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Brain-derived neurotrophic factor (BDNF) mediates bone morphogenetic protein-2 (BMP-2) effects on cultured striatal neurones

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Abstract

Bone morphogenetic proteins are members of the transforming growth factor-β superfamily that have multiple functions in the developing nervous system. One of them, bone morphogenetic protein-2 (BMP-2), promotes the differentiation of cultured striatal neurones, enhancing dendrite growth and calbindin-positive phenotype. Bone morphogenetic proteins have been implicated in cooperative interactions with other neurotrophic factors. Here we examined whether the effects of BMP-2 on cultured striatal neurones are mediated or enhanced by other neurotrophic factors. BMP-2 had a cooperative effect with low doses of brain-derived neurotrophic factor or neurotrophin-3 (but not with other neurotrophic factors such as glial cell line-derived neurotrophic factor, neurturin or transforming growth factor-β2) on the number of calbindin-positive striatal neurones. Moreover, BMP-2 induced phosphorylated Trk immunoreactivity in cultured striatal neurones, suggesting that neurotrophins are involved in BMP-2 neurotrophic effects. The addition of TrkB-IgG or antibodies against brain-derived neurotrophic factor abolished the effects of BMP-2 on the number and degree of differentiation of calbindin-positive striatal neurones. Indeed, BMP-2 treatment increased brain-derived neurotrophic factor protein levels in treated cultures media and BDNF immunocytochemistry revealed that this neurotrophin was produced by neuronal cells. Taken together, these results indicate that brain-derived neurotrophic factor mediates the effects of BMP-2 on striatal neurones.

Keywords: bone morphogenetic protein, brain-derived neurotrophic factor, calbindin, differentiation, striatum, TrkB-IgG.

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Neurotrophic signals within a cell result from a balanced interaction of several pathways (Springer and Kitzman 1999). Indeed, CNS neurones are commonly supported by several neurotrophic factors, and this redundancy of trophic support accounts for the lack of striking effects in many single knockouts for these molecules (reviewed in Snider 1994; Henderson 1996). Neurones within the striatum, the major component of the basal ganglia, are supported *in vitro* by brain-derived neurotrophic factor (BDNF; Mizuno *et al*. 1994), neurotrophin-3 (NT-3; Ventimiglia *et al*. 1995; Nakao *et al*. 1996) and glial cell line-derived neurotrophic factor (GDNF; Humpel *et al*. 1996; Farkas *et al*. 1997). More recently, a member of the bone morphogenetic protein family (BMPs), BMP-2, has been described as promoting the differentiation of cultured striatal cells, increasing their dendritic growth and promoting calbindin phenotype (Hattori *et al*. 1999; Gratacos *et al*. 2001).

BMPs, initially identified by their ability to induce bone formation (Wozney *et al*. 1988), belong to the transforming growth factor-β (TGF-β) superfamily and have been linked recently to a wide array of nervous system events including neurulation, morphogenesis, lineage elaboration and phenotypic maturation (reviewed in Mehler *et al*. 1997; Ebendal *et al*. 1998). BMP-2 helps to establish the dorsoventral axis of the vertebrate embryo (reviewed in DeRobertis and Sasai 1996), controls the switch from neurogenesis to gliogenesis

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Abbreviations used: BMP, bone morphogenetic protein; BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; GDNF, glial cell line-derived neurotrophic factor; NT-3, neurotrophin-3; NTN, neurturin; pTrk, phosphorylated Trk; PBS, phosphate buffered saline; TGF-β, transforming growth factor-β.

in neural progenitors (Gross *et al*. 1996; Mabie *et al*. 1997, 1999; Li *et al*. 1998; Morrison *et al*. 2000) and induces the differentiation of several populations of post-mitotic neurones (Jordan *et al*. 1997; Guo *et al*. 1998; Galter *et al*. 1999; Reiriz *et al*. 1999; Gratacos *et al*. 2001). BMP-2 signals via heterotetrameric serine/threonine kinase receptors comprising two molecules each of type-I and -II receptor components (reviewed in Massague 1996; Josso and di Clemente 1997; Raftery and Sutherland 1999). The type-II receptor is a constitutively active kinase, whereas the type-I receptor kinase, activated when phosphorylated by the type-II receptor, is the downstream signalling component. Activated BMP-2 receptors phosphorylate the transcription factor *Smad*1, enabling its association with the common mediator *Smad*4 and thus leading to transactivation of target genes (Heldin *et al*. 1997).

