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Glial cell line-derived neurotrophic factor up-regulates GTP-cyclohydrolase I activity and tetrahydrobiopterin levels in primary dopaminergic neurones

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) protects dopaminergic neurones against toxic and physical damage. In addition, GDNF promotes differentiation and structural integrity of dopaminergic neurones. Here we show that GDNF can support the function of primary dopaminergic neurones by triggering activation of GTP-cyclohydrolase I (GTPCH I), a key enzyme in catecholamine biosynthesis. GDNF stimulation of primary dopaminergic neurones expressing both tyrosine 3-monooxygenase and GTPCH I resulted in a dose-dependent doubling of GTPCH I activity, and a concomitant increase in tetrahydrobiopterin levels whereas tyrosine 3-monooxygenase activity was not altered. Actinomycin D, as an inhibitor of *de novo* biosynthesis, abolished any GDNF-mediated up-regulation of GTPCH I activity. However, GTPCH I mRNA levels in primary dopaminergic neurones were not altered by GDNF treatment, suggesting that the mode of action for that up-regulation is not directly connected to the regulation of GTPCH I transcription. We conclude that GDNF, in addition to its action in structural differentiation, also promotes differentiation regarding expression and enzymatic activity of a crucial component in the dopaminergic biosynthetic pathway.

Keywords: dopaminergic neurones, glial cell line-derived neurotrophic factor, GTP-cyclohydrolase I, ventral mesencephalon.

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Glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-β superfamily (Lin *et al*. 1993), is a potent survival factor for midbrain dopaminergic neurones both *in vitro* and *in vivo*. GDNF treatment of dopaminergic neurones results in increased cell survival *in vitro* (Lin *et al*. 1993) and protection against toxin and injury-induced cell death (Beck *et al*. 1995; Bowenkamp *et al*. 1995; Choi-Lundberg *et al*. 1997; Burke *et al*. 1998). In addition, dopaminergic neurones exposed to GDNF show morphological changes, including more extensive fibre outgrowth as well as increased cell body size (Akerud *et al*. 1999).

Except for morphological changes, knowledge on the functional effects of GDNF regarding the metabolism of

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Abbreviations used: AD, actinomycin D; BH_4, 5,6,7,8-tetrahydrobiopterin; CNTF, ciliary neurotrophic factor; DA, dopamine; DIV5, days *in vitro* 5; 3,4-DOPAC, dihydroxyphenylacetic acid; DMEM, Dulbecco's modified Eagle's medium; E14, embryonic day 14; FFRT, free floating roller tube; GNDF, glial cell line-derived neurotrophic factor; GTPCH I, GTP-cyclohydrolase I; HBSS, Hank's balanced salt solution; HVA, homovanillic acid; NGF, nerve growth factor; NO, nitric oxide; PB, phosphate buffer; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TH, tyrosine 3-monooxygenase; VM, ventral mesencephalic.

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primary dopaminergic neurones is thus far limited. There is evidence that GDNF accelerates high-affinity dopamine (DA) uptake in fetal rat ventral mesencephalic (VM) cultures *in vitro* (Lin *et al*. 1993). In addition, *in vivo* application of GDNF into the *substantia nigra* increased both DA storage and the amount of available DA to be released after *d*-amphetamine stimulation (Hebert *et al*. 1996). However, the important question of whether GDNF can also affect DA production remains unanswered.

DA production depends on two rate-limiting enzymes, tyrosine 3-monooxygenase (TH, EC 1.14.16.2) and GTP-cyclohydrolase I (GTPCH I, EC 3.5.4.16), expressed in VM neurones. TH catalyses the rate-limiting, first essential step in the synthesis of the catecholamines DA, norepinephrine and epinephrine (Nichol *et al*. 1985; Fitzpatrick 2000). TH activity, as well as the enzyme's expression and protein stability, are regulated by signalling through discrete, receptor-mediated pathways that include the cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) (reviewed by Kumer and Vrana 1996). For its activation, TH depends strictly on 5,6,7,8-tetrahydrobiopterin (BH_4) as a cofactor (Kettler *et al*. 1974; Abou-Donia *et al*. 1986). The first and rate-limiting step for BH_4 synthesis is catalyzed by the enzyme GTPCH I; BH_4 serves as cofactor for TH, phenylalanine 4-monooxygenase (EC 1.14.16.1), tryptophan 5-monooxygenase (EC 1.14.16.4) and nitric oxide (NO) synthase (EC 1.14.13.39) (Fitzpatrick 1999; Mayer and Hemmens 1997). Besides its properties as cofactor in biochemical pathways, BH_4 application has been linked to proliferation and survival of neuronal cells *in vitro* (Anastasiadis *et al*. 1996; Anastasiadis *et al*. 2001). Treatment with (6*R*)-BH_4 enhances survival of PC12 pheochromocytoma cells through the activation of Ca^{2+} channels via the cAMP pathway (Koshimura *et al*. 1999). Furthermore, (6*R*)-BH_4 has been shown to protect PC12 cells from NO-mediated toxicity (Koshimura *et al*. 1998). It has been previously demonstrated that nerve growth factor (NGF) treatment of superior cervical ganglia tissue results in elevated TH and GTPCH I gene expression and enzyme activities (Hirayama and Kapatos 1995). On the other hand, ciliary neurotrophic factor (CNTF) application causes a decrease in GTPCH I enzyme activity, associated with decreased GTPCH I mRNA levels (Stegenga *et al*. 1996). These findings suggest a more general scheme for neurotrophic factor-mediated regulation of these two rate-limiting enzymes in catecholamine synthesis. Similar to the role of cytokines in the immune system that act not only as survival factors but also as inducers and enhancers of discrete decisions for function, neurotrophic factors might play an analogous role for discrete sets of neurones.

In light of the above-mentioned findings on the regulation of the two key enzymes involved in DA production, it seemed worthwhile to investigate whether GDNF, besides its effects on DA storage and release, also influences molecular components of the catecholamine biosynthesis machinery. As a cellular model system for this study, we have chosen fetal dopaminergic neurones within organotypic VM cultures, using the previously established 'free floating roller tube' (FFRT) culture system (Spenger *et al*. 1994). Organotypic tissue preserves many of the physiological features of intact *in situ* tissue. Here we report that GDNF can induce GTPCH I activity and generation of BH_4, thus suggesting a mechanism as to how GDNF can influence DA production.

