Tercer trabajo

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Bone Morphogenetic Protein-6 Is a Neurotrophic Factor for Calbindin-Positive Striatal Neurons

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Bone morphogenetic proteins (BMPs) are a set of members of the transforming growth factor-β superfamily recently described as promoting the differentiation of several neuronal populations within the basal ganglia. This study examined whether a member of this family, BMP-6, could exert neurotrophic effects on the neurons of the striatum, in which BMP-6 mRNA had been previously detected during development. Here we show that BMP-6 increases the number and differentiation of calbindin-positive neurons in vitro. Indeed, BMP-6 increased the total area, the perimeter, and the degree of arborization of this neuronal population. This trophic factor promoted dendritic growth without modifying axonal length or soma area. Furthermore, BMP-6 increased the number of glial fibrillary acidic protein-positive cells while decreasing the number of nestin-positive cells. The suppression of cell proliferation or glial development by the antimitotic fluorodeoxyuridine removed the effects on striatal neurons, suggesting the involvement of astroglial cells in the differentiation induced by BMP-6. The current results confirm the relevance of BMPs in the development of the striatum and emphasize the crucial importance of the trophic interaction between glial and neuronal cells.

Key words: striatum; differentiation; in vitro; glial cells

Bone morphogenetic proteins (BMPs) are a group of proteins belonging to the transforming growth factor-β (TGF-β) superfamily. BMPs were first identified by the ability to induce bone formation from mesenchymal cells at extraskeletal ectopic sites (Wozney et al., 1988). However, in recent years, more functions have been attributed to this family, including regulation of the early development of the nervous system and control of the differentiation of postmitotic neurons (for review see Mehler et al., 1997; Ebendal et al., 1998).

Sequence homology divides the BMP family into several subgroups: the dpp subgroup (BMP-2 and BMP-4), the 60A subgroup (BMP-6 and BMP-7), and other unrelated proteins, such as BMP-9 and BMP-3. There are overlapping and differing biological actions of members of these subgroups (Mehler et al., 1997). BMPs have been described as exerting neurotrophic effects within selective neuronal populations of the central nervous system. Members of the dpp subgroup, BMP-2 and BMP-4, promote the survival or differentiation of mesencephalic neurons (Jordan et al., 1997; Reiriz et al., 1999; Stull et al., 2001) and regulate cortical cell number and phenotype (Mabie et al., 1999). However, BMP-9 is a potent inducer of the cholinergic phenotype in septum and spinal cord (Lopez-Coviella et al., 2000). With regard to the 60A subgroup, BMP-7 is a neurotrophic molecule for cortical (Le Roux et al., 1999), hippocampal (Withers et al., 2000), septal (Nonner et al., 2001), and raphe (Galter et al., 1999) neurons, whereas BMP-6 promotes the survival of mesencephalic neurons (Jordan et al., 1997), the activity of septal cholinergic neurons (Nonner et al., 2001), and the serotonergic phenotype of raphe neurons (Galter et al., 1999). BMP-6 is an atypical member of the BMP family, in that, unlike other BMPs, it does not induce bone formation (Celeste et al., 1990) and is present not only in the fetal mouse brain but also in adult stages (Wall et al., 1993). During early development, BMP-6 is expressed in radial glia cells (Schluesener and Meyermann, 1994). However, at postnatal stages, BMP-6 expression is specific to neurons (Tomizawa et al., 1995). In addition to its function during brain development, BMP-6 regulates neuronal resistance to insults; it can protect septal and hippocampal neurons against ischemia (Martinez et al., 2001; Nonner et al., 2001; Wang et al., 2001).

