000).

In mesencephalic primary cultures, NT-4/5 has been shown to increase the neuronal soma size and the dendritic branching pattern and complexity (Studer et al., 1995). NT-4/5 also stimulates survival of cultured dopaminergic neurons (Hynes et al., 1994), and it has been shown that NT-4/5 infusions can enhance the efficacy of nigral grafts in a rat model of Parkinson's disease (PD; Haque et al., 1996).

We previously reported that both human and rat mesencephalic tissue can survive transient *in vitro* storage as free-floating roller-tube (FFRT) cultures before grafting into the striatum of hemiparkinsonian rats (Spenger et al., 1994, 1995, 1996; Meyer et al., 1998), and that BDNF treatment both increased the number of cultured dopaminergic neurons and elevated the spontaneous dopamine release to the culture medium (Spenger et al., 1995; Höglinger et al., 1998). More recently, a moderate but beneficial survival-promoting effect of combined GDNF and BDNF treatment on fetal nigral tissue was found (Sautter et al., 1998).

In the present study we investigated the effect of combined exposure to recombinant human GDNF and NT-4/5 on dopaminergic cell survival and on various biochemical parameters in cultured fetal nigral tissue. The results are discussed in relation to the need for improving graft survival and function in the ongoing clinical cell replacement trials in PD.

## EXPERIMENTAL PROCEDURES

### *Preparation of cultures*

Under deep pentobarbital anesthesia, embryos (embryonic day (E) 14) were removed by Cesarean section from pregnant Sprague–Dawley rats (BRL Biological Research, Füllinsdorf, Switzerland) and killed by decapitation (all efforts were made to minimize both the suffering and number of animals used). From the isolated brains the ventral mesencephali were dissected out according to standard techniques (Dunnett and Björklund, 1992), and grown as FFRT cultures (Spenger et al., 1994). In brief, each ventral mesencephalic tissue block was divided into four equally sized pieces by a sagittal and a coronal incision. Each piece was then transferred into a labeled conical plastic tube (Falcon) with 1 ml of culture medium [55% Dulbecco's modified Eagle medium (Gibco), 32.5% Hanks' balanced salt solution (HBSS; Gibco), 1.5% glucose, 10% heat inactivated calf serum (Gibco), 1% 0.01 M HEPES (Merck)] and placed in a roller-drum (60 rev/h) in an incubator (36.5–37°C) with 5% $CO_2$ for 8 days. The culture tubes were randomly assigned to one of four groups: a control group (no neurotrophic factors added); a GDNF group, receiving 10 ng/ml GDNF (Promega) at day 0 and at each medium change at every second day; a NT-4/5 group, receiving 10 ng/ml NT-4/5 (kindly provided by Regeneron Pharmaceuticals, Tarrytown, NY, USA) according to the schedule for GDNF; and a NT4/5+GDNF group, receiving both GDNF (10 ng/ml) and NT-4/5 (10 ng/ml).

### *Dopamine measurements by high-performance liquid chromatography (HPLC)*

After 8 days, the culture medium was collected and pooled for each experimental group for dopamine analysis ($n = 9$ per group; three independent experiments). To prevent oxidative degradation of dopamine, 0.22 mg metabisulfite (Sigma) in 50 μl orthophosphoric acid (7.5%) was added per ml culture medium. Dopamine concentrations were determined by reversed phase HPLC with electrochemical detection as described previously (Studer et al., 1996). In brief, samples of stabilized medium were centrifuged at $3000 \times g$ through ultrafilters (Ultracent-10, Bio-Rad Laboratories; Ca, USA) in order to remove proteins and macromolecular constituents from the probes. The dopamine was extracted by alumina adsorption and eluted in 120 μl elution buffer per ml medium (Chromosystems). Samples were injected by means of an autosampler (ASI-5; Talbot, Switzerland) using a 100-μl injection loop. Separation was achieved by isocratic elution using a reverse-phase C18 column (Geom-Sil 80, ODS 2.5 μ, 250×4.6 mm, Grom, Germany) in a commercially available mobile phase (Chromosystems). The flow rate was adjusted to 1 ml/min (HPLC-pump, S1020, Sykam, Switzerland) resulting in a working pressure of 150 bar and leading to an elution time of 24 min for dopamine. Electroactive impurities in the mobile phase were eliminated by a guard cell (ESA Mod. 5020) at a potential of 500 mV. Quantification of the dopamine content was carried out by electrochemical detection with a dual electrode analytical cell (ESA 5011) with an oxidation potential of 400 mV.

### *Tissue processing and immunohistochemistry*

Cultures for immunohistochemistry were washed twice with ice cold HBSS before fixation in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.16% picric acid for 45 min at room temperature. Following a brief wash in PBS, cultures were cryoprotected for 24 h in 20% sucrose in 0.1 M PBS containing 0.01% $NaN_3$, frozen in gaseous $CO_2$, sectioned at 20 μm on a freezing microtome (Frigocut 2800 N, Reichert-Jung) and mounted on gelatin chrome–alum-coated glass slides. Sections were washed in PBS (3×20 min) and preincubated for 60 min in PBS containing 0.3% Triton X-100 and 10% horse serum. After a brief wash in PBS, sections were incubated for 48 h at 4°C with the primary antibodies (rabbit

anti-tyrosine hydroxylase (TH), 1:500, Pel Freez Biologicals; mouse anti-calbindin (CB)-D28k, 1:2500, Swant, Bellinzona, Switzerland) diluted in PBS containing 0.1% Triton X-100 and 2.5% horse serum. Sections were then washed for 3×15 min in PBS and incubated for 90 min with biotinylated secondary antibodies (anti-rabbit antibody, TH) or anti-mouse antibody glial fibrillary acidic protein (GFAP) and CB (Vector Laboratories, Burlingame, CA, USA) dilute 1:200 in PBS containing 0.1% Triton-X-100 and 2.5% horse serum. Endogenous tissue peroxidase was blocked by incubation in 3.3% $H_2O_2$ and 10% methanol in PBS for 10 min. Sections were then washed in PBS (3×10 min) and incubated with an avidin–biotin conjugate of peroxidase (Vector Laboratories, Burlingame, CA, USA) for 90 min. Visualization of tissue-bound antibodies was performed using a metal-enhanced 3,3′-diaminobenzidine (DAB) substrate kit (Pierce, No 34065, IL, USA). Sections were dehydrated in alcohol, cleared in xylene and coverslipped.