Materials and methods

Tissue culture

VM was explanted from embryonic day 14 (E14) Sprague–Dawley rats (BRL Biological Research, Füllinsdorf, Switzerland). Dissection was performed under standardized conditions (Dunnett and Bjorklund 1992). VM explants were divided into four equally sized pieces and transferred to conical tubes (Falcon) containing 1 mL culture medium [100 mL of medium consisted of 55% Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA), 32.5% Hank's balanced salt solution (HBSS) (Gibco BRL), 1.5% glucose (20% solution), 2.4% Hepes-solution and 10% fetal calf serum (Gibco BRL)]. Tubes were transferred into a roller drum (Gähwiler 1981) and maintained for 7 days *in vitro* (DIV7) as organotypic FFRT cultures, as described by Spenger *et al*. (1994). Culture medium was replaced every second day during the culture period. In treatment groups, a single application of GDNF (Promega, Madison, WI, USA) (0.01–10 ng/mL culture medium) was done starting at DIV5 for a maximal period of 2 days.

Immunohistochemistry

Cultures were fixed for 60 min in 0.1 phosphate buffer (PB) containing 4% paraformaldehyde. After equilibration in 10% sucrose–PB for 24 h, cultures were sectioned frozen at 20 μm on a microtome, and groups of three to six sections were mounted on prepared glass slide. For *in vitro* cell counts of tyrosine hydroxylase immunoreactive neurones, sections were first washed in several PB rinses and incubated in 0.3% Triton X-100 PB solution containing 10% horse serum (Gibco BRL) for 60 min. After washing, sections were incubated with anti-TH antibody (1 : 500, Roche Molecular Biochemicals, Indianapolis, IN, USA), diluted in 0.1% Triton X-100 PB containing 2.5% horse serum at 4°C for 12 h. The sections where then washed again in PB and incubated for 2 h with a biotinylated anti-mouse antibody (Vectastain, 1 : 200, Vector Laboratories, Burlingame, CA, USA) and an avidin-biotinylated horseradish peroxidase complex according to the manufacturer's instructions (Vector Laboratories). Finally, TH-positive cells were visualized by incubation with 10% metal-enhanced diaminobenzidine in stable peroxide substrate buffer (Pierce, Rockford, IL, USA).

Immunofluorescence techniques were used for the double detection of TH and GTPCH I, as well as 5-HT neurones. Sections were first covered with 10 mM citrate-buffer and microwaved prior to antibody exposure, washed in 0.1 M PB for 30 min, preincubated for 60 min in PB containing 0.3% Triton X-100 and 10% horse serum, and then treated with polyclonal rabbit anti-TH antibody (1 : 1000, Pel Freez Bio, Rogers, AR, USA) and a monoclonal rat

anti-GTPCH I antibody, MGTP-6H11 (Hesslinger *et al.* 1998) overnight at 4°C. Sections were incubated for 2 h at room temperature with cy3-conjugated donkey anti-rabbit IgG (1 : 100, Jackson Immuno-Research, West Grove, PA, USA) and cy2-conjugated donkey anti-mouse IgGs (1 : 100, Jackson Immuno-Research). After washing, sections were cover-slipped in 25% glycerol containing PB. TH and GTPCH I immunoreactive cells were visualized under a fluorescence microscope. Specificity of immunostaining was determined by omission of primary and secondary antibodies.

Western blot analysis of GTPCH I and TH

Aliquots of cell extracts prepared as described below (see GTPCH I activity) were separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immediately transferred onto nitrocellulose membranes. For detection of GTPCH I, 10 μg protein extract and an aliquot of GTPCH I, bacterially expressed as a GTPCH I-fusion construct, was loaded. Membranes were blocked, probed with biotinylated MGTP-6H11 rat monoclonal antibody in a 1 : 100 dilution and visualized using peroxidase-conjugated streptavidin and enhanced chemiluminescence.

For detection of TH by western blot analysis equal volumes (instead of equal protein amounts; see also GTPCH I activity) of VM extract in the range of 10 μg were loaded. The quality of the antibody was tested using adrenal gland extract showing high level of TH expression (not shown). Membranes were blocked, probed with mouse TH antibody (Boehringer Mannheim, Mannheim, Germany) in a 1 : 100 dilution and visualized using alkaline phosphatase conjugated goat anti-mouse antibody (Tropix, Bedford, MA, USA).

MPP^+ and actinomycin D treatment

Cultures were treated twice at DIV4 and DIV6 by adding MPP^+ [stock solution: 30 mM MPP^+ solubilized in DMSO (Sigma)] to a final concentration of 15 μM (Sanches-Ramos *et al.* 1986).

Actinomycin D (AD) treatment was performed as described by Zhu *et al.* (1994). Briefly, a single application of AD to the culture medium (final concentration of 2 μg/mL medium) was carried out at DIV5. Depending on the experimental design, GDNF (10 ng/mL) and AD were added concurrently. To monitor adverse effects due to solvent, ethanol (0.2%) was added to the medium of the control groups.

Cell counts and volume estimation

In order to investigate the influence of GDNF on numbers of TH-ir neurones *in vitro*, cell counts were performed by treating single cultures as individual cases. To be quantified, cells had to fulfil the following criteria: (i) show a dark staining pattern; (ii) have at least one visible process; and (iii) a well-defined pericaryon. Numbers have been corrected according to Abercrombie's formula (Abercrombie 1946). Both cell counts and area measurements were done in a blinded experimental design under bright-field illumination, using a microscope connected to a three-dimensional neurone tracing system (NTS, Eutectic). Estimations of cell numbers were carried out as described previously (Höglinger *et al.* 1998; Meyer *et al.* 1999). In brief, the density of cultured TH-ir neurones was estimated by counting three 20-μm sections within a 240-μm range, representing the central portion of the culture. The volume of one culture was approximated using the area of the section with the greatest diameter assuming that the culture had a spherical shape. To determine the percentage of TH-ir neurones that were also GTPCH I positive, three central sections (within a 240-μm range) of four individual cultures each were investigated under the fluorescence microscope, using the double-labelling technique described above.

GTPCH I activity measurements

Whole VM sections were homogenized in 80 μL Tris buffer (50 mM, pH 8.0) containing 2.5 mM EDTA and 0.1 mg/mL phenylmethylsulphonyl fluoride (PMSF) by mechanical stirring (80 g) in a tissue grinder (Braun, Melsungen, Germany). After centrifugation (20 000 *g* for 15 min), the supernatant was assayed for enzyme activity or pterin content. Protein concentrations were determined by Bradford analysis (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

GTPCH I activity was assayed by incubating cell extracts with GTP (0.75 mM final concentration) at 37°C for 90 min in the dark. The reaction product was oxidized to neopterin triphosphate by acidic iodine (0.1 M). After reduction of excessive iodine by ascorbic acid, reaction products were monitored by ion pair reverse-phase HPLC with fluorescence detection. The detailed chromatographic procedure has been described elsewhere (Kerler *et al.* 1989; Kerler *et al.* 1990). As further evidence of the GTP origin of detected neopterin triphosphates, one additional VM cell extract was tested for GTPCH I activity with the substrate omitted. A further control included dephosphorylation of the neutralized neopterin triphosphate by alkaline phosphatase (0.8 U/200 μL), and subsequent identification of neopterin by reverse-phase HPLC. Neopterin phosphate levels correlated with neopterine levels in all cases.