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BMPs exert their trophic effects through the heteromeric complexes of transmembrane receptors with serine-threonine kinase activity. The finding that different receptor subtypes of BMPs are located in the striatum (Soderstrom et al., 1996; Zhang et al., 1998; Charytoniuk et al., 2000) indicates that BMPs may have an important function in the survival and maturation of striatal cells. Indeed, BMP-2, but not BMP-7, exerts a powerful neurotrophic effect on γ-aminobutyric acid (GABA)-ergic striatal neurons (Hattori et al., 1999; Gratacòs et al., 2001a), although these two trophic factors increase the number of astrocytes in striatal cultures (Gratacòs et al., 2001a). Furthermore, BMP-2 selectively induces the calbindin phenotype in this neuronal population (Gratacòs et al., 2001a). Therefore, in the present study, we investigated whether another member of the BMP family, BMP-6, might also be neurotrophic for the striatal population that expresses calbindin as a marker for the differentiation of striatal projection neurons and the contribution of other trophic factors and astroglial cells to this effect.

MATERIALS AND METHODS

Cell Culture

Animal handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat (Autonomous Government) of Catalonia (1094/99), in accordance with Directive 86/609/EU of the European Commission. Certified time-pregnant Sprague-Dawley dams (Iberfauna Spain) were deeply anesthetized on gestational day 19, and fetuses were rapidly removed from the uterus, as previously described (Gratacòs et al., 2001a). 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 precoated with 0.1 mg/ml poly-D-lysine (Sigma, St. Louis, MO) at a density of 50,000 cells/cm^2. Eagle's minimum essential medium (Gibco BRL, Renfrewshire, Scotland) supplemented with B-27 (Gibco BRL) was used to grow the cells under serum-free conditions.

Various doses of human recombinant BMP-6 (CBM Creative Biomolecules, Hopkinton, MA) were added to the cultures, following either a "chronic" (added at the time of plating and left for 8 days in vitro; DIV) or an "acute" (for 24 hr, from DIV 7 to DIV 8) treatment. Some cultures were treated with 20 μM 5-fluorodeoxyuridine (FdU; Sigma) combined with 20 μM uridine (Sigma) from DIV 2. In another set of experiments, the cultures were treated at the time of plating with 250 ng/ml of TrkB IgG, generously provided by Genentech Inc. (San Francisco, CA). Plated cell cultures were maintained in an incubator with 5% CO_2 at 37°C.

Immunocytochemistry

Striatal cultures were fixed with 4% paraformaldehyde for 1 hr 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 (Gibco) at room temperature. Cultures were then incubated (overnight at 4°C) with antibodies directed against calbindin$_{D28k}$ (1:10,000; SWant, Bellinzona, Switzerland), nestin (1:40; S. Hockfield, Developmental Studies Hybridoma Bank, Iowa City, IA), or glial fibrillary acidic protein (GFAP; 1:400; Sigma) diluted in PBS containing 0.3% Triton X-100 and 1% normal horse serum. Cells were then incubated in biotinylated secondary antibodies followed by the Vectastain Elite ABC Kit (Vector, Burlingame, CA) and then reacted with diaminobenzidine.

Bromodeoxyuridine Labeling

To determine whether cells were dividing in the presence of BMP-6, bromodeoxyuridine (BrdU; Boehringer Mannheim, Mannheim, Germany; 3 μg/ml) was added at 5 DIV for 24 hr, and then the cells were fixed. Double immunocytochemistry for BrdU (1:1; Amersham Life Science, Arlington Heights, IL) and calbindin or GFAP was performed using the Vector Peroxidase Substrate Kit AEC (red, SK-4200) and Vector-SG (blue-gray, SK-4700).

Detection of Cell Death

TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed at 4 DIV to determine the number of dying neurons, by using the Promega (Madison, WI) kit. Neurons were then incubated with calbindin$_{D28k}$ antibody overnight and visualized with a rhodamine-conjugated secondary antibody (1:200; Jackson Immunoresearch, West Grove, PA), and, finally, mounted with mowiol (Calbiochem, San Diego, CA).