*Protein measurements*

The protein content in the samples was analyzed according to the method of Bradford (Bradford, 1976). Measurements were performed using a Kontron Spectrophotometer (UVIKON® 810) according to the manufacturer's instructions (Bio-Rad, Munich, Germany). Bovine serum albumin was used as a standard (Bio-Rad, Munich, Germany).

Four independent protein determinations were performed (control $n=23$, GDNF $n=16$, NT-4/5 $n=17$, GDNF+NT-4/5 $n=16$).

*TH-activity*

TH-activity was measured according to the method of Reinhard et al. (1986) with modifications. After washing with PBS, sets of four cultures corresponding to one ventral mesencephalon were pooled and the cells were lysed by sonication in 170 μl 30 mM Tris–acetic acid containing 0.1% Triton X-100, pH 7.5. The lysate was diluted 1:5 (v/v) and incubated with 2.5 nmol tyrosine HCl containing 0.4 μCi/nmol L-[ring-3,5-$^3$H]tyrosine, 50 nmol of the cofactor 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (Schricks Laboratories, Switzerland), 5000 units of catalase and 5 nM dithiothreitol in 100 mM potassium phosphate, pH 6.0, for 20 min at 37°C. TH-activity measurements in tissue culture homogenates were based on the conversion of one molecule of tyrosine to one molecule of DOPA. Tyrosine and DOPA were adsorbed using an aqueous slurry of activated charcoal in 0.1 M HCl, and the released [$^3$H]$H_2O$ was analyzed by liquid scintillation counting. Data are expressed as pmol DOPA/h. Three independent TH-activity measurements were performed (control $n=15$, GDNF $n=8$, NT-4/5 $n=9$, GDNF+NT-4/5 $n=8$).

*Western blotting*

Proteins were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide minigel electrophoresis according to the method of Laemmli (1970) with modifications. The cell lysates were analyzed for the presence of TH, neuron-specific enolase (NSE), GFAP, vimentin, and CB by immunoblotting technique. In brief, sets of control, GDNF-, NT-4/5-, and GDNF+NT-4/5-exposed cultures corresponding to one rat ventral mesencephalon were pooled for each group, washed twice in sterile, ice cold HBSS, transferred to a tube with 160 μl HBSS solution containing leupeptin (10 μl/ml) and phenylmethylsulfonyl fluoride (1 mM) and stored at −80°C until further analysis. After thawing on ice, the samples were sonicated and centrifuged. A sample of 140 μl of the supernatant was removed and two 20-μl aliquots were collected for protein determination (Bradford, 1976). To the remaining 100 μl, 50 μl sample buffer (0.2 M Tris–HCl, pH 7.0, 30% glycerol, 6% SDS, 15% 2-mercaptoethanol and 0.0075% Bromophenol Blue) were added. Samples were stored at −40°C until use. The lysates (5–10 μg/lane) were subjected to a 12% SDS–polyacrylamide separating gel as described previously (Widmer et al., 1993b). One gel was stained with Coomassie Brilliant Blue R-250 and the sister gels subjected to western blotting. Proteins were blotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Electrophoretic transfer was performed at a constant current of 0.25 A for 2 h at 4°C in 0.03 M Tris buffer (pH 8.3) containing 0.2 M glycine, 20% methanol and 0.02% SDS. Blotting efficiency was investigated by staining the gels for residual protein bands with Coomassie Blue. After transfer, the PVDF membrane was blocked with 0.15% Casein PBS. After washing, the blots were incubated overnight at 4°C with mouse anti-TH (1:500, Boehringer Mannheim), rabbit anti-NSE (1:700, Chemicon), mouse anti-GFAP (1:1000, Boehringer Mannheim), mouse anti-vimentin (1:1000, Sigma), and mouse anti-CB (1:300, Swant, Bellinzona, Switzerland) antibodies. The blots were then washed with PBS containing 0.2% Tween 20 and subsequently incubated at room temperature with preformed antibody-peroxidase conjugates (anti-mouse antibody, 1:20 000, Nordic Immunological Laboratories, USA; anti-rabbit antibody, 1:10000, Jackson Immunoresearch Laboratories, USA) for 2 h. The blots were washed in PBS containing 0.2% Tween 20 followed by a rinse with distilled water. Visualization of bound antibody was performed using the Chemiluminescent substrate kit (Pierce). All experiments were done in triplicate.

*Lactate dehydrogenase (LDH) efflux*

Lactate dehydrogenase (LDH), released from dead or degenerating cells with permeabilized membranes into the culture medium, was analyzed at day 8 according to the method of Vassault (1983) by a fully automatic spectrophotometer (COBAS MIRA, Roche). The medium collected for LDH determination was always frozen immediately and stored at −20°C until analysis. Before each session of sample measurements, the changes in absorbance of standard LDH solutions (Boehringer Mannheim) were measured for calculation of a standard curve. Samples of media (20 μl), pyruvate (Sigma) and NADH; Sigma) dissolved in TBS were prepared, and the absorbance (340 nm; 37°C) of the reaction mixture, an index of NADH concentration, recorded automatically for a period of 120 s, starting with a lagtime of 30 s. The LDH activity was calculated from the slope of the linear portion of the absorbance curve.