Several lines of evidence suggest that BMPs may require cooperative interactions with other neurotrophic factors in order to exert their effects. BMP-2 has been shown to induce GDNF and NT-3 dependence in developing rat sympathetic neurones (Kobayashi *et al*. 1998; Zhang *et al*. 1998; Thang *et al*. 2000) and sympathoadrenal progenitor cells (Song *et al*. 1998). Moreover, BMP-2 effects on cultured serotoninergic neurones are mediated by endogenous BDNF (Galter *et al*. 1999). Interestingly, these strong interaction patterns among trophic factors have also been reported for other members of TGF-β superfamily. For instance, GDNF requires TGF-β to induce the survival of both mesencephalic and peripheral ganglia neurones (Krieglstein *et al*. 1998) and its protective effect on corticospinal neurones is mediated via an endogenous release of BDNF (Giehl *et al*. 1998). BDNF has also been proposed as mediating TGF-β pro-survival effects on sensory neurones (Chalazonitis *et al*. 1992).

Given the cooperative effects described for BMPs, the present study examined whether BMP-2 neurotrophic effects on cultured striatal neurones could be mediated or enhanced by other neurotrophic factors.

Materials and methods

Materials

Certified time pregnant Sprague–Dawley dams were purchased from Iberfauna, Spain. Eagle's minimum essential medium, B-27 supplement and normal horse serum were purchased from Gibco-BRL (Paisley, UK). Poly-D-lysine was obtained from Sigma Chemical Co. (St Louis, MO, USA). BMP-2 and TrkB-IgG (fusion proteins between human IgG and the BDNF receptor TrkB) were generously provided by Genetics Institute (Cambridge, MA, USA) and Genentech Inc. (San Francisco, CA, USA), respectively. BDNF, NT-3, GDNF and neurturin (NTN) were purchased from Peprotech EC Ltd. (London, UK) and TGF-β2 was obtained from R & D Systems Ltd. (Abingdon, UK). For uptake experiments, [^{3}H]GABA was purchased from Amersham (Arlington Heights, VA, USA) and OptiPhase Hisafe scintillation fluid from Pharmacia (Uppsala, Sweden). Anti-calbindin$_{d28K}$ was obtained from Swant (Bellinzona, Switzerland) and antiphosphorylated Trk Py-490 (pTrk) from New England BioLabs Inc. (Beverly, MA, USA). BDNF antibody and BDNF E_{max} immunoassay system were purchased from Promega (Madison, WI, USA). The Vectastain Elite ABC kit was obtained from Vector Laboratories (Burlingame, CA, USA).

Cell culture

All procedures using animals were approved by Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99) in accordance with the European Commission Directive 86/609/EU. Certified time pregnant Sprague–Dawley dams were deeply anaesthetized on gestational day 19 and fetuses were rapidly removed from the uterus. Fetal brains were then excised and placed in sterile phosphate-buffered saline (PBS), pH 7.4. The striatum was dissected, pooled and gently dissociated with a fire-polished Pasteur pipette. Cells were plated onto 24-well plates or onto 100-mm culture dishes (for ELISA measurements) precoated with 0.1 mg/mL poly D-lysine at a density of 50 000 cells/cm^2. Eagle's minimum essential medium supplemented with B-27 was used to grow the cells in serum-free conditions. Plated cell cultures were maintained in an incubator with 5% CO_2 at 37°C. Using this technique, neuronal cells represented more than 95% of the cell population, as previously described (Gratacos *et al*. 2001). At 8 days *in vitro* (DIV), cells were either fixed for immunocytochemistry or biochemical assays were performed.

BMP-2, at a concentration of 10 ng/mL, was added to the cultures, following either a 'chronic' (added at the time of plating and remaining for 8 DIV) or an 'acute' (for 24 h, from 7 to 8 DIV) treatment, as described elsewhere (Gratacos *et al*. 2001). In order to assess the putative synergistic effect of BMP-2 in our culture system, BMP-2 (10 ng/mL) was added either alone or in combination with 0.05 or 5 ng/mL of BDNF, NT-3, GDNF, NTN or TGF-β2 at the time of plating. In one set of experiments, 250 ng/mL of TrkB-IgG or 15 μg/mL of an antibody against BDNF were administered simultaneously with BMP-2, following either chronic or acute treatment. For phosphorylated Trk (pTrk) immunocytochemistry, BMP-2 or BDNF (5 ng/mL) were added to the culture at 8 DIV for 30 min, 1 or 4 h.

