

Segundo trabajo

“Glial cell line-derived neurotrophic factor promotes the arborization of cultured striatal neurons through the p42/p44 mitogen activated protein kinase pathway”

Sometido a revisión en *Journal of Neuroscience Research*

Glial Cell Line-Derived Neurotrophic Factor Promotes the Arborization of Cultured Striatal Neurons Through the p42/p44 Mitogen Activated Protein Kinase Pathway

Juan M. García-Martínez, Núria Gavaldà, Esther Pérez-Navarro and Jordi Alberch*

Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona, IDIBAPS, Casanova 143, E-08036 Barcelona, Spain.

* Correspondence to: Jordi Alberch

Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona, IDIBAPS, Casanova 143, E-08036 Barcelona, Spain.

alberch@ub.edu

Phone number: 34 93 4035285

Fax number: 34 93 4021907

Running title: p42/p44 MAPK pathway mediates GDNF effects

Financial support was obtained from the Ministerio de Educación y Ciencia, Grant SAF2002-00314; Redes Temáticas de Investigación Cooperativa (G03/167; G03/210, Ministerio de Sanidad y Consumo), Fondo de Investigaciones Sanitarias (Instituto de Salud Carlos III) and Fundació La Caixa. J.M. G-M is a fellow of the Ministerio de Educación y Ciencia.

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) promotes the survival and/or differentiation of several types of neurons. In this study we examined GDNF-induced signal transduction and biological effects in cultured striatal neurons. Our results show that GDNF addition to striatal cultures transiently increased the protein levels of phosphorylated p42/p44, but did not change the levels of phosphorylated Akt. GDNF effects on phosphorylated p42/p44 levels were blocked by the mitogen-activated protein kinase (MAPK) pathway specific inhibitors (PD98059 and U0126). Activation of the p42/p44 MAPK pathway by GDNF led to an increase in the degree of dendritic arborization and axon length of both GABA- and calbindin-positive neurons but had no effect on their survival and maturation. These GDNF-mediated effects were suppressed in the presence of the inhibitor of the MAPK pathway (PD98059). Furthermore, the addition of the phosphatidylinositol 3-kinase pathway specific inhibitor (LY294002) blocked GDNF-mediated striatal cell differentiation suggesting that the basal activity of this pathway is needed for the effects of GDNF. Therefore, our results indicate that treatment of cultured striatal cells with GDNF specifically activates the p42/p44 MAPK pathway, leading to an increase in the arborization of GABA- and calbindin-positive neurons.

Key words: Calbindin, GABA, differentiation, PI3-K

INTRODUCTION

Neurotrophic factors are essential proteins for the regulation of neuronal survival, growth and differentiation during development (Baloh et al., 2000; Huang and Reichardt, 2001; Davies, 2003). Most of them stimulate a receptor tyrosine kinase, which activates several well-defined signaling cascades (Airaksinen and Saarma, 2002; Huang and Reichardt, 2003; Segal, 2003). The receptor tyrosine kinase Ret (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996) is an important component in the signaling cascade activated by members of the glial cell line-derived neurotrophic factor (GDNF) family, a group of structurally and functionally related polypeptides. This receptor is activated only if GDNF ligands are bound to an accessory protein linked to the plasma membrane by a glycosyl phosphatidylinositol anchor named GDNF family receptor α (GFR α ; Airaksinen and Saarma, 2002). Stimulation of Ret initiates several downstream intracellular pathways, of which the phosphatidylinositol 3-kinase (PI3-K) and the p42/p44 mitogen-activated protein kinase (MAPK) pathways are the most extensively studied (Airaksinen and Saarma, 2002). The activation of these pathways may promote neuronal survival and/or differentiation (Pong et al., 1998; Van Weering et al., 1998; Soler et al., 1999; Couplier et al., 2002; Pelicci et al., 2002).

GDNF, the first member of the GDNF family to be discovered, was initially characterized as a neurotrophic factor for midbrain dopaminergic neurons (Lin et al., 1993). In agreement with its role on nigrostriatal dopaminergic neurons, GDNF is highly expressed in the striatum during development (Schaar et al., 1993; Choi-Lundberg and Bohn, 1995; Golden et al., 1999). The GDNF receptors, Ret and GFR α 1, are also expressed by striatal neurons (Golden et al., 1999; Perez-Navarro et al., 1999; Marco et al., 2002; Cho et al., 2004) suggesting that these neurons can also respond to GDNF (Alberch et al., 2004). Indeed, GDNF protects striatal neurons against excitotoxicity (Perez-Navarro et al., 1996; 1999; Araujo and Hilt, 1997; Gratacos et al., 2001a; Kells et al., 2004) or 3-nitropropionic acid lesion (Araujo and Hilt, 1998).

