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Basic fibroblast growth factor protects cerebellar neurons in primary culture from NMDA and non-NMDA receptor mediated neurotoxicity

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We have investigated the ability of bFGF to protect cerebellar neurons from neurotoxicity by excitatory amino acids. We have found that preincubation with 1–2.5 nM bFGF for 1–6 days significantly protected neurons from excitotoxic damage via NMDA receptors as well as ionotropic non-NMDA receptors. bFGF neuroprotection appeared not to be dependent upon neuronal differentiation and was not mimicked by other neurotrophins including BDNF, NT-3 and NGF. A greater rise in extracellular calcium-dependent cGMP formation, following either depolarization or excitatory amino acid receptor activation was observed in bFGF-pretreated neurons. We suggest that neuroprotection from excitotoxicity following bFGF treatment may be associated to the modulation of neurochemical pathways dependent upon extracellular calcium influx.

Cerebellar granular cell; Cyclic GMP; Excitatory amino acid receptor; Basic fibroblast growth factor; Neurotoxicity

1. INTRODUCTION

Fibroblast growth factors (FGFs) are polypeptide mitogens that induce proliferation in a wide variety of cell types [1]. Of the seven family members, the best characterized are basic and acidic FGF. In addition to their mitogenic effects, FGFs may also promote neuronal survival and outgrowth in the CNS. High levels of basic FGF (bFGF) can be found in the developing rat brain [2,3], and both basic and acidic FGF have been found to be potent neurotrophic factors for cultured neurons from multiple CNS regions [4].

bFGF has been shown to support the survival in primary culture of spinal cord neurons [5,6], mesencephalic dopaminergic and GABAergic neurons [7], cerebellar granule cells [8], hypothalamic [9], hippocampal [10,11], cortical [12,13] and neocortical neurons [14].

Beyond its role in normal brain development, bFGF may play an important role in the brain response to injury and neurodegenerative events. bFGF mRNA has been shown to increase in lesioned rat brain and it may prevent the degeneration of cholinergic neurons in animal models of neurodegenerative disorders [15,16]. On the other hand, bFGF has been demonstrated to protect cultured rat hippocampal neurons against excitotoxicity by glutamic acid [11], suggesting a protective role of this growth factor in regions particularly vulnerable to both acute (e.g. stroke) and chronic (e.g. Alzheimer's disease) neurodegenerative insults.

Glutamic acid may activate three main receptor categories: ionotropic NMDA (activated also by *N*-methyl-D-aspartic acid), ionotropic non-NMDA and metabotropic receptors [17]. Recently, bFGF has been suggested to protect striatal neurons from glutamic acid toxicity by attenuating NMDA receptor function, but not from kainic acid toxicity via the ionotropic non-NMDA receptor [18]. Here, we have evaluated the neuroprotective effects of bFGF on cerebellar granule cells in primary culture. We have found that bFGF strongly protects cultured cerebellar neurons from NMDA receptor-mediated glutamic acid toxicity as well as from neurotoxicity by domoic acid, a tricarboxylic amino acid structurally related to kainic acid, acting as a potent agonist at the ionotropic non-NMDA receptor [19].

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-methyl-D-aspartate; ANP, atrial natriuretic peptide; APV, D-(−)-2-amino-5-phosphovaleric acid; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; cGMP, cyclic GMP; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIC, days in culture; Dom, domoic acid; Glu, glutamic acid; NGF, nerve growth factor; NT-3, neurotrophin-3; NMDA, *N*-methyl-D-aspartic acid; MK-801, (+)-10,11-dihydro-5-methyl-5*H*-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate; SNP, sodium nitroprusside; VET, veratrine; VSCC, voltage-sensitive calcium channels.

2. EXPERIMENTAL

2.1. *Cell cultures*

Primary culture of rat cerebellar neurons were prepared as previously described [19]. Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated and suspended in basal Eagle's medium with 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly-L-lysine coated (5 μg/ml) 35 mm dishes at 2.5×10^5 cells/cm^2 and incubated at 37°C in a 5% CO_2, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added

after 20–24 h of culture to inhibit the replication of non-neuronal cells. After 8 days in vitro, morphologically identified granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation [19].