GABA-uptake

[^{3}H]GABA uptake was assessed following Weiss (1988), with minor modifications. Cultures were incubated for 20 min at 37°C with [^{3}H]GABA (80 Ci/mmol) which was added to a final concentration of 20 nM. Uptake was halted by rinsing three times in a Krebs–Ringers–HEPES solution at 0°C. Cells were lysed with 800 μL of NaOH (0.1 M). This volume of lysate was added to 10 mL of OptiPhase Hisafe scintillation fluid and counted.

Immunocytochemistry

Striatal cultures were fixed with 4% paraformaldehyde for 1 h at room temperature, followed by three rinses in PBS. Cells were preincubated for 30 min with PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. Cultures were then incubated overnight at 4°C with an antibody directed against calbindin$_{d28K}$ (1 : 10 000) or BDNF (1 : 100) diluted in PBS containing 0.3% Triton X-100 and 1% normal horse serum as described elsewhere (Gratacos *et al*. 2001). Protocol for pTrk

immunocytochemistry was performed as previously described (Watson *et al*. 1999). Striatal neurones were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) with 1 mM orthovanadate. After fixation, cells were washed in TBS, blocked and permeabilized (5% normal goat serum, 0.5% NP-40) for 1 h at room temperature. Cells were rinsed in TBS and incubated with anti-pTrk (1 : 50) for 48 h at 4°C. After incubation for 1 h at room temperature with the respective peroxidase-labelled secondary antibodies, immunoreactivity for calbindin or pTrk was detected using the Vectastain Elite ABC kit and developed with diaminobenzidine.

Quantitative analysis of cell cultures

Cells were counted within 33 fields at 200×. Results are given as percentages of control cultures, analysing six wells per condition from three different experiments. Morphological parameters were assessed using a PC-Image analysis system from Foster Findlay on a computer attached to an Olympus microscope. Calbindin-positive neurones (90–120) were randomly chosen from three wells and traced in a phase-contrast image using the mouse hookup. Total and soma area, perimeter and degree of arborization ($Perimeter^2/4\pi Area$) were determined as described by Fujita *et al*. (1996). All experiments were performed in triplicate. Statistical significance was assessed by ANOVA followed by Neuman–Keul's test for comparison of multiple means.

Determination of BDNF protein levels by ELISA

After acute exposure to 10 ng/mL of BMP-2 or vehicle from 7 DIV to 8 DIV, BDNF protein content was analysed in cultured striatal cells and in media extracts. Striatal cells were collected in lysis buffer [137 mM NaCl, 20 mM Tris, pH 8; 1% NP40, 10% glycerol, 1 mM phenylmethysulphonyl fluoride (PMSF), 10 μg/mL aprotinin, 1 μg/mL leupeptin and 0.5 mM sodium orthovanadate], whereas striatal media were concentrated by tricarboxylic acid precipitation. Samples were analysed using the BDNF E_{max} immunoassay system following the manufacturer's recommendations. Extracts were diluted in 100 μL of the 'block & sample' buffer provided and incubated on a plate coated with a BDNF antibody. Standard curve of pure BDNF protein, provided by the kit, was used to quantify BDNF contents. Absorbance values were determined at 450 nm in a plate reader. For control wells in which anti-BDNF monoclonal antibody was omitted, absorbance values were not significantly different from the absorbance of blank wells.

Results

BMP-2 and neurotrophins cooperatively increase the number of striatal calbindin-positive neurones *in vitro*

In order to test whether BMP-2 could have a cooperative effect with neurotrophins (BDNF and NT-3) or other members of the TGF-β superfamily (GDNF, NTN, TGF-β2), combinations of BMP-2 and these neurotrophic factors were added to the culture at the time of plating. The overall GABAergic population or the calbindin-positive subpopulation was evaluated at DIV8. GABA uptake analysis showed that only BDNF or NT-3 increased this biochemical parameter (100 and 44%, respectively), in line with previous studies (Ventimiglia *et al*. 1995; Nakao *et al*. 1996). The combined addition of 5 ng/mL of BDNF, NT-3, TGF-β2, GDNF or NTN together with 10 ng/mL of BMP-2 did not induce any change in the GABA uptake compared with the addition of these factors alone (Table 1).