In the striatum, projection neurons account for 90% of the overall population (Smith and Bolam, 1990). They are GABAergic and they also express calbindin in a late stage of maturation (Liu and Graybiel, 1992). Several neurotrophic factors have been shown to promote the survival and/or maturation of striatal GABAergic

neurons *in vitro* (Mizuno et al., 1994; Ventimiglia et al., 1995; Ivkovic et al., 1997; Gratacos et al., 2001b, 2001c; Gavalda et al., 2004) but very little is known about the biological effects of GDNF (Humpel et al., 1996; Farkas et al., 1997). Furthermore, there are no data about the intracellular signaling pathways activated by this neurotrophic factor in striatal neurons. Therefore, here we examined whether GDNF activates the p42/p44 MAPK or PI3-K pathways, and the functional meaning of this activation in the survival, maturation or differentiation of striatal GABAergic neurons *in vitro*.

MATERIALS AND METHODS

Cell culture

Animal handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the Directive 86/609/EU of the European Commission. Certified time-pregnant Sprague–Dawley dams (Charles River Laboratories, France) were deeply anesthetized on gestational day 19, fetuses were rapidly removed from the uterus and striatal cells were obtained as described elsewhere (Gratacos et al., 2001b). Cells were plated at a density of 50,000 cells/cm² onto 24-well plates or 60-mm culture dishes, which were precoated with 0.1 mg/ml poly-D lysine (Sigma Chemical Co., St. Louis, MO, USA), for morphological or Western blot analysis, respectively. Eagle's minimum essential medium (MEM; Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B-27 (Gibco-BRL) was used to grow the cells in serum-free conditions. To study the activation of PI3-K and p42/p44 MAPK pathways, medium was removed at 3 days *in vitro* (DIV) and replaced by N2-supplemented medium to deprive cells for 3h. Then, GDNF (Peprotech EC Ltd., London, UK) was added to the cultures and Akt and p42/p44 phosphorylation was examined at different time points. In another set of experiments, cultures were treated with various inhibitors, such as PD98059 (25 or 50 μ M; Calbiochem, San Diego, CA), U0126 (5 or 10 μ M; Calbiochem) or LY294002 (25 or 50 μ M; Biomol Research Laboratories, USA). They were dissolved in N2-supplemented medium containing bovine serum albumin (6.6 mg/ml; Sigma), and added to cell cultures 1 h before GDNF treatment. For morphological analysis, MEM supplemented with B-27 was used to grow the cells and at 3DIV GDNF (50 ng/ml) was added alone or in combination with LY294002 or PD98059. Two days after treatments

the medium was removed and replaced by MEM supplemented with B-27 until 7DIV, when the cultures were fixed. Plated cell cultures were maintained in an incubator with 5% CO₂ at 37 °C.

Western blot analysis

After GDNF exposure, cells were rinsed rapidly in ice-cold phosphate-buffered saline (PBS), and lysed with buffer as described elsewhere (Gavalda et al., 2004). Membranes were incubated overnight at 4 °C with antibodies against phospho-p42/p44 (1:5,000; Cell signaling Technology, Beverly, MA) or phospho-Akt (1:2000; Cell Signaling Technology). To standardize total protein content in each lane, membranes were incubated for 1 h at room temperature (r.t.) with a mouse monoclonal antibody against panERK (1:5000; BD Transduction Laboratories) or against panAkt (1:500; Cell Signaling Technology). After addition of the corresponding secondary antibody conjugated to horseradish peroxidase (1:2000; Promega), membranes were developed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, California, USA). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, UK).

Immunocytochemistry

Striatal cultures were fixed with 4% paraformaldehyde for 1 h at r. t., followed by three rinses in PBS. Cells were then preincubated for 15 min with PBS containing 0.3% Triton X-100 (Sigma) and 30% normal horse serum (Gibco-BRL) at r.t. Cultures were then incubated overnight at 4 °C with antibodies directed against calbindinD28K (1:10,000; Swant) or GABA (1:1000; Sigma) diluted in PBS containing 0.3% Triton X-100 and 5% normal horse serum. Cells were then incubated in biotinylated secondary antibodies, then with avidin-biotin complex (Pierce ABC Kit) and finally developed with 0.05 % diaminobenzidine and 0.02 % H₂O₂.

Detection of cell death

At 3DIV, cultures were treated with GDNF (50 ng/ml) and dying neurons were detected 2 days later. Cells were fixed with 4% paraformaldehyde for 1 h at r.t., followed by three rinses in PBS. Neurons were incubated with DAPI (1:100; Sigma) for 5 min and then rinsed twice with PBS.