Quantitative Analysis of Cell Cultures

Counts of cell number were performed within an area of 0.19 cm^2 corresponding to 20% of the total culture area as described previously (Gratacòs et al., 2001a,b). Numbers are given as percentages of control cultures from three different experiments, with four to six wells analyzed in each. Morphological parameters were assessed using a PC-Image analysis system from Foster Findlay on a computer attached to an Olympus microscope. Calbindin-positive neurons (60 per condition) were randomly chosen and traced in a phase-contrast image using the mouse. Total and soma area, perimeter, and degree of arborization (perimeter^2/4πarea) were determined as described by Fujita et al. (1996). Axon length was also measured, with the axon defined as the longest emerging neurite from the soma, as previously described (Gratacòs et al., 2001a). In the statistical analysis for comparison of multiple means, ANOVA and then the Neuman-Keuls test were performed.

RESULTS

BMP-6 Specifically Increases the Number of Calbindin-Positive Striatal Neurons

To assess the putative neurotrophic effect of BMP-6 on cultured striatal neurons, a range of concentrations of this factor was added at the time of plating, and the number of calbindin-positive neurons was analyzed at 8 DIV. BMP-6 induced a dose-dependent increase in the number of calbindin-positive cells. The maximal effect of BMP-6 (142% ± 6% of control values) was reached at 10 ng/ml, and this effect was not surpassed with higher doses of BMP-6 (at 50 ng/ml: 145% ± 9% of control; Fig.

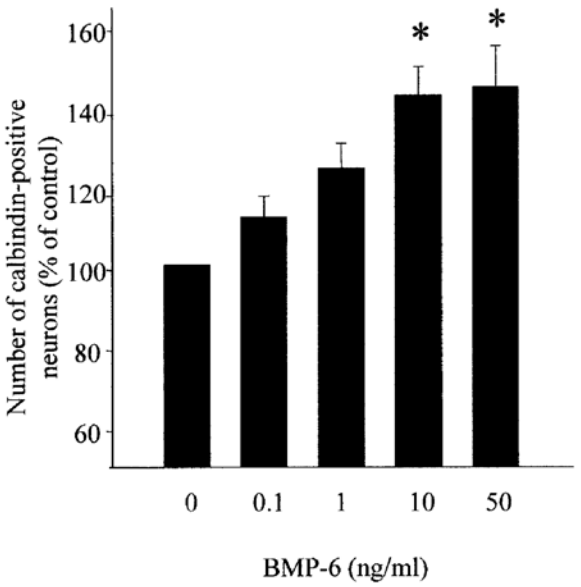

Fig. 1. BMP-6 increased the number of calbindin-positive neurons. BMP-6 was added to the culture at the time of plating at different concentrations. Calbindin-positive neurons were assessed immunocytochemically after 8 DIV. Results are expressed as a percentage of control values. Treatment with BMP-6 caused a 42% increase in the number of calbindin-positive neurons when added at 10 and 50 ng/ml. Results are the mean ± SEM of six determinations from three different experiments. $*P < 0.05$ compared with control values (Neuman-Keuls test).

1). We also tested the effects of BMP-6 administered through an "acute treatment" from DIV 7 to DIV 8 on the number of calbindin-immunoreactive neurons. However, this treatment did not increase significantly the number of calbindin-positive cells (BMP-6 10 ng/ml: 105% ± 6% of control).

To clarify whether this increase in the number of calbindin-positive cells was related to changes in survival, proliferation, or differentiation, other parameters were also evaluated. Our results show that this effect was specific for the calbindin neuronal population; BMP-6 treatment did not modify the total number of neurons at 10 ng/ml (control: 21,656 ± 2,815 cell/cm^2; BMP-6: 22,522 ± 1,024 cell/cm^2). According to this result, the number of TUNEL-positive cells was not modified after a 4 DIV treatment (control: 18,397 ± 897 cells/cm^2; BMP-6: 17,756 ± 769 cells/cm^2). Furthermore, a colocalization of calbindin and TUNEL or calbindin and BrdU was not observed (data not shown). We also analyzed whether BMP-6 induced changes in the proliferation of neuronal precursors. Nestin was used as a marker for undifferentiated neuroepithelial cells. Our results show that BMP-6 induced a decrease (58%) in nestin-positive cells after a 7 DIV treatment (Fig. 2). All these results point out that BMP-6 could be involved in the differentiation of striatal calbindin-positive cells.