Two independent LDH measurements were performed (control $n=6$, GDNF $n=6$, NT-4/5 $n=6$, GDNF+NT-4/5 $n=6$).

*Morphometric analysis and cell counts*

To analyze culture volumes, the area of every third section was measured using the NIH Image 1.49 analysis program for Macintosh. In brief, video microscope images of culture sections were captured, the culture boundaries traced in a calibrated image window, and the area calculated. To estimate total culture volumes ($n=14$ per experimental group), the areas of the sections were multiplied by the section thickness and by 3 (every third section analyzed). The numbers of TH-immunoreactive (IR) and CB-IR neurons were counted on coded slides, to allow analysis by experimenter's 'blinded' to sample identity, using bright field microscopy with a ×20 objective. Only cells displaying an intense staining with a well preserved cellular structure were counted. The estimation of total cell numbers was based on cell counts performed on every sixth culture section throughout the explant, corrected for double counting according to Abercrombie's formula (Abercrombie, 1946), using the average diameter of the nuclei in the cells (Meyer et al., 1999), which did not differ significantly between the experimental groups. The size of the nuclei was estimated using a neuron tracing system (Neurolucida, MicroBrightField). Counts of TH-IR and CB-IR cells were performed using cultures from five and four independent experiments, respectively (TH-IR cells, $n=34$–62; CB-IR cells, $n=20$–38 per experimental group).

*Statistical analysis*

The statistical comparisons were performed by means of com-

mercially available software packages (Statistica 5.0, StatSoft; Instat, GraphPad Software). The experimental groups were compared using analysis of variance (ANOVA) followed by Scheffes's post-hoc test. Differences were considered statistically significant at $P < 0.05$. All values are presented as mean ± S.E.M.

## RESULTS

### *Effect of growth factor treatment on culture volume and protein content*

Cultures from all experimental groups showed a steady increase in volume during the culture period as observed by routine inspection. At day 4, the cultured mesencephalic tissue had formed small spheres, and at day 8 the culture diameter ranged between 0.9 and 1.5 mm. At that time-point, cultures treated with NT-4/5 or GDNF+NT-4/5 were significantly larger than controls [59% ($P < 0.05$) and 101% ($P < 0.01$), respectively], whereas GDNF-treated cultures were not different from controls ($P > 0.05$; Table 1). Similarly, cultures treated with NT-4/5 or GDNF+NT-4/5 contained significantly more protein, expressed per one ventral mesencephalon, than control cultures [62.6% ($P < 0.05$) and 63.1% ($P < 0.05$), respectively]. GDNF-treated cultures displayed a non-significant tendency towards higher protein levels than controls ($P > 0.05$; Table 1).

### *Dopamine release, survival and morphology of TH-IR neurons*

HPLC analysis of medium from 8-day-old cultures (Table 1) revealed that the dopamine content was significantly increased in all neurotrophic factor-treated groups compared to untreated controls [GDNF = 147% ($P < 0.01$); NT-4/5 = 253% ($P < 0.01$); GDNF+NT-4/5 = 469% ($P < 0.001$)]. The culture medium of the GDNF+NT-4/5 group contained significantly more dopamine than any of the other experimental groups. When the dopamine values obtained after single GDNF or NT-4/5 treatments were added and compared with the dopamine content obtained with the combination treatment, there was no significant difference ($P > 0.05$) pointing to an additive effect of the combined treatment.