Quantitative analysis of cell cultures

Total cell number, GABAergic neurons, calbindin-positive neurons and pyknotic/fragmented nuclei stained by DAPI were counted in 20 fields at 200X. Cell number was analyzed in four to six wells per condition and in four independent experiments. Morphological parameters were assessed using a PC-Image analysis system from Foster Findlay on a computer attached to an Olympus microscope. GABA- and calbindin- positive neurons (60 per condition) were chosen at random and traced in a phase-contrast image using the mouse hook up. Total and soma area, perimeter and degree of arborization ($\text{Perimeter}^2 / 4\pi\text{Area}$) were determined as described by Fujita et al. (1996). Axon length was also measured, considering the axon as the longest emerging neurite from the soma, as previously described (Gratacos et al., 2001b).

Statistical significance was assessed by ANOVA followed by the L.S.D. post hoc test.

RESULTS

GDNF specifically activates the p42/p44 MAPK pathway.

In order to identify which intracellular pathways were activated by GDNF in striatal neurons, medium was removed on 3DIV and replaced by N2-supplemented medium to deprive cells for 3 hours before GDNF (50 ng/ml) addition. Phospho-p42/p44 levels rose sharply (by 2-fold, Fig. 1A) five minutes after GDNF treatment. In contrast, levels of phospho-Akt were not affected by GDNF at any time (Fig. 1B). However, after cell culture deprivation basal phospho-Akt levels increased in both control and GDNF-treated cultures (Fig. 1B), while phospho-p42/p44 levels did not change (data not shown). The same membranes were reprobed for total Akt, showing that total levels of the protein were not modified (Fig. 1B).

We next examined whether activation of the PI3-K pathway by GDNF was dose dependent. Addition of 100 ng/ml of GDNF did not affect phospho-Akt levels (GDNF 50 ng/ml: 106 ± 20 ; GDNF 100 ng/ml: 82 ± 10 ; results obtained five minutes after GDNF addition and expressed as a percentage of phospho-Akt control values). In contrast, p42/p44 phosphorylation levels were higher than after treatment with 50 ng/ml

of GDNF (GDNF 50 ng/ml: phospho-p44, 213 ± 58 ; phospho-p42, 625 ± 89 ; GDNF 100 ng/ml: phospho-p44, 389 ± 28 ; phospho-p42, 1035 ± 117 ; results obtained five minutes after GDNF addition and expressed as a percentage of phospho-p44 levels in control condition).

To further characterize the activation of the p42/p44 MAPK pathway by GDNF in striatal cells, cultures were treated for 1h with specific inhibitors before the addition of the trophic factor (50 ng/ml). Pretreatment with PD98059 (25-50 μ M) or U0126 (5-10 μ M) reduced GDNF-induced p42/p44 phosphorylation (Fig. 2A). Furthermore, we analyzed the effect of PI3-K inhibitors in p42/p44 activation by GDNF. In cultures treated with LY294002 (25 μ M) alone basal levels of phospho-Akt decreased but phospho-p42/p44 was unaffected (Fig. 2B), showing that this inhibitor selectively blocks PI3-K activation. Abrogation of PI3-K by pretreatment of cells with LY294002 (25 μ M) did not modify GDNF-induced phosphorylation of p42/p44 (Fig. 2B), but addition of a higher dose of LY294002 (50 μ M) slightly inhibited (by 30 %) phospho-p42/p44 levels (Fig. 2B).

GDNF treatment has no effect on neuronal survival.

The next step was to investigate the biological effects resulting from GDNF-induced activation of the p42/p44 MAPK pathway in striatal neurons. The percentage of dying cells was similar in control (29 ± 1 %) and in GDNF-treated (23 ± 4 %) cultures. Similarly, the total number of cells at 7DIV was not modified by GDNF (50 ng/ml) addition at 3DIV (Control: $18,865 \pm 2,609$ cells/cm²; GDNF: $17,287 \pm 2,273$ cells/cm²).