2.2. *Neurotoxicology*

Primary cultures of cerebellar granule cells were used for toxicological studies at the indicated days in culture. Drugs were added in the growth medium. After exposure to the drugs for the indicated time, the growth medium was removed and cultures were incubated for 5 min with 1 ml of incubation buffer containing (in mM): 154 NaCl, 5.6 glucose, 5.6 KCl, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$, pH 7.4, to which the vital stain fluorescein diacetate (5 mg/ml) was added [19]. The staining mixture was then aspirated, replaced with incubation buffer, and cultures were examined for neurotoxicity. Under 480 nm light, live neurons showed a bright green colour in the cell body and the neurites, while dead neurons did not retain any fluorescein and their bare nuclei could be stained in red by 1 min exposure to 50 mg/ml ethidium bromide. Photographs of randomly selected culture fields were taken, live and dead neurons were counted, and neuronal survival percentage was calculated. Total number of neurons per dish could be calculated considering the ratio between the area of the dish and the area of the picture (~ 2000). In control cultures, live neurons = 1,364,000 ± 240,000 (mean ± S.D., n = 50).

2.3. *Biochemistry*

Intracellular cGMP concentration was determined as previously reported [20]. Briefly, cultures were washed twice with 1 ml of prewarmed (37°C) incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$, pH 7.4 ($MgCl_2$ was omitted when indicated). Dishes (control or pretreated with bFGF as indicated) were incubated at 37°C for 10 min with 1 ml of fresh incubation buffer and for an additional 20 min with a second 1 ml of fresh incubation buffer. Drugs were added at the end of the 20 min incubation period for 1 min (unless otherwise indicated). Antagonists were added 1 min before agonists. Incubation was stopped by aspiration of the solution and addition of 1 ml $HClO_4$ (0.4 N). After neutralizing the perchlorate extract, cGMP content was determined by radioimmunoassay. Protein content was determined on the membrane pellet from the same sample.

2.4. *Data presentation and analysis*

Results are presented as indicated in each figure. For statistical analysis the one-way (Figs. 1,3,4) or the two-way analysis of variance (ANOVA) (Fig. 5) was used to identify overall treatment effects, followed by the unpaired two-tailed Student's t-test for selective comparison of individual data groups. Data in Fig. 6 was analyzed by the unpaired two-tailed Student's t-test. Percentage data was transformed to arcsin data. Only significances relevant for the discussion of the data are indicated in each figure.

2.5. *Materials*

Basic FGF was from UBI; Nifedipine, verapamil and (+)-10,11-dihydro-5-methyl-5*H*-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801) were a generous gift from Dr. G.J. Kaczarowski (Merck Sharp and Dohme Laboratories, NJ, USA); CNQX was from Tocris Neuramin; Nerve Growth Factor and Atrial Natriuretic Peptide were from Peninsula Laboratories Inc.; Domoic acid was from Diagnostic Chemicals Ltd., West Royalty Industrial Park, Charlottetown, Canada. All other drugs were from Sigma.

3. RESULTS

Excitatory amino acids (EAAs) are potent neurotoxins for cultured cerebellar neurons [19]. In order to activate all EAA receptors we used glutamic acid (Glu), which was directly added to the culture growth medium.