The 8-day-old cultures from all experimental groups

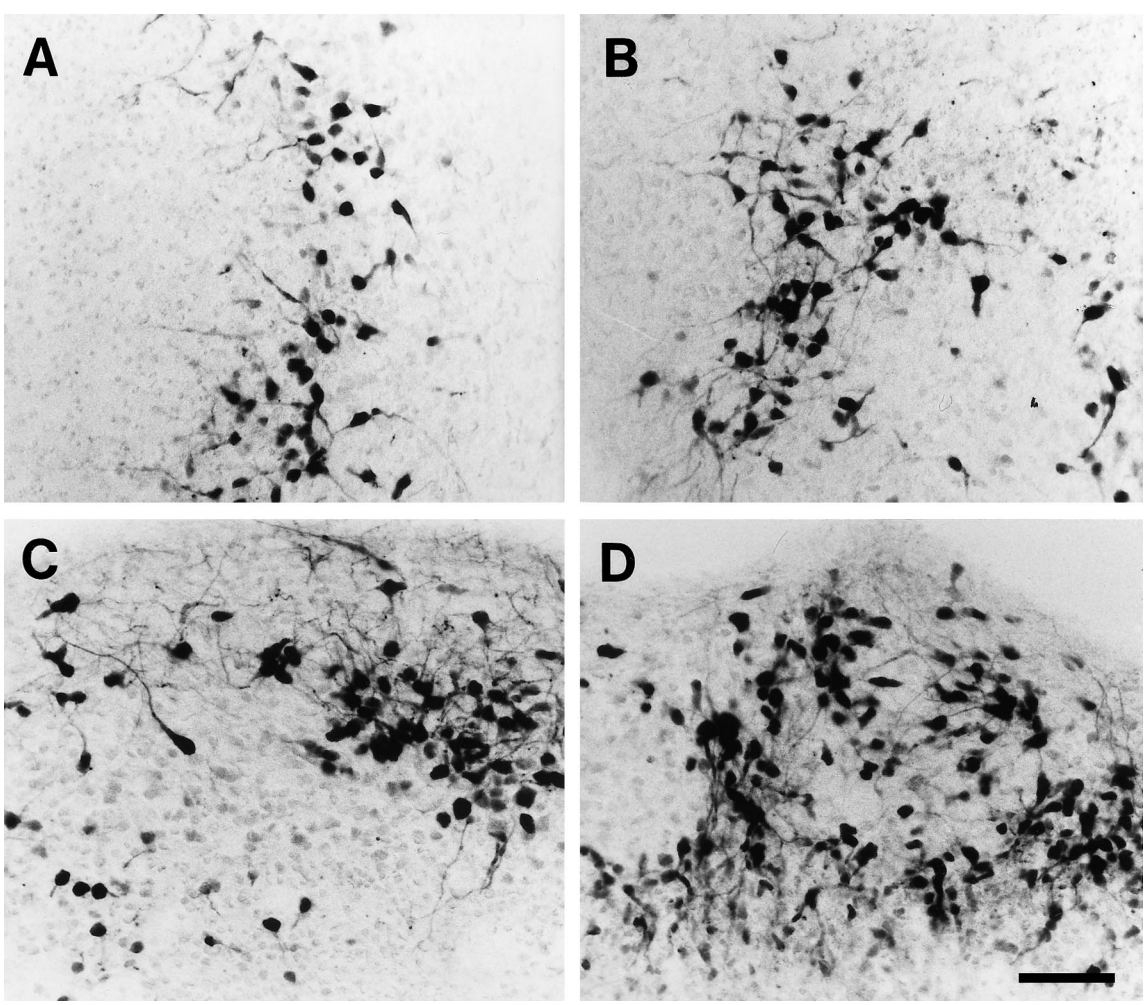


Fig. 1. TH-IR cells in sections of rat ventral mesencephalic FFRT cultures grown for 8 days without (A) and with addition of GDNF (B), NT-4/5 (C), or GDNF+NT-4/5 (D). Both GDNF (10 ng/ml) and NT-4/5 (10 ng/ml) improved TH-IR cell survival, and when applied together (10 ng/ml of each factor) they had an additive effect (see Fig. 2B). Scale bar = 50 μm.

Table 1. Summary of data (mean ± S.E.M.)

| | Control | GDNF | NT-4/5 | GDNF+NT-4/5 |
|---|---|---|---|---|
| Culture volume ($mm^3$) | 0.71 ± 0.08 | 0.79 ± 0.08 | 1.13 ± 0.10$^{\#}$ | 1.43 ± 0.13$^{\#\#}$ |
| Protein (μg/ventral mesencephalon) | 94.4 ± 5.2 | 119.8 ± 8.0 | 153.5 ± 11.5$^{\#}$ | 154.0 ± 9.7$^{\#}$ |
| Dopamine release (pg/ml) | 44.7 ± 8.8 | 110.4 ± 21.7$^{\#\#}$ | 157.8 ± 17.2$^{\#\#}$ | 254.3 ± 34.4$^{\#\#\#}$ |
| TH-IR cells | 1051 ± 189 | 1725 ± 213$^{\#}$ | 2272 ± 241$^{\#\#}$ | 3594 ± 354$^{\#\#\#}$ |
| TH-activity (pmol/h) | 127.9 ± 9.7 | 188.3 ± 16.3$^{\#}$ | 179.9 ± 7.2$^{\#}$ | 169.8 ± 19.1$^{\#}$ |
| Dopamine/TH-IR cell (fg/ml) | 42 ± 6 | 64 ± 6$^{\#}$ | 69 ± 5$^{\#}$ | 71 ± 6$^{\#}$ |
| TH-IR ($cells/mm^3$) | 1480 ± 29 | 2184 ± 31$^{\#\#}$ | 2011 ± 28$^{\#}$ | 2513 ± 31$^{\#\#}$ |
| Western blotting | | | | |
| NSE | + | + | ++ | +++ |
| TH | + | + | + | + |
| CB | (+) | + | + | ++ |
| CB-IR cells (percent of controls) | 100 ± 10 | 162 ± 13$^{\#}$ | 159 ± 15$^{\#}$ | 217 ± 29$^{\#\#}$ |
| Western blotting | | | | |
| GFAP | + | + | + | + |
| Vimentin | +++ | +++ | +++ | +++ |
| LDH release (U/l/μg protein, percent of controls) | 100 ± 6 | 79 ± 8 | 70 ± 10 | 56 ± 9$^{\#}$ |

$^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$. (+), very weak signal; +, weak signal; ++, moderate signal; +++, strong signal.

contained many TH-IR neurons (Table 1) with one to four primary neurites and polygonal or oval shaped somata. No marked morphological differences between TH-IR neurons in control and GDNF-treated cultures were observed, but in NT-4/5 or combination-treated cultures, the TH-IR cells appeared to display a more differentiated morphology, although a detailed analysis of TH-IR cell morphology was beyond the scope of the present study. For cultures from all experimental groups, a gradient of differentiation was found according to location of the cells with TH-IR cells located in the periphery of the spherical cultures displaying a more mature morphology than cells located centrally. In particular for cultures treated with NT-4/5 or GDNF+NT-4/5, this gradient of differentiation was less pronounced, and the TH-IR cells and the TH-IR cell clusters appeared more homogeneously distributed than in control cultures. GDNF, NT-4/5 and GDNF+NT-4/5-treated cultures contained significantly more TH-IR neurons compared to controls [GDNF = 64% ($P < 0.05$); NT-4/5 = 116% ($P < 0.01$); GDNF+NT-4/5 = 242% ($P < 0.001$); Table 1, Fig. 1]. Simple addition of the numbers of TH-IR neurons for the groups of cultures treated with GDNF or NT-4/5 did not differ from the number of cells found after combination treatment ($P > 0.05$), indicating a regular additive effect of the two neurotrophic factors. There was no difference in TH-IR cell numbers between cultures treated with GDNF or NT-4/5.