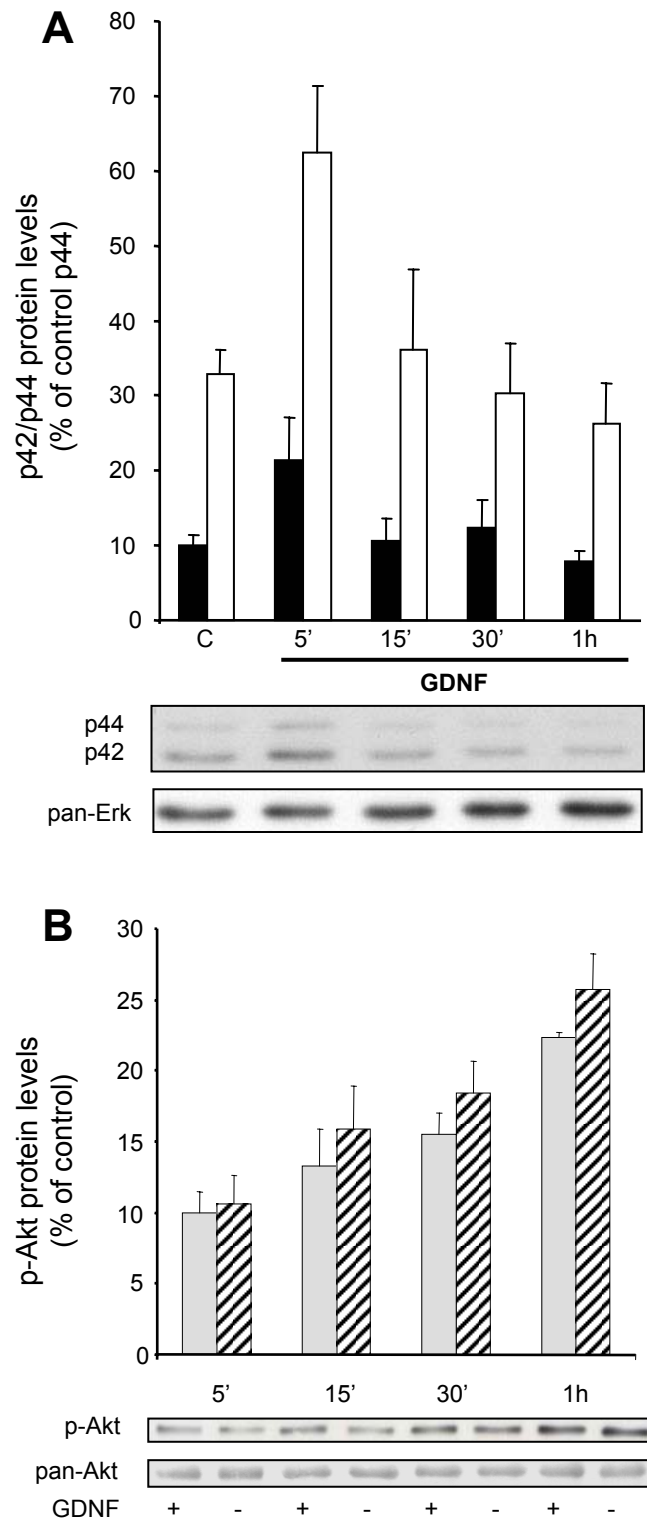


Figure 1.- GDNF treatment activates the p42/p44 MAPK but not the PI3-K pathway. GDNF (50 ng/ml) was added to cultures, and p42/p44 and Akt phosphorylation were examined by Western blot at different time points. Immunoblots were obtained from representative experiments. (A) Bars showing phosphor-p42/p44 protein levels. Results were obtained from densitometric analysis (n=4) and expressed as a percentage of phospho-p44 protein levels in control condition. p42: open bars; p44: filled bars. (B) Bars showing phospho-Akt protein levels. Results obtained from densitometric analysis (n=4) were expressed as a percentage of control (gray bars); GDNF-treated: hatched bars.