Activities were expressed as pmol/min per VM. In E14 rat embryos the dopaminergic neurones lay packed adjacent the rostral-caudal midline. Therefore, the tissue is dissected such that the dopaminergic cell cluster is located within the middle of the explant with a safety margin around it to make sure that all dopaminergic neurones are included. Variations in total protein amount of VM extracts are the consequence (45 μg to 94 μg protein per VM with an average amount of 60 μg per VM). There is evidence from immunohistochemical data that the number of dopaminergic neurones is similar for each explant (Meyer *et al.* 1999). As a consequence, as GTPCH I expression is limited to these cells, activity units are presented as pmol/min per VM based on the observation that only then are all dopaminergic neurones from one individual embryo are represented. Cellular BH_4 was determined after acidic oxidation of pterins by iodine. After protein precipitation by trichloroacetic acid (TCA) and cation exchange chromatography purification, the samples were analysed by reverse-phase HPLC as described previously (Ziegler 1985).

Tyrosine hydroxylase activity and DA measurements

TH enzyme measurements were performed according to the method described by Bostwick and Le (1991) with some modifications (Waldmeier *et al.* 2000). The tissue pieces were lysed on ice in 70 μL lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100) by sonification (Vibracell, 50% power, 10 s; Sonics and Materials, Danbury, CT, USA). After centrifugation for 15 min at 4°C and

16 000 g, 25 μL of supernatant was pipetted in duplicates into a precooled 96-well microtitre plate on ice. For the standard curve, an extract was prepared from adult rat striatum lysed in 10 volumes of lysis buffer. After centrifugation for 15 min at 4°C and 16 000 g the supernatant was stored in aliquots at −20°C. The standard curve was prepared by 1 : 2 dilution of the striatal extract and pipetting 25 μL each in triplicate to the appropriate wells. The reaction was started by pipetting 17 μL reaction buffer [450 mM morpholinoethanesulphonic acid, pH 6.1, 2.3 mM dithiothreitol, 4.7 mM ascorbic acid, 2.3 mM 2-amino-4-hydroxy-6-methyltetrahydropterine (Sigma M4758); 2500 μ/reaction catalase (Sigma C100); 45 μM [^{14}C]L-tyrosine (NEC-170; NEN Life Science, Brussels, Belgium)] to each well. The plate was sealed and incubated at 37°C for 60 min. TH activity was stopped by chilling the plate on ice. A piece of Whatman 3MM paper was cut to a size to completely cover the plate, soaked with benzethonium hydroxide and air dried for 5 min. Then 41.6 μL of ice-cold cyanide buffer [16.5 mM potassium ferricyanide, 10 mM *p*-chloromercuribenzenesulphonic acid (Sigma C4503)] was added to each well to initiate the nonenzymatic decarboxylation of DOPA. After quickly covering the plate with the prepared benzethonium hydroxide (Sigma B2156)-soaked filter, a weight was placed on top of the plate and the decarboxylation reaction was allowed to proceed for 60 min at 60°C. The filter was cut and radioactivity counted in 3 mL Ecoscint-0 (National Diagnostics, Atlanta, GA, USA) in a scintillation counter. The values detected for the VM tissue samples (range from 6000 to 10 000 cpm) were in all experiments within the linear range of the corresponding standard curves. TH activity measurements without addition of 2-amino-4-hydroxy-6-methyltetrahydropterine to the incubation medium resulted in baseline levels only (blanks ranged from 300 to 800 cpm). Assays performed in the presence of a 10-fold lower level of 2-amino-4-hydroxy-6-methyltetrahydropterine in the incubation medium (final concentration: 0.23 mM), however, revealed linear standard curves (with a five-fold lower yield of radioactivity recovered as compared to the standard incubation medium) and allowed for reproducible activity measurements in the tissue samples.

DA and 3,4-dihydroxyphenylacetic acid (DOPAC) as well as homovanillic acid (HVA) were measured in cell preparations using HPLC with electrochemical detection. In brief, cultures were collected, washed in cold buffer, centrifuged, and cold 1 N perchloric acid (PCA; Merck) with antioxidants (0.2 g/L $Na_2S_2O_5$, 0.05 g/L Na_2-EDTA) was added (200 μL/sample). Subsequently, samples were sonicated and centrifuged at 20 000 g for 20 min at 4°C. The supernatant was collected and 50 μL used for HPLC analysis. Samples were injected by means of an autosampler (Merck-Hitachi), and separation was achieved by a Waters Spherisorb S5 ODS2 guard column (4.6 × 30 mm) and a Waters Spherisorb S3 ODS2 cartridge analytical column (4.6 × 150 mm). The flow rate of the mobile phase [0.1 M sodium acetate (Sigma), 6% methanol (Merck, Darmstadt, Germany), 18 mg/mL *n*-octyl sodium sulphate (Merck), 13 mg/mL EDTA (Sigma) dissolved in Milli-Q-water and adjusted to pH 4.1 with glacial acetic acid (Merck)], which was degassed with helium for 1 h before use, was adjusted to 0.9 mL/min. The electrochemical detector within the build column oven (Decade) was set at an oxidation potential of + 0.75 V against an Ag/AgCl reference electrode. A mixture of DA and DA metabolites was used as external standards. Two independent experiments were done.