One-time addition of 1 nM bFGF to cerebellar neu-

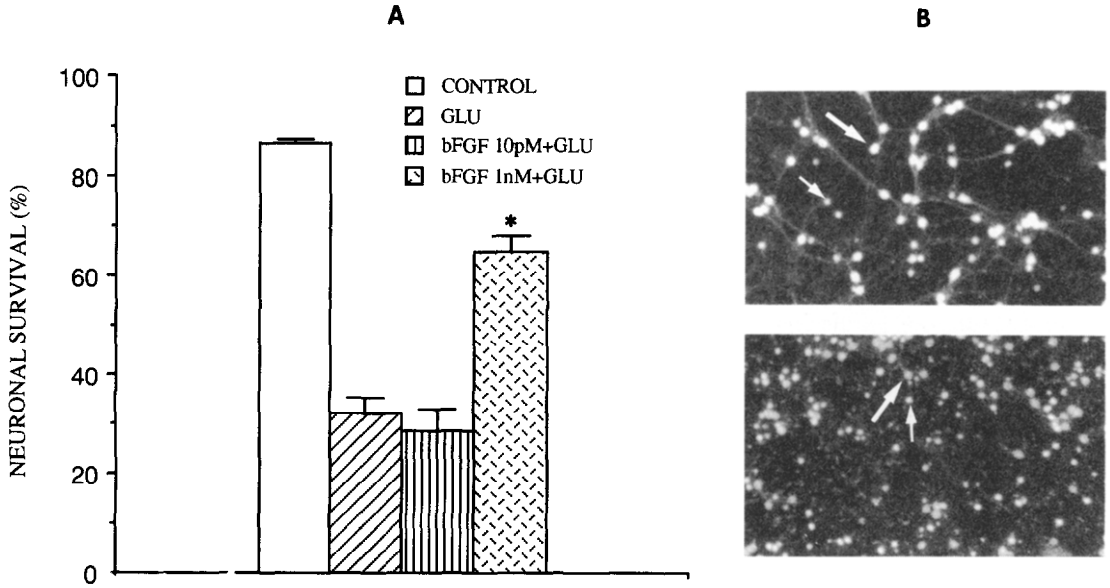

Fig. 1. Neuroprotective effect of bFGF against glutamic acid toxicity in cultured cerebellar neurons. (A) Neuronal survival after 24 h exposure to 20 μM glutamic acid (GLU) was determined in untreated neurons and in neurons pretreated for 6 DIC (from 5 to 11DIC) with 10 pM or 1 nM bFGF. Represented values are the mean ± S.D. of data from 5 independent experiments (n = 5). *P < 0.001 vs. Glu. (B) Cultured neurons were simultaneously exposed to fluorescein diacetate (5 min) and to ethidium bromide (1 min). Under 480 nm light, live neurons showed a bright green color in the soma and in the neurites (big arrow), and bare nuclei of dead neurons were stained in red (small arrow). Upper panel: control neurons exposed to 20 μM glutamic acid for 24 h. Lower panel: neurons pretreated with 1 nM bFGF for 6DIC prior to exposure to 20 μM glutamic acid for 24 h.

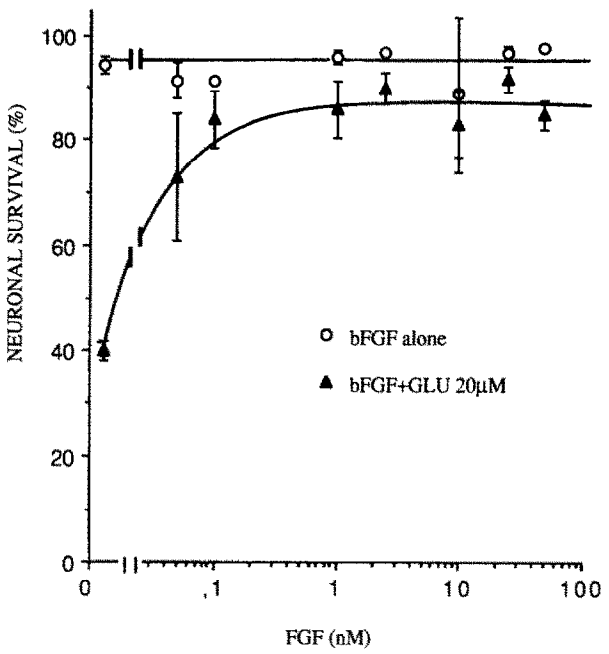

Fig. 2. Dose–response curve of neuroprotective activity of bFGF against excitatory amino acid toxicity. Neurons were preincubated with the indicated concentrations of bFGF for 7DIC (from 4 to 11 DIC). Neuronal survival was determined after 24 h exposure to 20 μM glutamic acid (GLU). Values represent the mean ± S.D. (n = 3).

rons at 5 days in culture (5 DIC), resulted in a 2.5-fold increase in the number of neurons surviving Glu-induced neurotoxicity at 11 DIC. Thus, while only 32 ± 3.2% of the neurons survived after 24 h exposure to 20 μM Glu, neuronal survival in the presence of 1 nM bFGF was 65 ± 3% (Fig. 1A). bFGF increased not only the number of neurons able to retain fluorescein (Fig. 1A,B), but also produced a remarkable increase in the number of neurites morphologically unaltered after the exposure to Glu (Fig. 1B). Lower concentrations of bFGF (10 pM) did not show any protective effect against Glu neurotoxicity (Fig. 1A). We have not observed any decrease in the biological activity of bFGF with time in culture previously reported by other authors [11].

bFGF protection against Glu toxicity was dependent upon the concentration of the growth factor. 50% protection was obtained at 20 pM bFGF and it reached a maximum at approximately 1 nM (Fig. 2). No further effect could be achieved by adding higher doses of the growth factor.