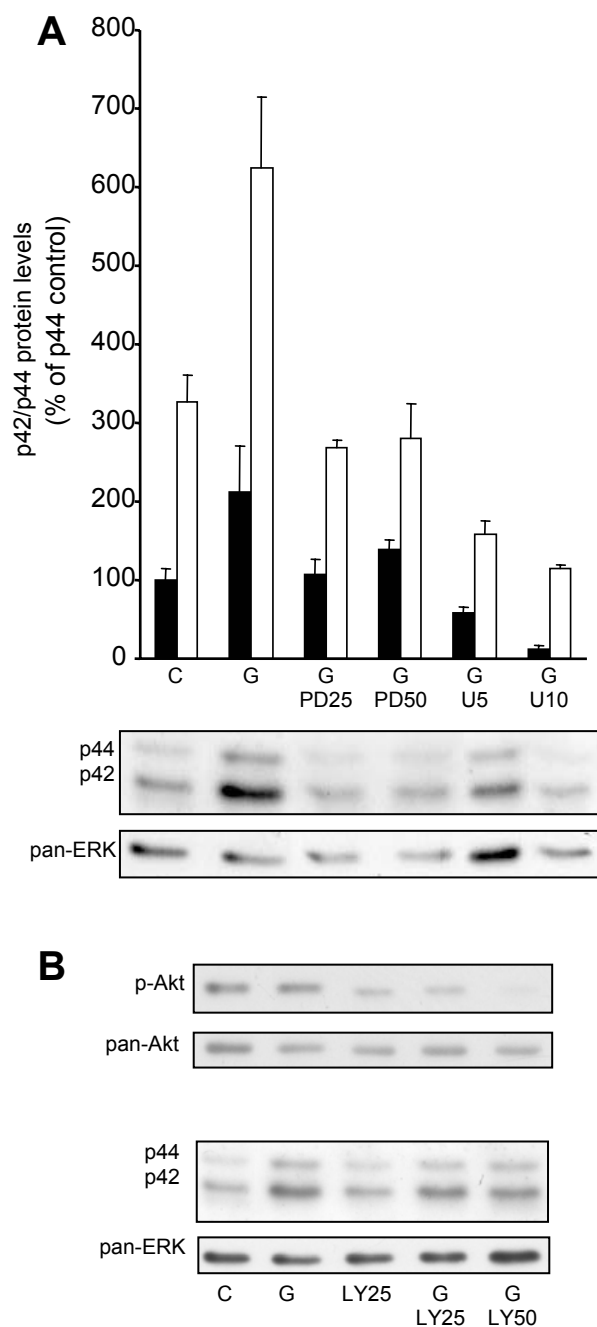
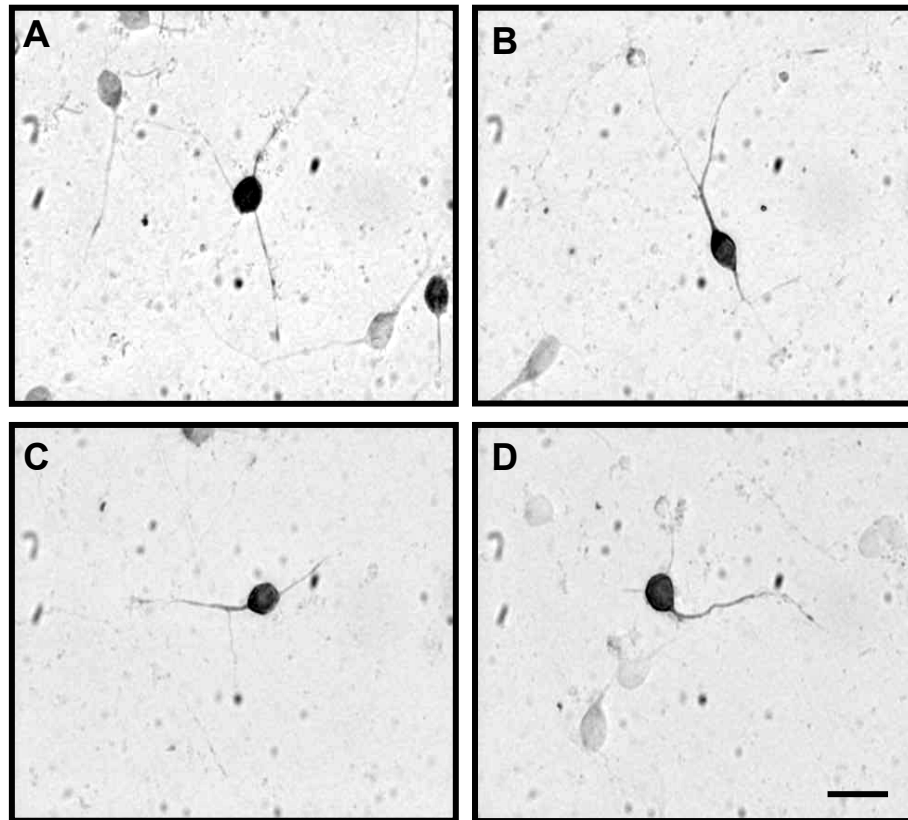


Figure 2.- GDNF-induced activation of the p42/p44 MAPK pathway is blocked by treatment with inhibitors of p42/p44 and PI3-K pathways. Cultures were treated with inhibitors for 1 hour before the addition of GDNF (50 ng/ml). Phospho-p42/p44 and phospho-Akt levels were measured by Western blot at 5 minutes after GDNF addition. (A) Figure showing the blockade of GDNF-induced increase in phospho-p42/p44 by treatment with specific inhibitors of the p42/p44 MAPK pathway. Results obtained from densitometric analysis (n=4) were expressed as a percentage of phospho-p44 protein levels in control condition. p42: open bars; p44: filled bars. PD25: PD98059 25 μ M; PD50: PD98059 50 μ M; U5: U0126 5 μ M; U10: U0126 10 μ M. Immunoblot was obtained from a representative experiment (B) Immunoblots showing the inhibition of phospho-Akt basal levels by treatment with the PI3-K pathway specific inhibitor LY294002 25 μ M (LY25), and the blockade of increased phospho-p42/p44 levels induced by GDNF by treatment with 25 μ M LY294002 (G/LY25) and 50 μ M LY294002 (G/LY50). C: control; G: GDNF (50 ng/ml).