RT-PCR quantification of GTPCH I mRNA

VM tissue obtained from E14 rat embryos were cultured for 5 days in standard medium and subsequently exposed to cAMP (5 mM final concentration) or GDNF (10 ng/mL medium) for 5 and 24 h, respectively. cAMP-treated cultures were used as positive controls, since it was previously shown that cAMP treatment of primary rat dopaminergic neurones results in up-regulation of GTPCH I mRNA (Hirayama *et al*. 1993); untreated VMs served as negative controls. After factor treatment, RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNAs were obtained by reverse transcription primed with polyT(15mer) primer using the First Strand cDNA-Synthesis Kit (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). PCR was performed with the primer GTPCH I (forward) (5′-GCGAGGAGGATAACGAGCTG-3′) and GTPCH I (reverse) primer (5′-GCTGCAAGGCTTCTGTGATGG-3′) and GAPDH (forward) (5′-CGGGAAGCTTGTGATCAATGG-3′) and GAPDH (reverse) (5′-GGCAGTGATGGCATGGACTG-3′) using LightCycler Fast Start DNA Master SYBR Green I Kit (Roche), according to the manufacturer's recommendations. The PCR protocol was as follows: Initial denaturation for 10 min at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 65°C and 50 s at 72°C. Relative quantification was done using an external standard (purified GTPCH I PCR product). The variation of mRNA amounts were normalized with the housekeeping gene GAPDH. Analysis was performed using LightCycler Software 3 ('Fit Points' Analysis) according to the manufacturer's recommendations. Data were obtained from two independent experiments for each treatment group and quantification was performed twice for each individual sample (Fig. 7b). Gel electrophoresis was performed with PCR products from representative samples. The PCR run was stopped after 32 cycles to obtain amplified PCR products in the log-linear phase (Fig. 7a).

Statistical analysis

Data analysis was performed using a commercially available statistical software package (SPSS 10.0.1). Cell counts and biochemical measurements were shown as mean ± SEM. For comparison of two treatment groups (cell counts of TH-ir neurones) the Student's *t*-test was used. For comparison of multiple treatment groups one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Tukey's test) (GTPCH I-activity measurements) and by Dunn's method (BH_4 measurement) were performed.

Results

Expression of TH and GTPCH in rat VM tissue

To determine the effects of GDNF on DA biosynthesis, primary dopaminergic VM neurones had to be stable in terms of cell numbers and developmental stage. Solid rat VM tissue, derived from E14 rat embryos was cultivated in FFRT for DIV5 and DIV7. Immunohistochemical investigation with TH antibodies as a marker for dopaminergic neurones showed mature TH-immunoreactive neurones scattered throughout the spherical cultures at DIV5 and DIV7

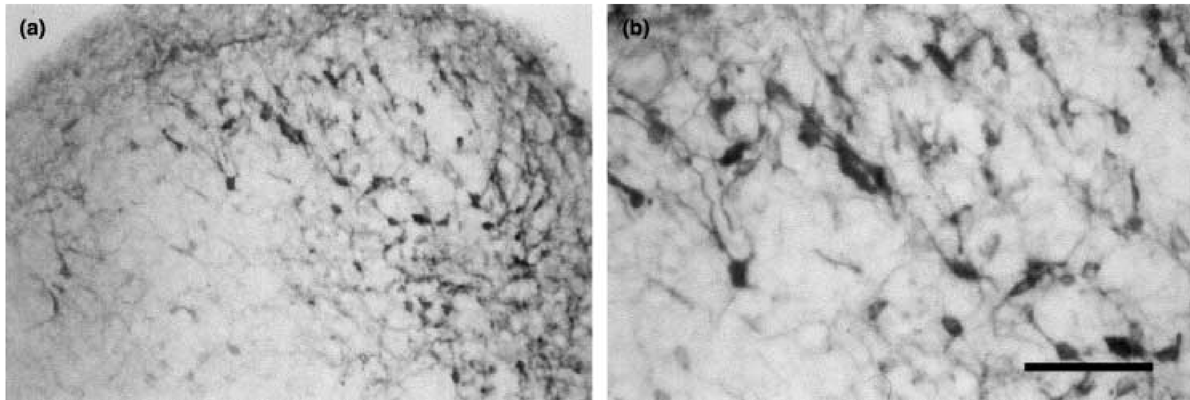

Fig. 1 TH-positive neurones in FFRT cultures after 7 days *in vitro* (DIV7). Ventral mesencephalon from E14 rat embryos was dissected and cultured as solid tissue pieces using the 'free floating roller tube' (FFRT) culture system. Cultures were processed for immunohistochemistry after DIV7. The antibody raised against tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine biosynthesis was used to detect mature dopaminergic neurones within the ventral mesencephalic region. TH-positive neurones show strongly stained processes forming network-like structures within the culture. Scale bar represents 100 μm in (a) and 50 μm in (b).

(Fig. 1). No morphological differences were seen in TH-positive neurones at both time points. Similarly, no differences were noted in number of TH-positive neurones in agreement with previous observations by Höglinger *et al.* (1998). Morphometric assessments at DIV7 in a representative number of VM tissue pieces ($n = 26$) revealed a mean volume of 0.72 ± 0.04 mm^3 and a mean number of 2029 ± 185 TH-positive neurones per culture. GTPCH I protein was detected by immunohistochemistry in TH positive neurones. Double labelling of a FFRT culture at DIV7 with cells expressing both TH and GTPCH I are shown in Fig. 2. Here 66% of the TH-ir neurones also showed GTPCH I-positive immunoreactivity.

GTP cyclohydrolase activity in VM FFRT cultures is restricted to TH-positive neurones

In agreement with earlier reports (Hirayama and Kapatos 1998), GTPCH I immunoreactivity was shown to be localized in TH-positive neurones in the VM tissue preparations. Assuming that this restriction is also valid for GTPCH I enzyme activity, GDNF-mediated effects on GTPCH I activity should exclusively affect TH-positive neurones in organotypic VM cultures. Tissue preparation procedures were optimized with the aim of obtaining a well-defined tissue region harbouring dopaminergic neurones as the main source of GTPCH I activity. In order to monitor and assess a potential contribution to overall enzyme activity by GTPCH I positive serotoninergic (5-HT positive) neurones of the pontine raphe nucleus lying caudally to the nigral anlage (Dunnett and Bjorklund 1992), as well as other non-dopaminergic neurones expressing GTPCH I (Dassesse *et al.* 1997), selective destruction of dopaminergic neurones by MPP^+, a neurotoxin highly specific to destroy dopaminergic neurones (Sanches-Ramos *et al.* 1986), was performed. Cultures were exposed to 15 μM MPP^+ (ICN Biomedicals, Costa Mesa, CA, USA) at both DIV4 and DIV6 and then processed for GTPCH I activity seven days later. Upon treatment with MPP^+, GTPCH I activity was barely detectable (data not shown), indicating that TH-positive neurones are the major source of GTPCH activity in FFRT cultures of the ventral mesencephalon.