BMP-6 Promotes Dendritic Growth of the Calbindin-Positive Population

The morphological evaluation of calbindin-positive neurons disclosed that 10 ng/ml of BMP-6 significantly induced their differentiation (cf. Fig. 3A–D). The quantification of these effects revealed that BMP-6 increased the total area (by 23%), perimeter (by 34%) and degree of arborization (by 43%) of this population of striatal neurons (Table I). However, the area of the soma was not affected by this factor.

In another set of experiments, we measured the axon length to discern whether BMP-6 could be acting on the overall neuritic branching or had a specific effect on the dendritic compartment. This parameter was not increased by BMP-6 at any of the doses tested (Table I), indicating that the effects assessed on the degree of arborization were restricted to increased dendritic branching.

Glial Cells Are Involved in BMP-6 Neurotrophic Effects

BMPs are well-known promoters of glial activation. Therefore, to study BMP-6 effects on astroglial cells, several doses of BMP-6 were added at the time of plating, and GFAP immunocytochemistry was performed at 8 DIV (Fig. 4). We rarely observed any astrocytes in control wells under our in vitro conditions. However, the addition of BMP-6 (10 ng/ml or higher) significantly increased the number of GFAP-positive cells (451% ± 114% of control values). This effect correlated with an increase in BrdU-positive cells (data not shown). BMP-6 not only increased the number of astrocytes but also significantly affected the morphology of these cells by increasing their branching (Fig. 4).

To test whether glial cells could be involved in BMP-6 neurotrophic effects on calbindin-positive neuronal population, the number and degree of arborization of these neurons in the presence of FdU were assessed. This factor completely suppressed the enhancement of GFAP-positive cells induced by BMP-6. Under this condition, BMP-6 did not cause any increase either in the number of calbindin-immunoreactive neurons (Fig. 5) or in their degree of differentiation (Fig. 3E, Table I). Moreover, we also tested the ability of BMP-6 to increase the number of GFAP-positive cells following an "acute treatment." The incubation of the cultures with 10 ng/ml of BMP-6 from DIV 7 to DIV 8 did not induce any significant increase in the number of astrocytes of treated wells.

Brain-Derived Neurotrophic Factor Does Not Mediate BMP-6 Effects

Because other members of the BMP family had previously been shown to exert their trophic effects through the endogenous release of brain-derived neurotrophic factor (BDNF; Galter et al., 1999; Gratacòs et al., 2001b), we tested whether the addition of TrkB-IgG, fusion proteins between human IgG and the BDNF receptor TrkB, could reverse BMP-6 neurotrophic effects. There were, however, no changes in BMP-6 neurotrophic effects in the presence of this TrkB blocker (Figs. 3F, 5, Table I).