Table 1 Effect of BMP-2 and other trophic factors on [^{3}H]GABA uptake

Growth factors	BMP (–)	BMP (+)
Control	100	96 ± 4
BDNF	191 ± 4*	198 ± 2*
NT-3	144 ± 5*	145 ± 3*
GDNF	105 ± 2	103 ± 1
NTN	102 ± 1	106 ± 1
TGF-β2	103 ± 1	105 ± 2

A total of 5 ng/mL BDNF, NT-3, GDNF, NTN or TGF-β2, alone or in combination with 10 ng/mL of BMP-2, were added at the time of plating. [^{3}H]GABA uptake was measured at DIV8. Values indicate the mean ± SEM of six different wells belonging to three different platings, and are expressed as a percentage of control. *$p < 0.05$ compared with control values.

When calbindin immunoreactivity in DIV8 cultures treated with the aforementioned factors was assessed, BMP-2 (10 ng/mL), BDNF (5 ng/mL) or NT-3 (5 ng/mL) were found to increase the number of calbindin-positive neurones by 42, 410 and 235%, respectively. Interestingly, the combination of BMP-2 (10 ng/mL) with 0.05 ng/mL of BDNF or NT-3 cooperatively increased the number of calbindin-positive neurones. In contrast, when higher doses (5 ng/mL) of BDNF or NT-3 were added to BMP-2

Table 2 Effect of BMP-2 and other trophic factors on the number of calbindin-positive striatal neurones

Growth factors	BMP (–)	BMP (+)
Control	100	142 ± 6*
0.05 ng/mL BDNF	165 ± 9*	185 ± 12*,†
5 ng/mL BDNF	510 ± 53*	485 ± 45*
0.05 ng/mL NT-3	164 ± 5*	185 ± 8*,†
5 ng/mL NT-3	335 ± 61*	375 ± 47*
5 ng/mL GDNF	105 ± 2	103 ± 1
5 ng/mL NTN	102 ± 1	106 ± 1
5 ng/mL TGF-β2	103 ± 1	105 ± 2

BDNF, NT-3, GDNF, TGF-β2 or NTN, alone or in combination with 10 ng/mL of BMP-2, were added at the time of plating. Calbindin-positive neurones were counted at DIV8. Values indicate the mean ± SEM of six different wells belonging to three different platings, and are expressed as a percentage of control. *$p < 0.05$ compared with control values, †$p < 0.05$ compared with BMP(–) values.