GDNF effects on enzymes and products of the DA biosynthesis pathway

Cultured VM tissue was treated with 10 ng/mL GDNF for 6 h (DIV7), 24 h (DIV6 to DIV7) and 48 h (DIV5 to DIV7), and GTPCH I activity was measured in four VM tissue pieces corresponding to one individual rat VM. GDNF exposure for 24 h and 48 h resulted in a 55% ($n = 7$) and 116% ($n = 14$, $p < 0.001$) increase of GTPCH I activity, compared with non-treated control cultures ($n = 15$) (Fig. 3a); no significant changes in GTPCH I activity were observed following a 6-h treatment (data not shown). Detection of GTPCH I protein by immunohistochemistry did not show any obvious differences in signal intensity in control versus GDNF-treated cultures. In order to analyse whether up-regulation of its activity would correlate with GTPCH I protein levels we carried out western blot analysis (data not shown). However, we were not able to detect GTPCH I protein using a monoclonal rat MGTP-6H11 mAB (Hesslinger *et al.* 1998). GTPCH I/TH-expressing dopaminergic neurones represent only a subpopulation (2–5%) of VM cells (immunohistochemical analysis, unpublished data) and GTPCH I is only expressed at low levels relative to TH (Nagatsu *et al.* 1997; Hirayama and Kapatos 1998). This explains why we failed to detect GTPCH I protein by western blot analysis in contrast to previously being able to detect it in highly expressing primary mast cells (Hesslinger *et al.* 1998).

As GTPCH I is the rate-limiting step in BH_4 *de novo* synthesis, an increase in enzyme activity would be expected

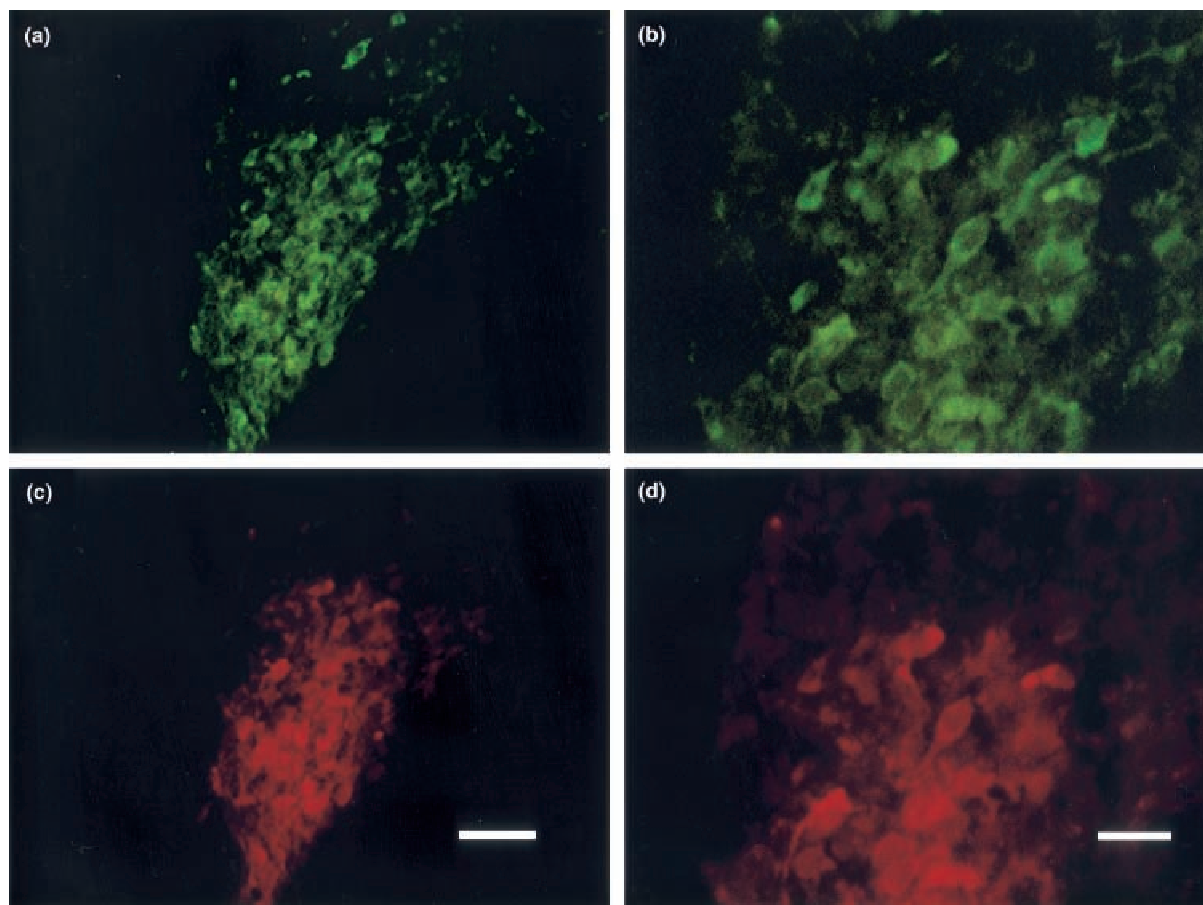

Fig. 2 Co-localization of tyrosine 3-monooxygenase (TH) (a and b) and GTP-cyclohydrolase I (GTPCH) I (c and d) in embryonic ventral mesencephalic (VM) tissue detected by double-label immunofluorescence. E14 rat VM tissue was cultured as free floating roller tube cultures and fixed for immunohistochemical investigation after DIV7. Antibodies raised against TH and GTPCH I were used to localize their expression within the VM tissue blocks. Anti-TH (a) and anti-GTPCH I (b) immunoreactivity was visualized using either Cy2 (green) or Cy3 (red) labelled secondary antibodies, respectively. Cluster of TH-positive neurones within the VM culture in low (a) and high (b) magnification. GTPCH I immunoreactive neurones (c and d). Scale bar represents 50 μm in (a and c) and 25 μm in (b and d), respectively).

to result in elevated BH_4 levels. BH_4 levels measured after 24 h ($n = 5$) and 48 h of GDNF ($n = 6$) exposure, resulted in 39% (not significant) and 48% ($p < 0.05$) higher BH_4 levels, as compared with controls ($n = 6$) (Fig. 3b). In order to examine dose dependency of the observed GDNF effect on GTPCH I activity, VM cultures were treated with 0, 0.01, 0.1, 1 and 10 ng/mL of GDNF ($n = 3$ for each) for 48 h, as described previously. Changes in GTPCH I activity due to varying doses of GDNF are summarized in Fig. 4. GTPCH I activity was not affected by 0.01 ng/mL GDNF and 0.1 ng/mL GDNF, whereas maximal stimulation occurred at 1 ng/mL to 10 ng/mL GDNF.