When the dopamine content in the culture medium was correlated with the number of TH-IR neurons (i.e., the average content of dopamine in the medium divided by the average number of TH-IR cells in the experimental group), the dopamine content per TH-IR neuron was increased in neurotrophic factor-treated groups compared to controls [GDNF = 52% ($P < 0.05$); NT-4/5 = 64% ($P < 0.05$); GDNF+NT-4/5 = 69% ($P < 0.05$)]. No difference between single factor-treated and combination-treated cultures was found with regard to dopamine content per TH-IR cell (Table 1). The density of TH-IR cells (average number of cells divided by the average culture volume in the experimental group) was higher in all growth factor-treated cultures compared to controls [GDNF = 48% ($P < 0.01$); NT-4/5 = 36% ($P < 0.05$); GDNF+NT-4/5 = 70% ($P < 0.01$); Table 1]. No significant additive effect of combined GDNF and NT-4/5 treatment was found.

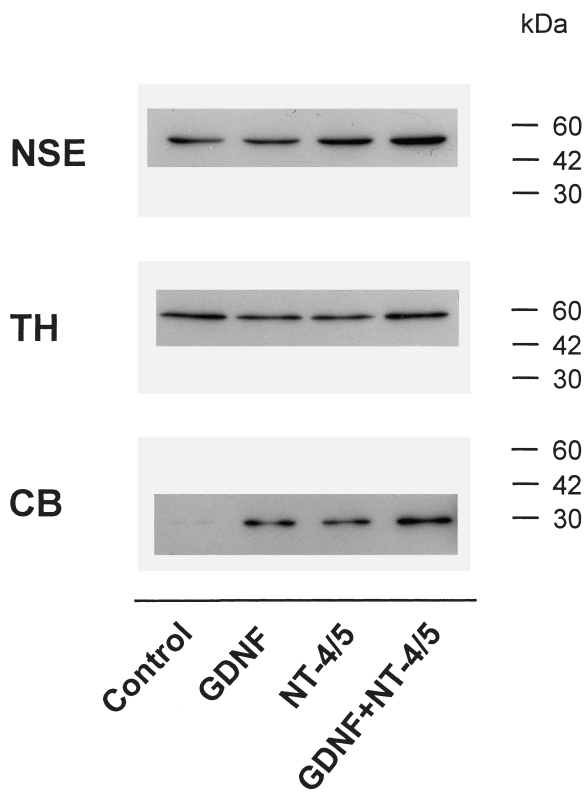


Fig. 2. Western blots of NSE, TH, and CB in tissue from rat ventral mesencephalic cultures grown for 8 days with or without addition of GDNF (10 ng/ml), NT-4/5 (10 ng/ml), or GDNF+NT-4/5 (10 ng/ml of each factor). NT-4/5 and GDNF+NT-4/5 induced NSE bands with higher signal intensity compared GDNF and controls. For GDNF+NT-4/5 the signal appeared stronger than for NT-4/5. The signal intensity for TH was not changed markedly by any of the growth factor treatments, but intensities of CB bands were much stronger after GDNF and NT-4/5 treatment. A pronounced additive effect in CB signal intensity was seen for the GDNF+NT-4/5 treatment group.

*TH-activity measurements and western blotting for NSE and TH*

Analysis of TH-activity in tissue from 8-day-old cultures (Table 1) revealed that the enzyme activity was moderately increased in all treatment groups compared to the activity in untreated control cultures [GDNF = 47% ($P < 0.05$), NT-4/5 = 41% ($P < 0.05$), GDNF+NT-4/5 = 33% ($P < 0.05$)]. No significant differences were found between the different treatment groups.

Western blotting for NSE in tissue from 8-day-old cultures (Fig. 2) showed the following ranking of NSE band intensities: control ~ GDNF < NT-4/5 < GDNF+NT-4/5, suggesting an additive or synergistic effect of combined GDNF and NT-4/5 treatment on neuronal elements in the cultures. In contrast, the TH bands derived from control, GDNF-, NT-4/5-, and GDNF+NT-4/5-treated cultures all had similar signal intensities.

*Effect of GDNF and NT-4/5 treatment on CB-containing cells*

Immunostaining for the calcium-binding protein, CB, which is expressed in a subpopulation of nigral dopaminergic neurons, showed the presence of this protein in a relatively high proportion of cells in the ventral mesencephalic cultures (Fig. 3). Almost all CB-IR cells had a neuronal morphology, and the distribution pattern of the cells within the cultures resembled that of the TH-IR cells. Cell counts showed that cultures in all treatment groups contained significantly more CB-IR neurons than the control cultures, with most CB-IR cells being found in the GDNF+NT-4/5-treated group, indicating an additive effect of the neurotrophic factors (GDNF = 62% ($P < 0.05$), NT-4/5 = 59% ($P < 0.05$), GDNF+NT-4/5 = 117% ($P < 0.01$) compared to controls; Table 1).

In accordance with the cell counts, western blots for CB of 8-day-old cultures showed a marked increase in signal intensity compared to control after both GDNF and NT-4/5 treatment, and an even stronger signal after combined GDNF and NT-4/5 treatment (Fig. 2), again pointing to an additive or synergistic effect of the two neurotrophic factors.