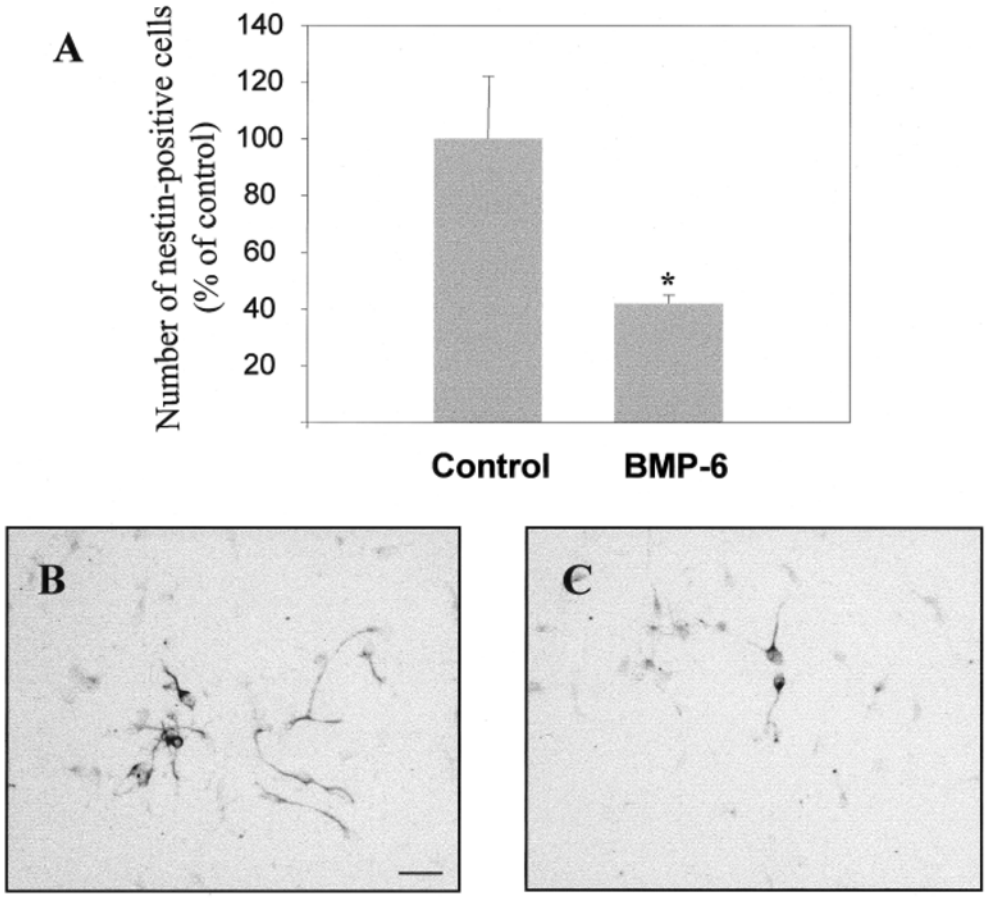

Fig. 2. Effect of BMP-6 on the number of nestin-positive cells. The addition of BMP-6 (10 ng/ml) at the time of plating caused a decrease in the number of nestin-positive cells after 8 DIV (**A**). Photomicrographs show the differences between control (**B**) and BMP-6 (**C**) in the number of nestin-positive cells. Results are the mean ± SEM of four determinations of three experiments. $*P < 0.05$ compared with control values (Neuman-Keuls test). Scale bar = 60 μm.

DISCUSSION

These results show that BMP-6 is a novel neurotrophic factor for the differentiation of striatal neurons, in that it induces the calbindin phenotype and promotes dendritic growth. Moreover, we demonstrate that BMP-6 effects are mediated by astroglial cells; the blockage of BMP-6-induced GFAP-positive cells removes the BMP-6 neurotrophic actions.

Recent years have seen great advances in the study of the trophic factors that regulate the development and maturation of the striatum. The final trophic support for each independent neuronal population is mediated by a complex interaction between different trophic factors. Indeed, these studies were at first restricted to neurotrophin and glial cell line-derived neurotrophic factor (GDNF) families. Among their members, BDNF, neurotrophin-3, and neurotrophin-4 promote the calbindin phenotype and differentiation of the overall GABAergic striatal population (Mizuno et al., 1994; Widmer and Hefti, 1994; Ventimiglia et al., 1995; Ivkovic et al., 1997; Ivkovic and Ehrlich, 1999), and GDNF induces the expression of calretinin (Farkas et al., 1997) and substance P (Humpel et al., 1996) in cultured striatal cells. Recently, BMPs have been shown to be crucial in regulating the differentiation and survival of several neuronal populations in the striatum (Hattori et al., 1999; Gratacòs et al., 2001a). BMP-2 exerts a trophic effect on striatal cells, inducing calbindin phenotype and dendritic growth (Gratacòs et al., 2001a,b). The present results indicate that, like BMP-2, BMP-6 also increases the number of calbindin-positive cells, and this effect is due to an induction of the calbindin phenotype, in that BMP-6 does not modify the number of TUNEL-positive cells. Furthemore, BMP-6 decreases the number of nestin-positive cells without modifying the total num-