In contrast, TH activity measurements in untreated and GDNF-treated (10 ng/mL, DIV5–7) rat VM tissue revealed no significant differences (100 ± 3% versus 104 ± 4%, $n = 9$ for each two independent experiments, respectively) (Fig. 5a). Due to methodical limitations in combination with low endogenous levels of the cofactor tetrahydropterin in the VM preparations (see Material and methods), we cannot rule out that the increased BH_4 levels after GDNF treatment (see above) also influenced TH activity (see Discussion). Nevertheless, the results indicate that GDNF has no direct effect on TH besides altering BH_4 cofactor levels.

Although not significant, there was a tendency for higher DA content within GDNF-treated tissue compared with controls (9.3 ± 1.6 versus 7.0 ± 0.6 pmol per VM, $n = 6$, $p = 0.2$) (Fig. 5b). DOPAC as well as HVA tissue content did not reach above baseline levels (data not shown). Western blot analysis was carried out and no increase in TH protein was observed after GDNF exposure (Fig. 6).

GTPCH I up-regulation is not due to increased numbers of TH-ir neurones

In order to rule out any significant survival-promoting effects on TH-positive neurones due to GDNF exposure (Lin *et al.* 1993), TH-positive neurone cell counts *in vitro* were

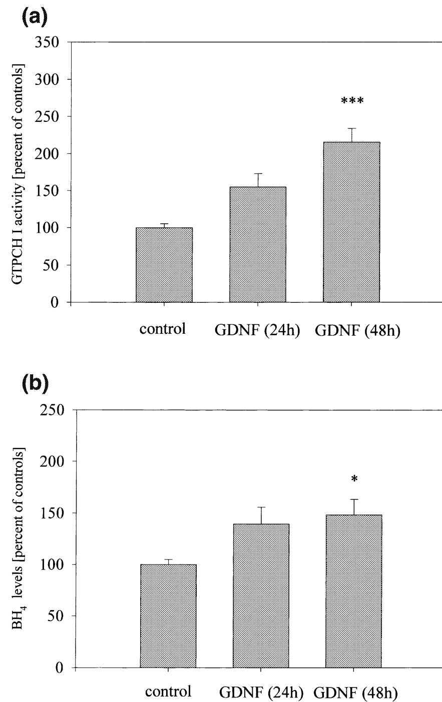

Fig. 3 Glial cell line-derived neurotrophic factor (GDNF) effects on GTP-cyclohydrolase I (GTPCH I) activity (a) and 5,6,7,8-tetrahydro-biopterin levels (BH_4) (b). Free floating roller tube cultures of rat ventral mesencephalon were treated with 10 ng/mL GDNF from day 6–7 (24 h) or from day 5–7 (48 h). Ventral mesencephalic (VM) tissue was prepared for HPLC analysis at DIV7 in all treatment groups. Results are expressed as percentage relative to control levels. Absolute baseline values (control) were 0.022 ± 0.00122 pmol/min GTPCH I per VM ($n = 15$). Neopterin phosphate levels correlated with neopterine levels in all cases (a). Base line value for BH_4 was 1.43 ± pmol biopterin per VM ($n = 6$). (b). ***$p < 0.001$ versus control, 6 h and 24 h (ANOVA, *post-hoc* test Student–Newman–Keuls), *$p < 0.05$ versus control (ANOVA on rank, Dunn's method). Values represent the mean ± SEM.

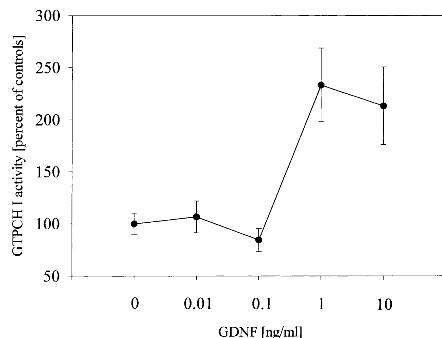

Fig. 4 Dose dependent up-regulation of GTP-cyclohydrolase I activity by glial cell line-derived neurotrophic factor. Free floating roller tube cultures of rat ventral mesencephalic were treated with 0, 0.01, 1 and 10 ng/mL glial cell line-derived neurotrophic factor for 48 h from day 5 to day 7 after dissection ($n = 3$, each group). Values represent the mean ± SEM.

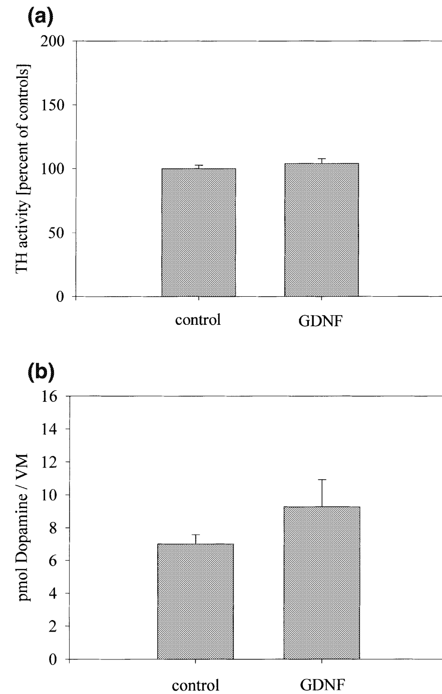

Fig. 5 Glial cell line-derived neurotrophic factor (GDNF) effects on tyrosine 3-monooxygenase (TH) activities and dopamine tissue levels. For TH-activities (a) and dopamine tissue levels (b) free floating roller tube cultures of rat ventral mesencephalon were treated with 10 ng/mL GDNF from day 5–7 (48 h). Ventral mesencephalic (VM) tissue was prepared for HPLC analysis at DIV7 in all treatment groups. Results are either expressed as percentage relative to control levels (a) or as pmol of one individual VM (comparison of the groups with Student's *t*-test, no significant differences). Values represent the mean ± SEM.