*Effect of GDNF and NT-4/5 on glial cells*

Western blotting for the astroglial marker GFAP and

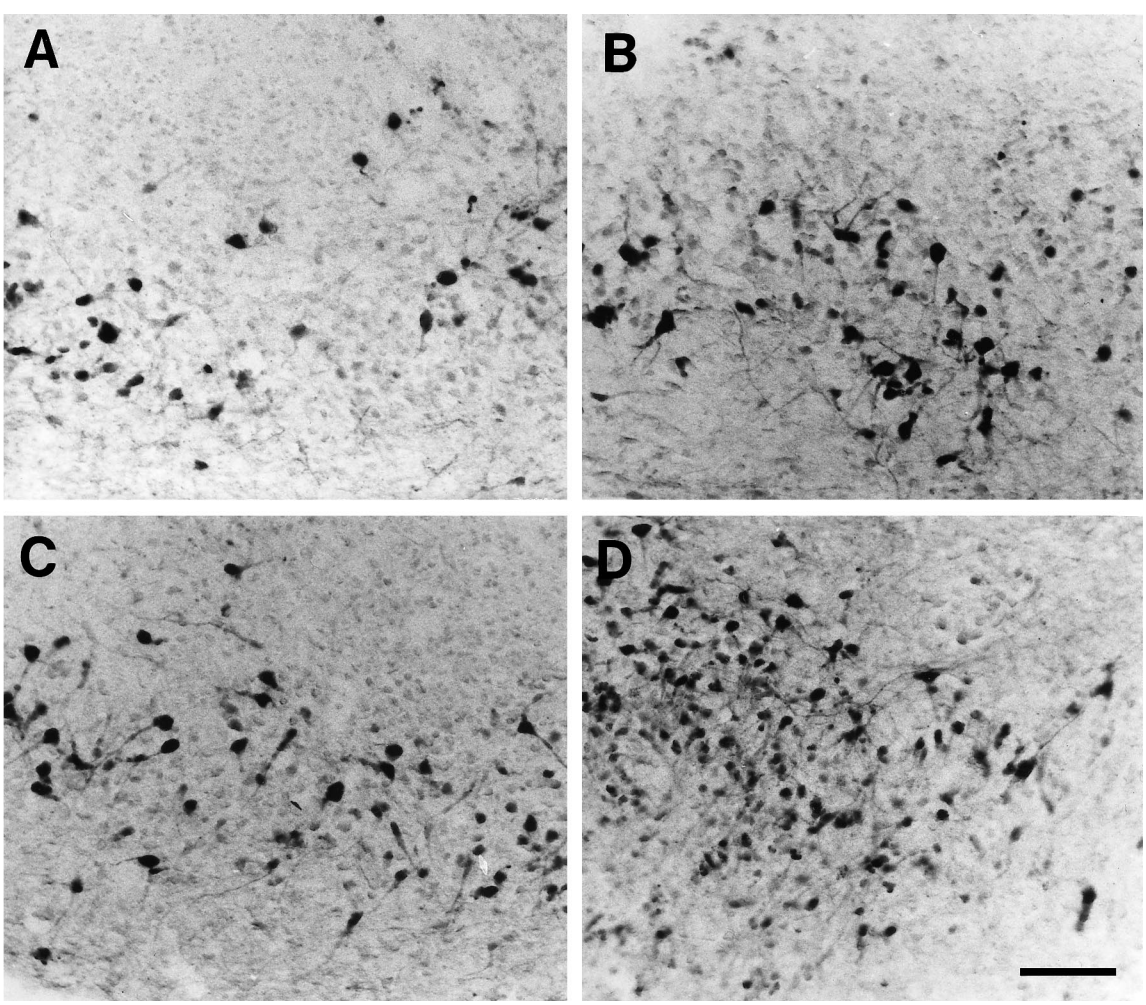


Fig. 3. CB-IR cells in sections of rat ventral mesencephalic FFRT cultures grown for 8 days without (A) or with addition of GDNF (B), NT-4/5 (C), or GDNF+NT-4/5 (D). Both GDNF and NT-4/5 improved CB-IR cell survival, and addition of the two factors together had an additive effect on the cell numbers (see also Table 1). Scale bar = 50 μm.

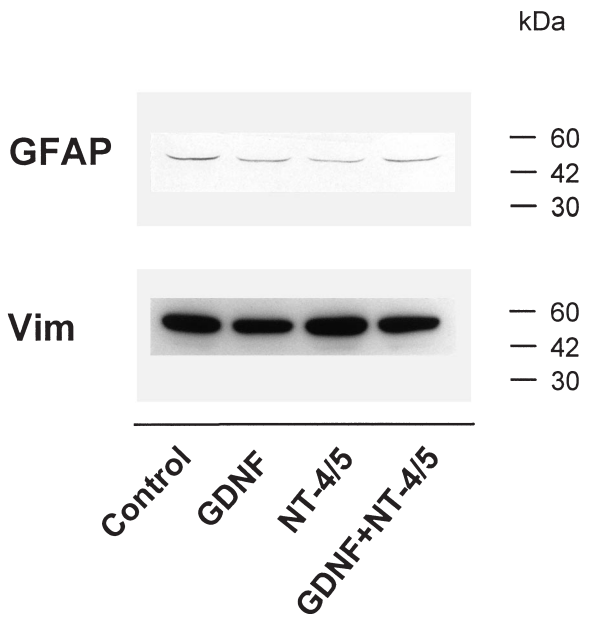


Fig. 4. Western blots of the astroglial marker GFAP and the cytoskeletal intermediate filament vimentin (Vim) in tissue derived from 8-day-old cultures grown with or without GDNF (10 ng/ml), NT-4/5 (10 ng/ml) or GDNF+NT-4/5 (10 ng/ml of each factor). No significant changes in signal intensity were observed after any of the growth factor treatments.

the intermediate filament protein vimentin, expressed by reactive or undifferentiated glial cells, did not reveal significant changes after GDNF, NT-4/5, or GDNF+NT-4/5 treatment (Fig. 4).