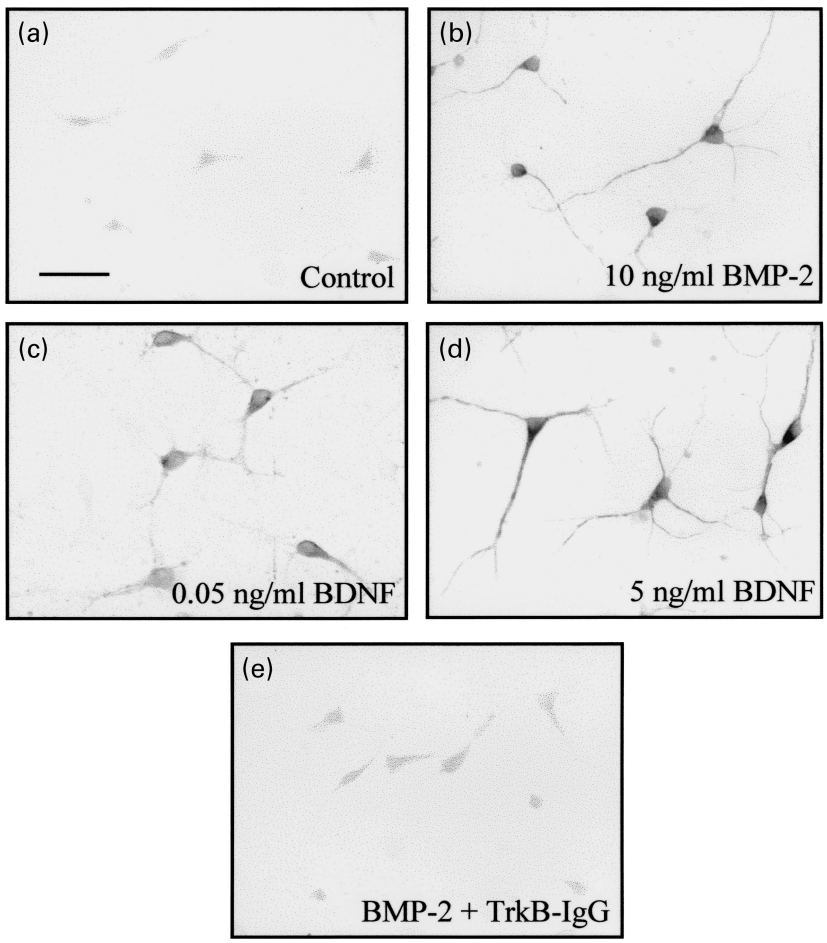

Fig. 1 BMP-2 increases pTrk staining in cultured striatal cells. At DIV8, cultures were exposed for 4 h to either (a) vehicle (b) 10 ng/mL BMP-2 (c) 0.05 ng/mL BDNF (d) 5 ng/mL BDNF or (e) BMP-2 (10 ng/mL) together with TrkB-IgG (250 ng/mL) and pTrk immunodetection was performed. Whereas control cultures displayed no signal for pTrk staining, cultures treated with either BMP-2 or BDNF showed a marked increase in the immunoreactivity for this activated form of Trk receptor both in soma and neurites. The effect of BMP-2 was abolished by the addition of TrkB-IgG. (Scale bar, 60 μm.)

(10 ng/mL), the potent effect of these neurotrophins masked BMP-2-induced increase in the number of calbindin-positive neurones (Table 2).

BDNF mediates BMP-2 effects on calbindin-positive striatal neurones

To determine whether neurotrophins could be involved in BMP-2 neurotrophic effects, we assessed immunoreactivity for the activated form of the neurotrophin receptor, pTrk, in cultures treated with 10 ng/mL of BMP-2 for 30 min, 1 or 4 h at DIV8. Interestingly, BMP-2 induced pTrk staining compared with control cultures (compare Figs 1a and b). Indeed, pTrk immunoreactivity was widespread among cultured striatal cells, this being consistent with previous data showing that most striatal neurones are positive for TrkB immunoreactivity *in vitro* (Nakao *et al*. 1995). The maximal signal for this activated form of Trk receptor was detected at 4 h, both in the soma area and neurites of cultured striatal cells. The addition of BDNF (5 ng/mL) to the cultures induced a similar though more intense pattern of pTrk staining than the one induced by BMP-2 (Fig. 1d).

Since our results point to BDNF as the most potent neurotrophic factor for striatal cultured neurones (Figs 1c and d), in line with previous studies (Mizuno *et al*. 1994; Ventimiglia *et al*. 1995), and it has previously been shown to mediate BMP-2 effects on cultured serotoninergic neurones (Galter *et al*. 1999), it was hypothesized that BDNF could be mediating BMP-2 effects on the number and degree of differentiation of striatal calbindin-positive neurones. Therefore, we simultaneously added BMP-2 (10 ng/mL) together with TrkB-IgG (250 ng/mL) or an antibody against BDNF (15 μg/mL) at the time of plating and analysed calbindin-positive population at DIV8. These doses of TrkB-IgG or anti-BDNF blocked BDNF (5 ng/mL) effects on cultured calbindin-positive striatal neurones (BDNF, 454 ± 83% of control; BDNF + TrkB–IgG, 91 ± 9% of control; BDNF + anti-BDNF, 98 ± 8% of control).

The addition of BMP-2 to the cultures promoted the differentiation of calbindin-immunoreactive cells (36% increase in total area, 48% increase in the perimeter and 58% increase in the degree of arborization; Fig. 2). In contrast, concomitant addition of TrkB-IgG together with BMP-2 completely blocked BMP-2 effects on the differentiation of calbindin-positive neurones (Figs 2c–e). Moreover, the addition of TrkB-IgG or a blocking antibody for BDNF together with BMP-2 at the time of plating was able