GDNF-mediated stimulation of the p42/p44 MAPK pathway promotes the arborization, but not the maturation, of GABA- and calbindin-positive striatal neurons

We studied whether GDNF induces GABA and calbindin phenotypes, and the differentiation of these neuronal populations. The number of GABA-positive neurons was not modified by GDNF (Control: $13,659 \pm 3,558$ cells/cm²; GDNF-treated: $13,248 \pm 3,291$ cells/cm²). Similarly, no differences were detected between the number of calbindin-positive neurons in control (684 ± 108 cells/cm²) and in GDNF-treated cultures (694 ± 119 cells/cm²).

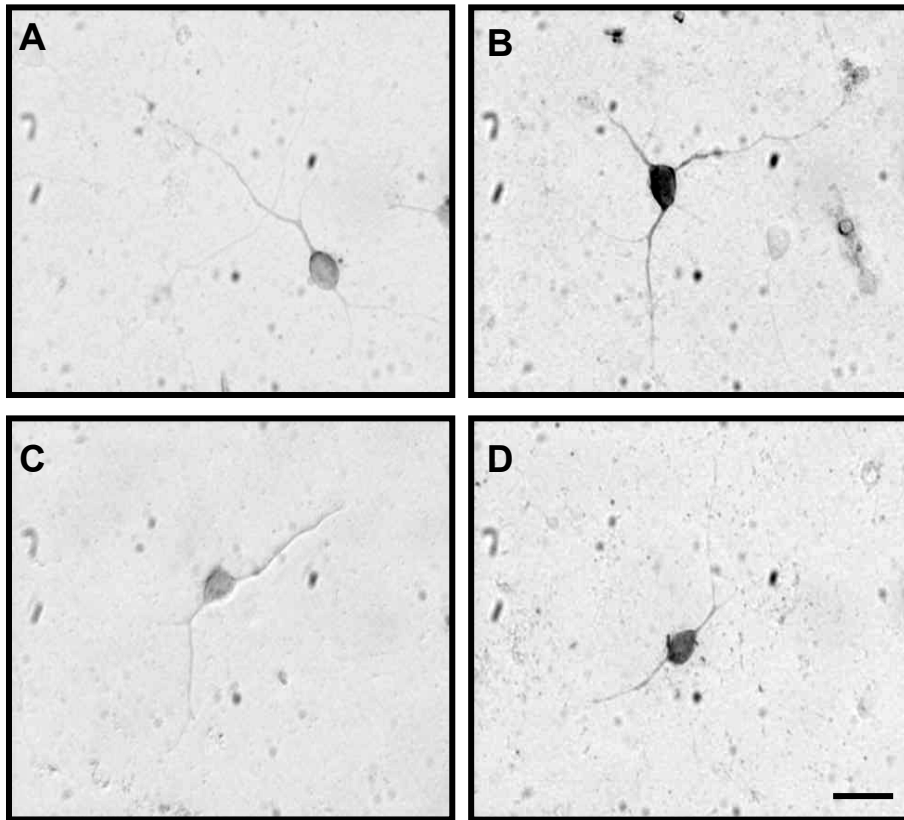
Morphological analysis was performed to investigate the involvement of GDNF in the differentiation of GABA- and calbindin-positive neurons. GDNF treatment increased the total area, perimeter, axon length and degree of arborization of both GABA- (Fig. 3) and calbindin-positive (Fig. 4) neurons, without modifying the soma area. The effects on the degree of arborization were higher in the calbindin-positive population (compare Fig. 3 and 4). These morphological parameters were also analyzed in the presence of the inhibitor PD98059 (50 μ M). The addition of PD98059 alone did not affect the differentiation of GABA- (axon length, in μ m: 40 ± 1 ; degree of arborization: 14 ± 1) or calbindin-positive neurons (axon length, in μ m: 53 ± 1 ; degree of arborization: 20 ± 1). In contrast, addition of PD98059 in combination with GDNF blocked the effects of the neurotrophic factor on the differentiation of both GABA- and calbindin-positive neurons (Fig. 3 and 4, respectively). In this condition, all the parameters analyzed were the same as control. The differentiation of GABA- and calbindin-positive neurons after treatment with LY294002 alone was similar to that observed in control (axon length in μ m: 45 ± 2 and 58 ± 4 ; degree of arborization: 16 ± 2 and 22 ± 1 , for GABA- and calbindin-positive neurons, respectively). Unexpectedly, GDNF-mediated effects on the differentiation of GABA- and calbindin-positive neurons were inhibited in the presence of LY294002 (Fig. 3 and 4).