*LDH release to culture medium*

LDH measurements of the medium from 8-day-old cultures, expressed as LDH/μg protein, indicated a decreased enzyme release from GDNF- and NT-4/5-treated cultures by 21% and 30%, respectively ($P > 0.05$), while the combined GDNF+NT-4/5 treatment gave a significant 44% decrease compared to control ($P < 0.05$; Table 1). These findings suggest a pronounced reduction of cellular degeneration by combined treatment.

## DISCUSSION

Intracerebral transplantation into severely diseased parkinsonian patients has provided promising results in several clinical trials, but suboptimal survival or differentiation of grafted dopaminergic neurons as well as ethical concerns and the limited availability of embryonic human donor tissue prevent a more widespread application as described in several papers (Lindvall et al., 1990; Freed et al., 1992; Freeman et al., 1995; Olanow et al., 1996; Kordower et al., 1998; Tabbal et al., 1998; Borlongan et al., 1999; Dunnett and Björklund, 1999). Transient storage of donor tissue in FFRT culture offers the possibility of pregrafting treatment of cells with growth factors as well as pooling of tissue from several donors in order to obtain larger and more functional grafts. Such storage would also facilitate planning and timing of the transplantation in relation to procurement of donor tissue and allow screening for infectious agents and/or the potential removal of undesirable subsets of cells.

The growth and differentiation of specified types of neurons are likely to require the presence of various neurotrophic factors and combinations thereof. Receptors for neurotrophic factors display discrete distribution patterns with apparent overlaps (Lindsay et al., 1994). A single neuron may accordingly be under the influence of several factors by way of different or common intracellular signal transduction pathways. Accordingly, several neurotrophic factors promote survival and differentiation of dopaminergic neurons (Lin et al., 1993; Hyman et al., 1994; Hynes et al., 1994; Höglinger et al., 1998; Meyer et al., 2000; Rosenblad et al., 2000). However, little is known about additive or synergistic effects of combinations of growth factors or other neuroprotective agents, despite the potential importance of such applications for optimization of cell replacement therapies in PD. There is evidence that combinations of GDNF, BDNF, and ciliary neurotrophic factor may be effective in improving motoneuron survival and differentiation (Zurn et al., 1996), and it has been reported that GDNF and cardiotrophin-1 have a synergistic survival-promoting effect on these cells (Arce et al., 1998).

In the present work we demonstrate that combined treatment with the non-competitive neurotrophic factors GDNF and NT-4/5 is superior to each of the single factors in improving the survival and expression of several structural and biochemical functional markers of cultured fetal dopaminergic neurons (Table 1).

*Effects of GDNF and NT-4/5 on volume, total protein content, and neuronal and glial markers*

The increase in culture volumes observed after addition of NT-4/5 or GDNF+NT-4/5 to the culture medium corresponded to the data on increased total protein after the same treatments. The lack of effect on culture volume after exposure to GDNF alone (with a non-significant tendency for higher protein content) may indicate that GDNF primarily acts on the dopaminergic cell population. By contrast, our results indicate that NT-4/5 treatment may stimulate the survival and growth of a wider range of neurons, which is supported by cell culture studies showing that NT-4/5 promotes survival, morphological and biochemical differentiation of striatal and cortical GABAergic neurons (Widmer and Hefti, 1994a,b; Ventimiglia et al., 1995). Moreover, it appears unlikely that the larger volume and higher protein levels in NT-4/5-treated cultures is due to glial proliferation since western blots for the astroglial marker GFAP and the marker for undifferentiated glial cells (vimentin) showed unchanged levels after treatment. Studies on striatal cultures showing that NT-4/5 promotes survival and differentiation of GABAergic neurons (Widmer and Hefti, 1994b) may suggest that stimulation of mesencephalic GABAergic neurons plays a role for the larger volume and the higher protein content, but verifica-

tion requires a detailed analysis of the GABAergic neurons.

*Survival and density of TH-IR cells, TH enzyme activity and release of dopamine*

The presence of dopamine in the tissue culture medium is interpreted to reflect a basal release, induced by spontaneous activity within the neuronal network (Blöchl and Sirrenberg, 1996). In the present study, HPLC measurements of dopamine in the culture medium revealed pronounced effects of GDNF and NT-4/5. Interestingly, GDNF and NT-4/5 had an additive effect. Knowing that TH-IR cell numbers correlate with dopamine content in the culture medium (Studer et al., 1996), it was to be expected from the dopamine measurements, that the GDNF- and/or NT-4/5-treated cultures had an increase in TH-IR cells. The higher number of TH-IR cells in GDNF-treated cultures compared to controls is in agreement with previous work (Sautter et al., 1998; Meyer et al., 1999). FFRT cultures exposed to both GDNF and NT-4/5 revealed an additive effect of combining the two factors. Two recent parallel experiments on dissociated cultures have confirmed that GDNF and NT-4/5 exert a significant additive effect on the number of dopaminergic neurons (data not shown). The effect was, however, less pronounced in these low-density cultures than in our solid FFRT cultures, suggesting that mechanisms partially requiring intimate cell contact also are involved.