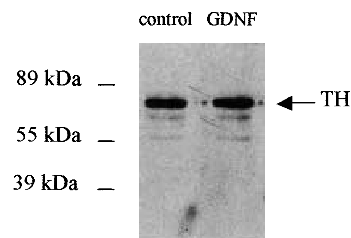

Fig. 6 Glial cell line-derived neurotrophic factor (GNDF) effects on tyrosine 3-monooxygenase protein level. Cultures of rat ventral mesencephalon were treated with 10 ng/mL GDNF from day 5–7 (48 h). Ventral mesencephalic tissue was prepared for western blot analysis at DIV7 in both treatment groups.

performed on GDNF-treated cultures (GDNF exposure for 48 h), and in non-treated controls after 7 days. No statistical difference between treatment ($n = 28$) and control ($n = 26$) groups were noted in respect to cell density (2919 ± 302 cells/mm^3 versus 2983 ± 313 cells/mm^3, respectively) and absolute numbers (2123 ± 245 cells/culture versus

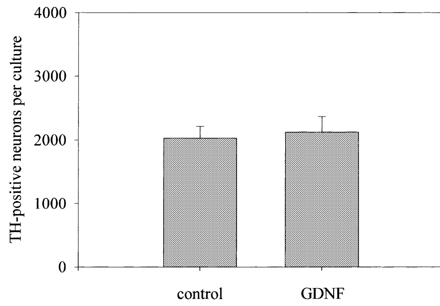

Fig. 7 Tyrosine 3-monooxygenase (TH)-positive cell counts in glial cell line-derived neurotrophic factor (GNDF)-treated ventral mesencephalic (VM) cultures and non-treated controls. E14 VM tissue was cultured with supplementation of GDNF starting at DIV5 (GD, $n = 28$) or without addition of GDNF (control, $n = 26$) or. Quantification of TH-positive neurones performed at DIV7 did not show a significant difference between GDNF-treated and control cultures. Counts are presented as numbers of TH-positive neurones per culture (corresponding to a quarter of a rat ventral mesencephalon). Values represent the mean ± SEM.

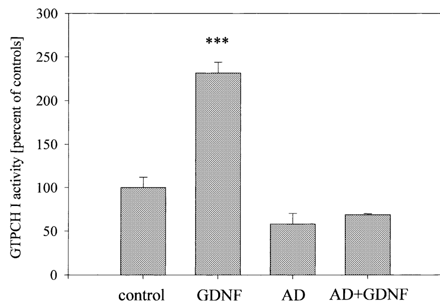

Fig. 8 Effects of actinomycin D on glial cell line-derived neurotrophic factor (GDNF)-mediated increases in GTP-cyclohydrolase I (GTPCH I) activity. Culture media received either 10 ng GDNF (GD) per millilitre of medium, 2 μg actinomycin D (AD) per millilitre of medium or both (AD + GD) at DIV5, and were cultured for two further additional days ($n = 4$, respectively). Untreated cultures served as controls (control, $n = 4$). Actinomycin D treatment blocked the GDNF-mediated increase in GTPCH I activity. $^{***}p < 0.001$ versus control, AD and AD + GD (ANOVA, Tukey's test). Values represent the mean ± SEM.

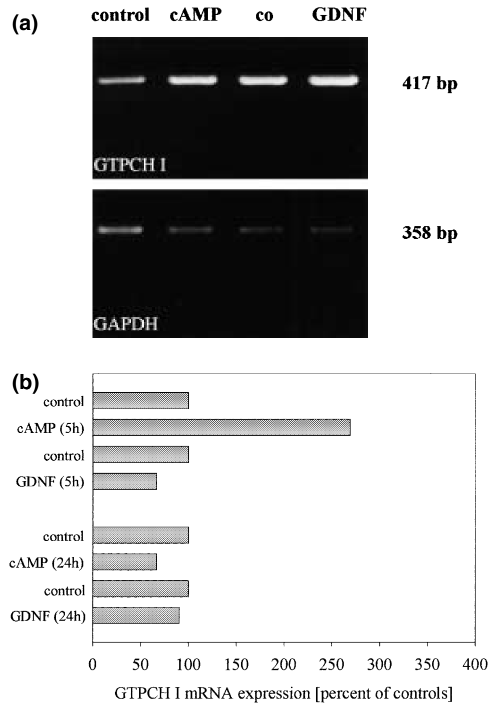

Fig. 9 Relative GTP-cyclohydrolase I (GTPCH I) mRNA levels in rat ventral mesencephalic (VM) tissue after factor treatment. Rat VM tissue was cultured for 5 days in free floating roller tubes using standard culture medium. Subsequently, the cultures were incubated in medium containing no additional factors (controls), cAMP (final concentration 5 mM, incubation time 5 and 24 h) as a positive control and glial cell line-derived neurotrophic factor (GDNF) (10 ng/mL, incubation time 5 and 24 h). cAMP-treated cultures were used as positive controls since it was shown previously that cAMP treatment of primary rat dopaminergic neurones resulted in an up-regulation of GTPCH I mRNA (Hirayama *et al.* 1993). After factor treatment, the tissue was processed for quantification by real-time PCR. (a) Ethidium bromide gel staining shows the amplified GTPCH I PCR product (factor treatment for 5 h) (upper panel). GAPDH was amplified to normalize for variations of mRNA amounts in the samples (lower panel). (b) Real-time PCR data from two independent experiments were blotted (in percentage) for different treatment groups. No up-regulation of GTPCH I mRNA in VM tissue after 5 h or 24 h GDNF exposure could be detected.

2028 ± 185 cells/culture, respectively) of TH-positive neurones (Fig. 7). Furthermore, determination of immunoreactivity to TH and GTPCH I in GDNF-treated (48 h) versus untreated cultures showed no difference in the relative number of GTPCH I positive cells in the TH-positive cell population (67 ± 7%, $n = 4$ versus 66 ± 3%, $n = 4$, respectively).

Molecular mechanisms of GDNF-induced increase of GTPCH I activity

To further investigate possible mechanisms of GDNF-mediated increase in GTPCH I activity, cultures were exposed to AD (2 μg/mL), a DNA transcription inhibitor, at DIV5. As depicted in Fig. 8, exposing untreated cultures to GDNF for 48 h resulted in a robust increase of GTPCH I activity. In contrast, stimulation of GTPCH I by GDNF was totally blocked by AD. A reduction of GTPCH I activity by 42% was observed in AD-treated cultures compared with non-AD-treated control cultures. This reduction probably reflects increased cell death of TH-positive neurones due to AD-mediated toxicity. This effect has recently been observed by Zhu *et al.* (1994).

Although AD experiments suggested a role of GDNF in GTPCH I transcription, semiquantitative RT-PCR did not

confirm an up-regulation of GTPCH I mRNA following GDNF exposure (Fig. 9). Therefore other mechanisms, including post-translational modifications or involvement of additional regulatory proteins, may play a role in the GDNF-mediated regulation of GTPCH I.

Discussion

Although the involvement of GDNF in promoting both the survival and morphological differentiation of dopaminergic neurones has been documented (Lin *et al.* 1993), little is thus far known about the functional effects of GDNF regarding the metabolism of primary dopaminergic neurones. Here we show that GDNF-mediated effects in primary dopaminergic neurones occur on one of the key enzymes of the DA biosynthesis, GTPCH I. We found that GDNF can selectively increase GTPCH I activity resulting in an increase of intracellular BH_4 in primary rat neurones.