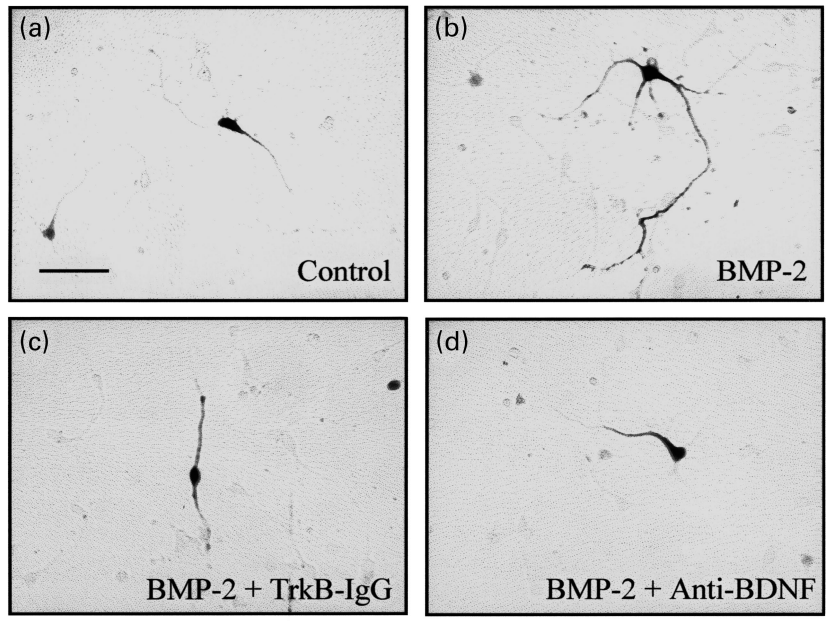

Fig. 2 TrkB-IgG and antibodies against BDNF block BMP-2 effects on the differentiation of calbindin-positive striatal neurones. Photomicrographs of striatal cultures treated with either (a) vehicle, (b) BMP-2 (10 ng/mL), (c) BMP-2 andr TrkB-IgG (250 ng/mL), or (d) BMP-2 and anti-BDNF (15 μg/mL) at the time of plating, and calbindin immunocytochemistry was assessed at DIV8 (scale bar, 40 μm). (e) Quantitative analysis of the effect of these treatments on the 8DIV striatal calbindin-positive neurone morphology. For each parameter and condition examined, 90–120 neurones were analysed in three experiments. Results are expressed as the mean ± SEM of the values. (*$p < 0.05$ compared with control values).

(e)

	Total area	Perimeter	Degree of arborization
Control	262 ± 15	497 ± 30	77 ± 6
BMP-2	358 ± 14*	738 ± 34*	122 ± 7*
BMP-2 + TrkB-IgG	271 ± 13	486 ± 23	75 ± 4

to abolish the BMP-2 induced increase (143% of control) in the number of calbindin-positive neurones assessed at DIV8 (Fig. 3). The addition of TrkB-IgG or anti-BDNF on their own did not induce changes compared with control cultures (data not shown). On the other hand, TrkB-IgG blocked BMP-2-induced pTrk immunoreactivity, returning it to control levels (Fig. 1e).

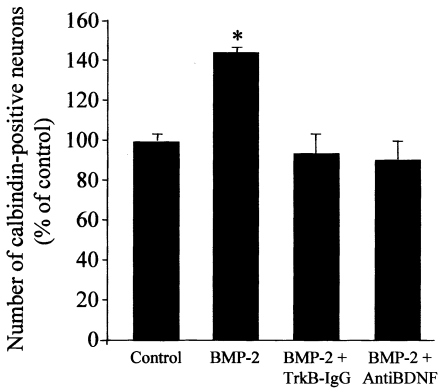

Fig. 3 TrkB-IgG and antibodies against BDNF block BMP-2 effects on the number of calbindin-positive neurones. BMP-2 (10 ng/mL) was added to the cultures with or without TrkB-IgG (250 ng/mL) or an antibody against BDNF (15 μg/mL), and calbindin immunoreactivity was assesed at DIV8. Both TrkB-IgG and anti-BDNF were able to abolish BMP-2-induced increase in the number of calbindin-positive neurones. Values indicate the mean ± SEM of six different wells belonging to three different platings, and are expressed as a percentage of control. (*$p < 0.05$ compared with control values).

BMP-2 increases BDNF protein levels on cultured striatal neurones

In an attempt to quantify this putative increase in endogenous BDNF synthesis or release induced by BMP-2, BDNF protein content was measured by ELISA. Since an exposure to 10 ng/mL of BMP-2 for 24 h from DIV7 to DIV8 is enough to increase calbindin content in striatal cultured neurones (Gratacos *et al.* 2001), we analysed the levels of BDNF in media and extracts of cells subjected to this acute treatment. While BDNF protein content was undetected in the control cells media, higher BDNF levels were found in the media of cells exposed to BMP-2 (Fig. 4a). In contrast, the levels of this neurotrophin in control cell extracts (8.2 ± 0.3 ng/mg protein) tended to decrease in cells treated with BMP-2, falling outside the sensitivity range of ELISA detection.

In order to identify the cells which produce BDNF we incubated striatal cultures at DIV8 with 10 ng/mL BMP-2 and BDNF immunocytochemical analysis was performed. BDNF immunoreactivity was expressed in most of striatal cultured neurones (Figs 4b and c).