Since cerebellar neurons have been shown to undergo differentiation with time in culture [21,22], we next asked whether bFGF protection against Glu toxicity could be a differentiation-dependent phenomenon. When bFGF (2.5 nM) was added to more differentiated cultured cerebellar neurons (9 DIC), it was still effective in protecting neurons against Glu neurotoxicity. As shown in Fig. 3, among neurons preincubated with 2.5 nM bFGF for 6 days (from 9 to 15 DIC), 72 ± 3% survived to 24 h exposure to 20 μM Glu (added to the cultures at 15 DIC), while only 34 ± 3% of the neurons survived in the absence of bFGF. Similar results were obtained when bFGF was added to neurons at 11 DIC and at 17 DIC, respectively (data not shown).

Glu neurotoxicity was completely blocked by the NMDA receptor antagonists APV (1 mM) and MK-801 (1 μM) ([19] and data not shown). In order to determine whether the protective action of bFGF was specific for NMDA-type EAA receptors, we explored the possibility that bFGF could be also effective in protecting neurons against neurotoxicity by domoic acid (DOM), a potent agonist at the ionotropic non-NMDA receptor [19]. AMPA (100 μM), an agonist at the ionotropic non-NMDA receptor, quisqualic acid (100 μM), an agonist at both ionotropic non-NMDA and metabotropic receptors, and the selective agonist at the metabotropic receptor t-ACPD (1 mM), did not produce any neurotoxicity in these cultures (data not shown). Neurotoxicity by DOM could not be prevented by the NMDA-receptor antagonists APV (1 mM) and MK-801 (1 μM) ([19] and data not shown), but it was fully abolished in the presence of the ionotropic non-NMDA receptor antagonist CNQX (20 μM) (37 ± 4% vs. 82 ± 5% neuronal survival). As it can be seen in Fig. 4, one time addition of bFGF at 5 DIC (Fig. 4A) or at 9 DIC (Fig. 4B) protected neurons from neurotoxicity by 24 h exposure to DOM (10 μM), added to the cultures at 11 or 15 DIC, respectively. Thus, 71 ± 2% and 75 ± 5% of the neurons that received bFGF at 5 DIC or at 9 DIC respectively survived after the exposure to DOM, while only 37 ± 4% and 40 ± 2% of the neurons survived to DOM in the absence of the growth factor.

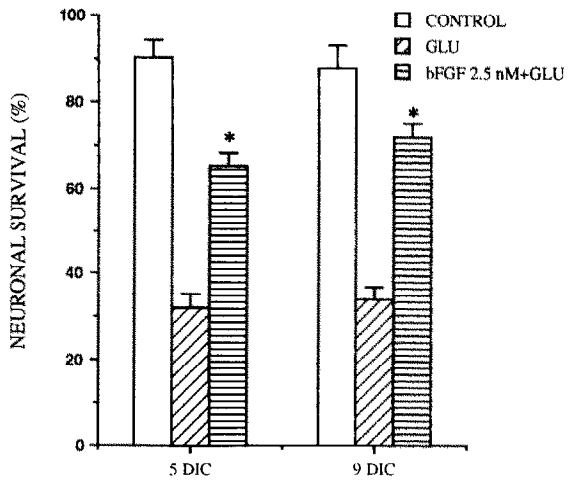

Fig. 3. bFGF protection from glutamic acid neurotoxicity is independent of neuronal age in culture. bFGF was added to cultured neurons at 5DIC or 9 DIC. Neuronal survival was determined after 24 h to 20 μM glutamic acid (GLU) exposure, added to the neurons at 11 DIC and 15 DIC respectively. Values represent the mean ± S.D. (n = 4). *P < 0.001 vs. GLU.