Maximal GTPCH I activity in VM dopaminergic neurones was observed when GDNF applied to the incubation medium reached 1 ng/mL at incubation times of at least 24 h. Similarly, these conditions were also effective in raising BH_4 levels in these cells. GDNF receptor saturation has been reported with 30–40 pg GDNF per millilitre of medium (Lin *et al.* 1993) and 30 pg GDNF per millilitre medium are sufficient for facilitating half maximal survival of primary neurones in monolayer cultures (Jing *et al.* 1996). We recently used a concentration of GDNF of 10 ng/mL for assessment of morphological differentiation in dissociated primary cultures and found a pronounced effect on dopaminergic neurones (Widmer *et al.* 2000). This observation is in agreement with previously published studies showing that GDNF treatment at a concentration of 10 ng/mL produced maximal levels of Ret phosphorylation, DA up-take and numbers of TH-positive cells and resulted in a profound increase in phosphatidyl 3-kinase activity (Beck *et al.* 1995; Pong *et al.* 1997; Pong *et al.* 1998). Different findings with various concentrations of GDNF can be explained by differences of the cellular system. Certain effects may thus depend on various parameters such as the developmental stage of the embryos used for preparation of the cultures, time in culture, the composition of the cell culture medium and the limited diffusion of GDNF within a solid tissue block (Giehl *et al.* 1997; Widmer *et al.* 2000).

GTPCH I but not TH activity was directly affected by GDNF treatment. *In vitro* investigations of BH_4 dependent up-regulation of TH activity was not possible since we could not detect TH activities above background levels when enzyme reactions were solely dependent on the BH_4 in the VM tissue, either with or without GDNF exposure. *In vivo* kinetic data on the affinity of adrenal TH for BH_4 show the existence of a phosphorylated form of the enzyme with a low K_M for BH_4 (10–30 μM), and a non-phosphorylated form of TH with a high K_M (500–600 μM) (Levine *et al.* 1981). Taking into account that approximately 80% of TH in CNS dopaminergic neurones exists in the non-phosphorylated form and that the estimated intraneuronal BH_4 concentration is about 100 μM (Levine *et al.* 1981), low BH_4 levels limit the activity of the non-phosphorylated enzyme (Miwa *et al.* 1985). Under *in vivo* conditions, GDNF-induced increase of BH_4 may therefore affect TH activity and catecholamine biosynthesis.

Following GDNF exposure, slightly higher DA levels were observed in VM cultures, although this effect was not statistically significant. In contrast, a tendency for higher DA levels in supernatant was detected in nonexposed samples (data not shown). This phenomenon may be explained by altered diffusion and release of DA (Höglinger *et al.* 1998) from VM dopaminergic neurones *in vitro*. Using an *in vivo* model, Beck *et al.* (1996) investigated GDNF action on DA synthesis, by injection of high doses into neonatal (P2) rat striatum and substantia nigra. Dystonia-like movement abnormalities accompanied by marked increases in TH-activity and DA levels in explanted tissue were observed following treatment. These findings may possibly be due to a GDNF-mediated increase of BH_4 resulting in increased TH activity.

The effect of GDNF on GTPCH I activity was blocked by AD, a DNA transcription inhibitor, in primary rat mesencephalic cultures (Zhu *et al.* 1994). However, PCR quantification did not show any GDNF-dependent up-regulation of GTPCH I mRNA. Therefore, other mechanisms regulating GTPCH I turnover, such as activity through as yet unknown regulatory proteins or post-transcriptional modification of GTPCH I, must be taken into consideration for GDNF-mediated effects on GTPCH I. GTPCH I feedback regulatory protein (GFRP) is one such protein known to interfere with GTPCH I activity which blocks the enzyme's activity by structural interaction (Yoneyama *et al.* 1997). GFRP is strongly expressed in the rat central nervous system including the ventral midbrain. In primary rat serotoninergic neurones, GTPCH I activity is either augmented by the interaction of GFRP with L-phenylalanine or negatively regulated by the presence of BH_4 (Kapatos *et al.* 1999). GFRP, however, is expressed only at low levels in primary dopaminergic neurones and no L-phenylalanine-dependent increase of GTPCH I activity is observed (Kapatos *et al.* 1999). Due to this observation, it is rather unlikely that GFRP does play a significant role in GTPCH I regulation in dopaminergic neurones. In addition, it was proposed that some other regulatory protein which remains so far unidentified may exist in dopaminergic neurones (Kapatos *et al.* 1999). A novel interacting protein, *catsup* (Stathakis *et al.* 1999; O'Donnell 2002), has been recently identified in Drosophila, indicating a rather complex concert of regulatory proteins involved in DA synthesis. To our knowledge, the mammalian homologue to *catsup* has not yet been

characterized. Thus, analysing mechanisms of GTPCH I regulation by protein–protein interaction based on a candidate approach are not feasible at this time.

Short-term activation of GTPCH I by phosphorylation through an IgE-triggered, PKC-dependent pathway has been reported for rodent mast cells (Hesslinger *et al*. 1998). This, however, affects GTPCH I activity only in a transient fashion. Since the present study did not include the effects of GDNF on GTPCH I phosphorylation status, the possibility that GDNF can induce short-term GTPCH I activity in dopaminergic neurones, or that phosphorylation contributes to enzyme activity after prolonged GDNF exposure cannot be ruled out.

DA, like other catecholamines can be converted by metabolic oxidation into reactive quinones and oxygen radicals that damage dopaminergic cells (Hastings and Zigmond 1994). Tight regulation of DA synthesis and the recycling of existing pools rather than a continuous *de novo* synthesis might have been evolved in neuronal tissue to cope with this potential hazard. Therefore, up-regulation of DA synthesis has been connected to activity and proper protection of the corresponding neurones, ensuring their survival and protection against damage concomitant to their activity. On the basis of our *in vitro* experiments, *in vivo* studies are necessary to clarify whether GDNF is physiologically involved in this regulation.

Taken together, our results indicate that GDNF, in addition to increasing cell survival and promoting the morphological integrity of DA neurones, enhances dopaminergic function by activating GTPCH I through an unknown transcription-dependent mechanism. This may also contribute to the beneficial effects seen with GDNF in experimental animal models of Parkinson's disease (Siegel and Chauhan 2000). Given the results obtained in this study, long-term expression of the GDNF gene in a moderate, physiological way in a cell transplantation setting (Bauer *et al*. 2000) may increase BH_4 production and thereby contribute to a better graft function. Thus, dopaminergic cell survival and function seems to be interrelated. Mediated by a single neurokine, enhancement of function, as well as cell protection of the cell against the risk of cell death, are thus coordinated. These features indicate that GDNF is suited for therapeutic use in Parkinson's disease.

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