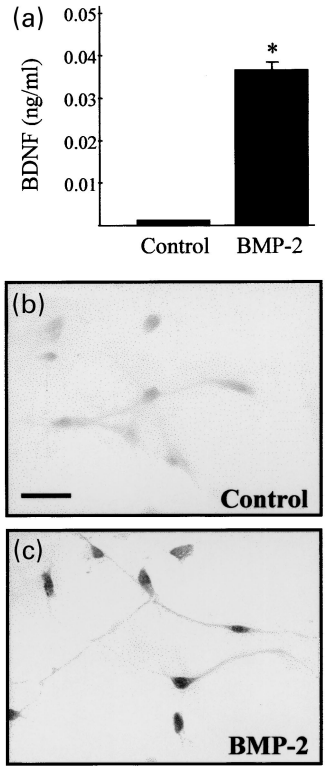

Fig. 4 BMP-2 treatment increases BDNF protein levels. (a) Cultured striatal neurones were incubated with 10 ng/mL BMP-2 from DIV7 to DIV8 and BDNF content was measured by ELISA in the media extracts. (*$p < 0.05$). (b,c) Cultured striatal neurones were incubated with 10 ng/mL BMP-2 at DIV7 for 4 h, and BDNF immunocytochemistry was performed. The photomicrographs show (a) control and (b) BMP-2-treated cultures. Scale bar, 60 μm.

Discussion

The present results identify BDNF as an essential component of BMP-2 neurotrophic effects on cultured striatal neurones. BMP-2 induced phosphoTrk immunoreactivity in striatal neurones *in vitro*. Moreover, the addition of TrkB-IgG and antibodies against BDNF abolish the effects of BMP-2 on the number and degree of differentiation of calbindin-positive striatal neurones. Indeed, we detected an increase in BDNF protein levels in the culture media of striatal cells treated with BMP-2. Taken together, these results suggest that BDNF mediates BMP-2 effects on striatal neurones.

The neurotrophic effects of BMP-2 on neuronal populations have been extended in recent years. After being identified as a neuronal phenotype inducer in PC12 cells (Iwasaki *et al*. 1996), neurotrophic effects of BMP-2 have been described on sympathetic (Guo *et al*. 1998), mesencephalic (Jordan *et al*. 1997; Reiriz *et al*. 1999), striatal (Hattori *et al*. 1999; Gratacos *et al*. 2001) and cortical (Mabie *et al*. 1997) neurones, where it promotes the survival and/or differentiation of these populations. Interestingly, the effects of BMP-2 have been extensively correlated with the neurotrophin family. BMP-2 induces NT-3 responsiveness in rat sympathetic neurones by regulating expression of the neurotrophin receptor TrkC, and its effects on neuronal survival are blocked by the addition of antibodies against NT-3 (Kobayashi *et al*. 1998; Zhang *et al*. 1998). Furthermore, BMP-2 treatment promotes nerve growth factor dependence in cultured sympathoadrenal progenitor cells (Song *et al*. 1998). In our *in vitro* system, BMP-2 increases the number of calbindin-positive striatal neurones (Gratacos *et al*. 2001). This effect was increased when BMP-2 was added in combination with low doses of the neurotrophins BDNF and NT-3. However, the combination of BMP-2 with these neurotrophins or with other neurotrophic factors (GDNF, NTN or TGF-β2) did not promote any synergistic effect on the overall GABAergic striatal population. In contrast, in cultured sympathetic neurones BMP-2 and BMP-7 require the co-addition of NT-3 or GDNF in order to induce a pro-survival effect, neither of them being effective when added on their own (Bengtsson *et al*. 1998; Thang *et al*. 2000).

BMP-2 increased striatal immunoreactivity for the activated form of Trk receptor, pTrk, confirming that neurotrophins are involved in BMP-2 effects. On the other hand, TrkB-IgG blocked BMP-2-induced increase in the degree of arborization and number of calbindin-positive neurones, pointing to TrkB ligands (i.e. BDNF or neurotrophin-4/5) as putative mediators of BMP-2 neurotrophic action. Actually, both BDNF and neurotrophin-4/5 have been previously reported to induce the differentiation of this subpopulation of GABAergic striatal neurones (Widmer and Hefti 1994; Ventimiglia *et al*. 1995). However, the addition of antibodies against BDNF mimicked the results obtained with the addition of TrkB-IgG, ruling out the putative involvement of neurotrophin-4/5 in the effects assessed on calbindin-positive neurones and thus suggesting that it is BDNF which mediates these effects.