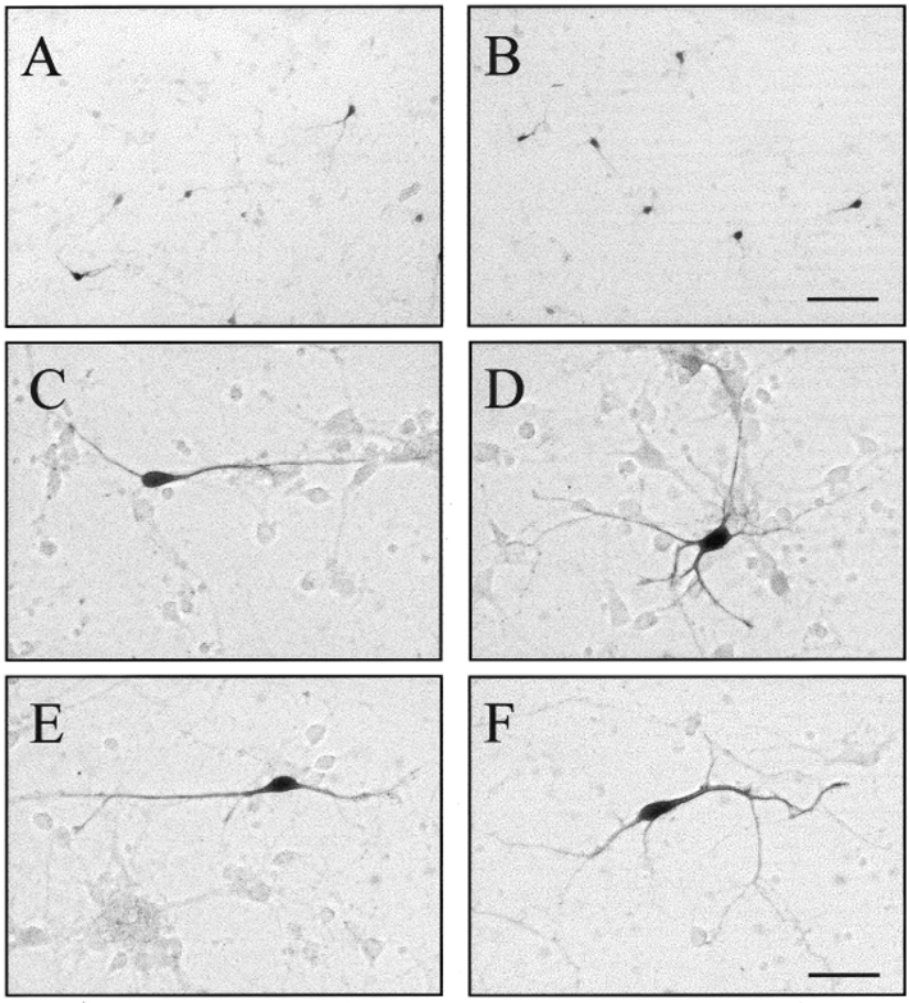

Fig. 3. BMP-6 caused the morphological differentiation of calbindin-positive striatal neurons. Photomicrographs show the differences in the number and pattern of arborization in cultures treated with vehicle (**A**) or 10 ng/ml BMP-6 (**B**). Detail of the neurons treated with PBS (**C**), 10 ng/ml BMP-6 (**D**), 10 ng/ml BMP-6 plus 20 μM FdU (**E**), and 10 ng/ml BMP-6 plus 250 ng/ml TrkB-IgG (**F**). All treatments were performed at the time of plating, and cultures were processed for calbindin immunoreactivity at 8 DIV. Scale bars = 180 μm in B (for A,B); 50 μm in F (for C–F).