E Quantitative analysis of the differentiation induced by GDNF on GABA-positive striatal neurons.

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
CONTROL	157 \pm 4	177 \pm 5	74 \pm 4	44 \pm 2	18 \pm 2
GDNF	201 \pm 6 *	246 \pm 9 *	79 \pm 5	61 \pm 4 *	25 \pm 1 *
GDNF+PD	158 \pm 11	165 \pm 16	79 \pm 3	44 \pm 3	14 \pm 2
GDNF+LY	153 \pm 5	172 \pm 1	71 \pm 2	44 \pm 2	16 \pm 0

Figure 3.- GDNF promotes GABA-positive neurons differentiation through the activation of the p42/p44 MAPK pathway. GABA immunocytochemistry was performed at 7DIV. Photomicrographs GABA-positive neurons from striatal cultures treated with either (A) vehicle, (B) GDNF (50 ng/ml), (C) GDNF plus PD98059 (50 μM) or (D) GDNF plus LY294002 (25 μM). Scale bar 40 μm . (E) Quantitative analysis of the effects of GDNF and the specific inhibitors on the morphology of striatal GABA-positive neurons. For each parameter and condition examined, 60 neurons were analyzed in three different experiments. Results are expressed as the mean \pm SEM. * $P < 0.001$ compared to control values; # $P < 0.001$ compared with GDNF values. ANOVA followed by the L.S.D. post hoc test.



E Quantitative analysis of the differentiation induced by GDNF on calbindin-positive striatal neurons.

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
CONTROL	193 \pm 6	213 \pm 7	89 \pm 3	51 \pm 3	19 \pm 1
GDNF	266 \pm 13 *	328 \pm 16 *	94 \pm 2	73 \pm 5*	33 \pm 2 *
GDNF+PD	194 \pm 11	218 \pm 14	88 \pm 3	53 \pm 4	21 \pm 2
GDNF+LY	196 \pm 15	204 \pm 16	92 \pm 6	55 \pm 7	19 \pm 1

Figure 4.- GDNF promotes the arborization of striatal calbindin-positive neurons through the activation of the p42/p44 MAPK pathway. Calbindin immunocytochemistry was performed at 7DIV. Photomicrographs shows GABA-positive neurons from striatal cultures treated with either (A) vehicle, (B) GDNF (50 ng/ml), (C) GDNF plus PD98059 (50 μM) or (D) GDNF plus LY294002 (25 μM). Scale bar 40 μm . (E) Quantitative analysis of the effects of GDNF and the specific inhibitors on the morphology of striatal calbindin-positive neurons. For each parameter and condition examined, 60 neurons were analyzed in three different experiments. Results are expressed as the mean \pm SEM. * P < 0.001 compared to control values; # P < 0.001 compared with GDNF values. ANOVA followed by the L.S.D. post hoc test.

DISCUSSION

In this study we show that GDNF specifically activates the p42/p44 MAPK pathway in cultured striatal cells. This activation leads to biological effects as GDNF treatment increases the degree of arborization and axon length in both GABA- and calbindin-positive striatal neurons. Although GDNF only activates the p42/p44 MAPK pathway, its biological effects are blocked in the presence of p42/p44 MAPK (PD98059) or PI3-K (LY294002) pathway specific inhibitors.

GDNF promotes both neuronal survival (Henderson et al., 1994; Oppenheim et al., 1995; Ha et al., 1996; Price et al., 1996; Burke et al., 1998) and morphological differentiation (Mount et al., 1995; Price et al., 1996; Widmer et al., 2000; Holm et al., 2002) depending on the neuronal type examined. In our culture conditions, GDNF increased the degree of arborization of both GABA- and calbindin-positive neurons without affecting either neuronal survival or maturation. We also show that GDNF only activated the p42/p44 MAPK pathway. Consistent with our results, the activation of the p42/p44 MAPK pathway induced by GDNF has mainly been implicated in neuronal differentiation (Chen et al., 2001; Park et al., 2005) while the activation of the PI3K pathway has been related to both survival (Miller et al., 1997; Soler et al., 1999; Encinas et al., 2001) and differentiation (van Weering and Bos, 1997; Pong et al., 1998). Therefore, we suggest that GDNF-induced neuronal differentiation of striatal neurons is mediated by the activation of the p42/p44 MAPK pathway.