The quantification of BDNF protein by ELISA analysis showed an increase in the levels of this neurotrophic factor after BMP-2 treatment. BDNF is the most potent factor inducing the differentiation of cultured GABAergic striatal cells, affecting parameters such as GABA uptake, morphological differentiation and calbindin expression (Mizuno *et al*. 1994). Interestingly, BDNF has been described as a powerful trophic agent for the overall GABAergic population, while the effect of BMP-2 on these neurones is weak (Gratacos *et al*. 2001). However, it is noteworthy that the levels of BDNF required in order to affect calbindin-positive population are two orders of magnitude lower than those required to affect the overall GABAergic population (Ventimiglia *et al*. 1995). This differential sensitivity to BDNF by calbindin subpopulation and by the overall GABAergic population would account for the slight effects

induced by BMP-2 on GABAergic neurones, since the amount of BDNF that we detected in the media of BMP-2-treated cultures was 0.04 ng/mL. In support of the present results, BDNF has been linked to the effects of other members of the TGF-β superfamily on the survival and/or differentiation of several neuronal populations. The treatment of cultured raphe cells with either BMP-6 or BMP-7 induced an increase in BDNF mRNA levels and the addition of TrkB-IgG, but not TrkC-IgG, completely abolished BMP-6 effects on the regulation of the serotoninergic marker tryptophan hydroxilase (Galter *et al*. 1999). Moreover, BDNF has been proposed as a mediator of the pro-survival effects of TGF-β1 on dorsal retinal ganglion (Chalazonitis *et al*. 1992) and on corticospinal neurones (Giehl *et al*. 1998).

BMPs are potent promoters of glial proliferation. On cultured striatal cells, BMP-2 induces a dose-dependent increase of GFAP-positive cells (Gratacos *et al*. 2001) and it also promotes astrogliosis on mesencephalic (Jordan *et al*. 1997; Reiriz *et al*. 1999) and cortical (Mabie *et al*. 1997) cultures. Indeed, astrocytes have been previously shown to express BDNF (Condorelli *et al*. 1994; Dougherty *et al*. 2000). However, in our *in vitro* conditions, where striatal neurones are plated in abscence of serum, there are virtually no GFAP immunoreactive cells ($< 5\%$, Gratacos *et al*. 2001). We detected an increase in BDNF levels 4 h after BMP-2 treatment. At this time point we could not detect any change in the number of astrocytes in the culture (data not shown). Moreover, BDNF immunocytochemistry revealed that the changes in the levels of this neurotrophin were located in neuronal cells. In accordance with these results, we had previously described that the treatment with the antimitotic fluorodeoxyuridine, which completely abolishes astroglial proliferation, did not block BMP-2 neurotrophic effects on striatal neurones (Gratacos *et al*. 2001).

Given that various neurotrophic factors coexist in physiological conditions, increasing importance has been ascribed to the interaction among members of different neurotrophic families. As such, the neurotrophic hypothesis has been extended by incorporating the possibility that the activity of established neurotrophic factors might result from endogenous factors (Krieglstein *et al*. 1998). Indeed, the involvement of BDNF as a mediator is not restricted to BMPs. For instance, BDNF signalling has been linked to the cascades started by other neurotrophic signals such as depolarization by potassium chloride (Meyer-Franke *et al*. 1998), calcium influx (Tao *et al*. 1998), cAMP elevation (Meyer-Franke *et al*. 1998; Galter and Unsicker 2000a,b), testosterone (Rasika *et al*. 1999) or subtoxic concentrations of NMDA (Marini *et al*. 1998). Moreover, endogenous BDNF participates in the protective effects of interleukin-6 on sensory neurones (Murphy *et al*. 2000) and of GDNF and NT-3 on axotomized rat corticospinal neurones (Schutte *et al*. 2000). Taken together, these data point to BDNF as being a crucial neurotrophin that, in addition to its own pro-survival and/or differentiation effects on several neuronal populations (reviewed in Barde 1994) and its effect on neuronal activity (reviewed in Thoenen 2000), could be involved in neurotrophic activities traditionally attributed to other factors.

Remarkable progress has been made over the last few years in terms of identifying neurotrophic molecules capable of enhancing the survival and/or differentiation of specific neuronal populations in order to use them as therapeutic tools against neurodegenerative diseases (reviewed in Connor and Dragunow 1998). In this context, the neurotrophin family, as well as members of the TGF-β superfamily, play an important role and the present results confirm the importance of considering the interactions between these families in order to reach an integrated understanding of their physiological effects.

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