TABLE I. Quantitative Analysis of the Differentiation Caused by BMP-6 in Calbindin-Positive Striatal Neurons†

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization (perimeter^2/4π area)
Control	260 ± 10	419 ± 23	106 ± 3	87 ± 4	54 ± 5
BMP-6 0.1	263 ± 7	427 ± 21	108 ± 3	98 ± 6	60 ± 7
BMP-6 1	278 ± 12	448 ± 25	105 ± 4	93 ± 5	58 ± 6
BMP-6 10	320 ± 13★	561 ± 28★	106 ± 4	99 ± 6	77 ± 7★
BMP-6 50	319 ± 8★	586 ± 32★	112 ± 6	93 ± 4	84 ± 5★
BMP-6 10 FdU	267 ± 9	421 ± 22	106 ± 3	94 ± 3	59 ± 3
BMP-6 10 TrkB	340 ± 20★	624 ± 42★	109 ± 3	97 ± 5	81 ± 5★

†Striatal neurons were treated with increasing doses (ng/ml) of BMP-6 at the time of plating, and calbindin immunoreactivity was assessed at 8 DIV. For each parameter examined, 60 neurons for each condition were analyzed. Results are expressed as the mean ± SEM of the values from a representative experiment. ★$P < 0.05$ compared with control values (Neuman-Keuls test).

ber of neurons. These results are in agreement with previous studies suggesting that the increase in cortical neurons after BMP treatment reflects enhanced neuronal differentiation of neuronal precursors rather than proliferation of cells already expressing the neuronal phenotype (Mabie et al., 1999). Therefore, together with the induction of dendritic growth, the BMP-6 effect is clearly related to the maturation of specific striatal neuronal pop-

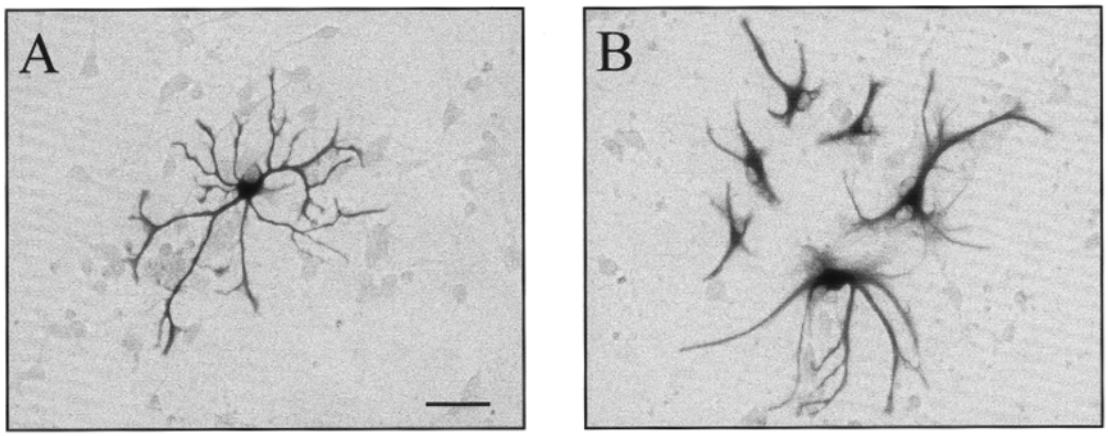

Fig. 4. BMP-6 causes astroglial activation. The addition of 10 ng/ml BMP-6 from the time of plating until 8 DIV increased the differentiation (**A**) and the number (**B**) of GFAP-immunoreactive cells. Scale bar = 60 μm.