Our results show that GDNF did not affect the number of calbindin-positive neurons. Previous studies have reported that treatment of striatal neurons with another trophic factor, BDNF, increases the number of calbindin-positive neurons (Gavalda et al., 2004). This BDNF-mediated effect depends on the activation of both PI3-K and p42/p44 MAPK pathways (Gavalda et al., 2004). Therefore, we can suggest that GDNF did not induce the calbindin phenotype, as it did not activate the PI3-K pathway in striatal neurons. Furthermore, both GDNF (present results) and BDNF (Gavalda et al., 2004) treatment increased the degree of arborization of GABA- and calbindin-positive neurons, but the effects of BDNF were higher. Since BDNF-induced neuronal differentiation was abolished in the presence of p42/p44 MAPK or PI3-K inhibitors, taken together our results could implicate PI3-K in neuronal differentiation. However, levels of phosphor-p42/p44 after BDNF addition are higher and more sustained (even 7

days after treatment, Gavalda et al., 2004) than after GDNF treatment (only at 5 minutes, present results). Thus, the strength and duration of the MAPK pathway activation may also be critical for these biological effects as has been previously described in other models (Mariathasan et al., 2001; Chang et al., 2003; Rossler et al., 2004; Whitehurst et al., 2004).

GDNF-induced p42/p44 MAPK pathway activation was blocked in the presence of the specific inhibitors PD98059 and U0126. As expected, treatment with PD98059 also prevented GDNF-mediated biological effects. However, our findings also showed that treatment with LY294002, a specific inhibitor of the PI3-K pathway, blocked the biological effects mediated by GDNF. This result could not be attributed to the type of cross-talk between p42/p44 MAPK and PI3-K pathways previously described in striatal cultures (Stropollo et al., 2001; Fuller et al., 2001; Perkinton et al., 2002) because GDNF failed to produce a direct activation of the PI3-K pathway, and the dose of LY294002 (25 μ M) used to analyze the biological effects did not inhibit GDNF-induced p42/p44 MAPK pathway activation. Furthermore, treatment with LY294002 blocked basal levels of phospho-Akt, underscoring that it is the basal activity of the PI3K pathway that is required for GDNF to exert its trophic effects on striatal neurons. Consistent with our data, it has been previously shown that weak stimulation, but not strong stimulation, of p42/p44 MAPK pathway could be dependent on the basal PI3-K pathway activity (Duckworth and Cantley, 1997; Wennström and Downward, 1999). In the present culture conditions, phospho-Akt, but not phospho-p42/p44 levels, gradually increased after changing the culture medium indicating that this pathway is important for neuronal survival, as previously described (Dudek et al., 1997; Miller et al., 1997; Soler et al., 1998; Kuruvilla et al., 2000; Gavalda et al., 2004).

Striatal neuron development, maturation and establishment of synaptic connections are regulated by different neurotrophic factors (Maisonpierre et al., 1990; Checa et al., 2000; Ciccolini et al., 2001). GDNF expression in the striatum varies during postnatal development with two peaks of expression on postnatal days 2 and 14 (Oo et al., 2005). This striatal GDNF has been mainly related with the survival of nigrostriatal dopaminergic neurons through a target-derived neurotrophic mechanism (Oo et al., 2003; Kholodilov et al., 2004). However, here we show that GDNF also regulates one aspect of striatal neuron development, the extension of neurites with

occurs late in postnatal development. Accordingly, GFR α 1 is expressed by striatal projection neurons with maximal levels between postnatal days 10 and 14 (Cho et al., 2004). Furthermore, our results show that GDNF-mediated effects were higher on mature striatal neurons, the calbindin-positive population, and that these effects were mediated by the activation of the p42/p44 MAPK pathway. Similarly, previous studies have related the activation of this pathway in the striatum with the regulation of mature neuronal functions such behavioral plasticity and drug addiction (Mazzucchelli et al., 2002).

In conclusion, our data demonstrate that GDNF, through the activation of the p42/p44 MAPK pathway, specifically promotes striatal neuron differentiation more strongly in the calbindin-positive population. This indicates that GDNF plays a main role in inducing late stages of striatal neuron maturation. Furthermore, GDNF-mediated effects require a basal activity of the PI3-K pathway.

Acknowledgments

We thank Maria Teresa Muñoz and Anna López for technical assistance. We are also very grateful to Dr. Amèrica Jiménez and the staff of the animal facility (Facultat de Medicina, Universitat de Barcelona) for their help.

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