A number of dose–response studies for GDNF (Thajeb et al., 1997; Lingor et al., 2000; Rosenblad et al., 2000) have shown that the optimal effect can be obtained at concentrations of around 10 ng/ml. Furthermore, it has been shown that GDNF treatment in a concentration of 10 ng/ml produces maximal levels of Ret phosphorylation, dopamine uptake, a profound increase in phosphatidylinositol 3-kinase activity, and morphological differentiation of mesencephalic neurons (Pong et al., 1997, 1998; Widmer et al., 2000). The study by Lingor and co-workers (Lingor et al., 2000) also reported TrkB receptor saturation at a NT-4/5 concentration of around 10 ng/ml. Based on these finding we used concentrations of GDNF and NT-4/5 of 10 ng/ml in the present studies. It cannot be excluded, however, that detailed and extended dose–response experiments for the combined growth factors treatment would have resulted in better survival-promoting effects.

All neurotrophic factor treatment groups contained populations of dopaminergic neurons at a higher cell density than controls, but no additive effect of combined GDNF and NT-4/5 treatment was found. The western blots for TH did not display the same changes in signal intensity after growth factor treatment. The reason for this remains unclear, but it might relate to the fact that TH-IR cells comprise only a relatively small fraction of the neurons in the cultures or limitations in the western blotting technique.

The marked effect of the neurotrophic factors on the dopamine content in the culture medium, expressed per TH-IR cell, is suggestive of a neurotrophic factor-induced increase of dopamine production and/or release per TH-IR cell, in line with previous findings for BDNF (Höglinger et al., 1998). However, there was no additive effect of GDNF and NT-4/5. Results on TH enzyme activity provide further evidence for an enhanced dopamine production, and the lack of further enhancement of TH-activity after combined treatment corresponds to calculations on dopamine release per TH-IR cell. One mechanism that should be considered here is that neurotrophic factors also affect the dendritic branching pattern of the cells and systems involved in dopamine re-uptake and turn-over (Studer et al., 1995; Kramer et al., 1999), and therefore future experiments should address, how these parameters are influenced by combined GDNF and NT-4/5 treatment. The enhanced TH-activity and dopamine production per TH-IR cell suggest that the functional capacity of dopaminergic neurons can be modulated and improved by neurotrophic factor treatment, which may be of potential importance for the functional outcome in cell replacement strategies.

*Effect of GDNF and NT-4/5 on CB-IR cells*

CB-D28k is a calcium-binding protein, which binds calcium ions with high affinity (Heizmann and Hunziker, 1991; Andressen et al., 1993). Such calcium-binding proteins are heterogeneously distributed throughout the mammalian CNS (Celio, 1990; Résibois and Rogers, 1992; Rogers, 1992), and the buffering of intracellular calcium overloads is thought to exert neuroprotective actions (Celio, 1990).

NT-4/5 has been shown to protect nigral CB-containing neurons in rats following medial forebrain bundle transections (Alexi and Hefti, 1996). Recently we reported that GDNF improves the survival and morphological differentiation of cultured nigral CB-containing cells (Meyer et al., 1999; Bauer et al., 2000; Widmer et al., 2000). This was confirmed in the present study, which moreover showed that NT-4/5 has a similar effect on CB-IR cell survival and/or phenotypic differentiation and that combined GDNF and NT-4/5 treatment had an additive effect. Whether the two factors act on distinct or overlapping subpopulations of TH-IR and CB-IR neurons remains to be elucidated. In a previous developmental study, we showed that there was no significant loss of TH-IR cells within the first week in culture (Höglinger et al., 1998). This suggests that the neurotrophic factors preferentially may regulate phenotypic differentiation rather than dopaminergic cell survival. Future studies will address these issues.

The effect of GDNF and NT-4/5 on CB-IR cells can be viewed in relation to the known heterogeneous distribution pattern of these cells within the dopaminergic subfields of the substantia nigra and the ventral tegmental area (Celio, 1990; Arai et al., 1991; Résibois and Rogers, 1992; Rogers, 1992; McRitchie et al., 1996; Alfahel-Kakunda and Silverman, 1997; Nemoto et al., 1999), and also in relation to the reported relative sparing of the CB-expressing dopaminergic neurons in PD (Yamada et al., 1990; Hirsch et al., 1992).

The functional and practical significance of dopaminergic CB-IR cells as donor cells for transplantation

therapies are not known, but given that CB-containing dopaminergic neurons might be more resistant to disease and injury, even though these findings are equivocal (Barrot et al., 2000), this subset of neurons deserves further study.

*Release of LDH*

LDH levels were reduced only in media from GDNF+NT-4/5-treated cultures. LDH is an abundant and relatively stable cytoplasmic enzyme that is released into the surrounding medium once the cell membrane is damaged. Measurements of LDH do not clarify the mode of injury or cell death which can be either necrotic or apoptotic. The detailed mechanisms underlying the higher numbers of TH-IR cells in cultures treated with GDNF and NT-4/5 are not well known, but the fact that GDNF can reduce apoptosis in dopaminergic neurons (Clarkson et al., 1995, 1997; Burke et al., 1998; Zawada et al., 1998), combined with our finding of reduced LDH efflux and higher levels of CB-IR cells, suggest that also anti-apoptotic and neuroprotective mechanisms have been operative.

*Conclusion*

The combined treatment of cultured fetal nigral tissue with the non-competitive growth factors GDNF and NT-4/5 appears to be very beneficial for dopaminergic cell survival and functionally related biochemical parameters. Ongoing and future experiments will investigate whether similar effects can be obtained for embryonic human tissue, and whether short-term combined GDNF and NT-4/5 pretreatment of donor tissue can improve subsequent graft survival and function in experimental animal models of PD.

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