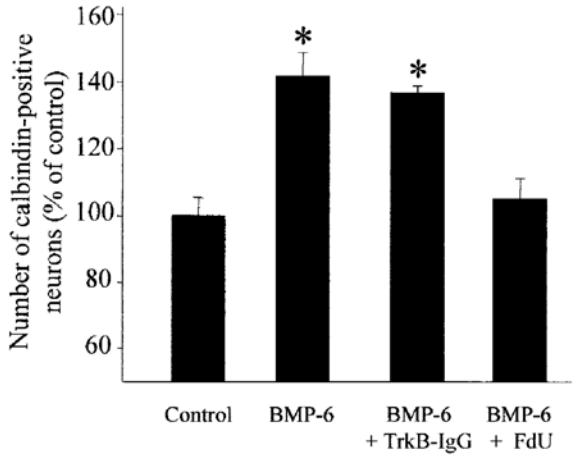

Fig. 5. FdU, but not TrkB-IgG, removes BMP-6 effects on the number of calbindin-positive neurons. BMP-6 (10 ng/ml) and TrkB IgG (250 ng/ml) were added at the time of plating and FdU (20 μM, combined with uridine 20 μM) at 2 DIV. Calbindin-positive neurons were assessed immunocytochemically at 8 DIV. Results are expressed as a percentage of control values. $*P < 0.05$ compared with control values (Neuman-Keuls test).

ulations. However, there are several striking differences in how BMP-2 and BMP-6 exert their effects on calbindin-positive striatal neurons. First, even though TrkB-IgG removes BMP-2 neurotrophic actions in our in vitro model (Gratacòs et al., 2001b), disclosing the BDNF involvement in its effects, it does not affect BMP-6-promoted differentiation (present results). In contrast, the antimitotic FdU is able to reverse the BMP-6 trophic effects on cultured calbindin-positive neurons (present results) but not the BMP-2 effects (Gratacòs et al., 2001a). Therefore, both BMPs can cause differentiation of striatal neurons, but their mechanisms of action are different, in that the BMP-2 effect is mediated by BDNF and is glia independent, whereas BMP-6 requires the induction of astroglial cells to produce the trophic effects.

BMPs are powerful promoters of astrocytic differentiation (Gros et al., 1996; Mabie et al., 1997), and our results show that BMP-6 as well as BMP-2 or BMP-7 significantly increased the number of GFAP-positive cells in striatal cultures. Under our in vitro conditions, BMP-2 and BMP-7 show a similar potency (tenfold increase compared with control; Gratacòs et al., 2001a), whereas BMP-6 promotes substantially less effect in the number of astrocytes (fourfold increase of control). Given that the addition of BMP-7 is able to increase the number of GFAP-positive cells without inducing striatal neuron differentiation, we can conclude that astroglial activation per se is not sufficient for a trophic response in calbindin-positive neurons to be observed. Similarly, BMP-9, as well as other BMPs, caused a significant increase in the number of GFAP-positive cells in mesencephalic cell cultures, but only the former failed to exert trophic effects on dopaminergic neurons (Jordan et al., 1997). Although we had previously shown that an acute treatment with BMP-2 from DIV 7 to DIV 8 was sufficient to mimic the effects of a chronic treatment with this BMP on calbindin-positive neurons (Gratacòs et al., 2001a), here we show that an acute exposure to BMP-6, which was not sufficient to increase the number of GFAP-positive cells, did not increase the number or the degree of differentiation of this striatal neuronal population, reinforcing the role of glial cells in the BMP-6 trophic effect. Along these lines, BMP-6 neurotrophic activities have also been related to glial mediation in mesencephalic (Jordan et al., 1997) and serotoninergic (Galter et al., 1999) cultured neurons. In conclusion, the present data provide evidence for the neurotrophic effects of BMP-6 on calbindin-positive striatal neurons in vitro and reinforce the role of this family of

proteins in the development of the striatum, in which they act through diverse signaling pathways.

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