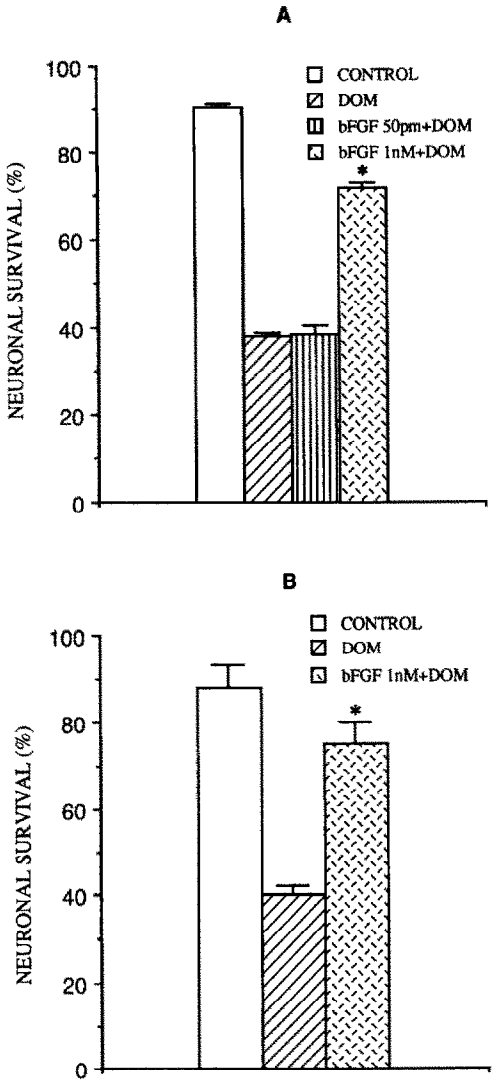

Fig. 4. Neuroprotective effect of bFGF against domoic acid toxicity in cultured cerebellar neurons. Neuronal survival after 24 h exposure to 10 μM domoic acid (DOM) was determined in untreated neurons and in neurons pretreated for 6 days with bFGF. bFGF was added at 5 DIC (A) or at 9 DIC (B). Values represent the mean ± S.D. (n = 3–5). *P < 0.001 vs. DOM.

We also tested the time dependence of exposure to bFGF on protection against Glu or DOM neurotoxicity. bFGF was added to the cultures 2, 5 or 24 h prior to exposure to either 20 μM Glu or 10 μm DOM. Cell survival 24 h following exposure to the toxins was partially but significantly enhanced in cultures pretreated with bFGF for 24 h but not in cultures pretreated with the growth factor for 2 or 5 h (data not shown). These findings indicate that the ability of bFGF to protect from EAA neurotoxicity required a relatively long-term exposure.

The protective effect of bFGF was specific in that preincubation of the neurons for 12 DIC (from 3 to 15 DIC) with BDNF (12 ng/ml) or NT3 (12 ng/ml) added to the cultures twice, at 3 DIC and at 10 DIC, not only did not result in any protection from either Glu or DOM toxicity (Fig. 5), but these neurotrophins significantly enhanced the toxic effect of the EAAs. In particular, BDNF increased Glu but not DOM toxicity, while NT-3 enhanced DOM toxicity to a higher extent than Glu toxicity. Pretreatment of the cultures with NGF (1–50 ng/ml) did not increase the number of neurons surviving to 20 μM Glu or 10 μM DOM (Fig. 5).

We then attempted to elucidate which mechanism could be possibly involved in bFGF neuroprotection. It has been suggested that bFGF may participate in the control of intracellular calcium concentration [11]. We therefore investigated whether bFGF protection against Glu and DOM neurotoxicity might be reflected in changes in extracellular calcium influx, by measuring the intracellular formation of cGMP stimulated by EAA, which depends upon extracellular calcium influx and nitric oxide synthase [20,23]. cGMP formation following exposure to 100 μM Glu in the absence of Mg^{2+} was significantly increased (165%) in cultures pretreated with bFGF (2.5 nM) for 6 DIC (Fig. 6A). Pretreatment with bFGF did not alter cGMP levels in unstimulated cultures, nor affected Mg^{2+} (1 mM) block of Glu-stimulated cGMP formation. MK-801 (1 μM) completely antagonized GLU stimulation of cGMP both in bFGF treated and untreated cultures. Selective NMDA receptor-mediated stimulation of cGMP formation by NMDA (100 μM) was unaffected by bFGF pretreatment (Fig. 6A), while non-NMDA stimulation of cGMP formation by DOM was significantly increased in cultures pretreated with bFGF (163%; Fig. 6B).

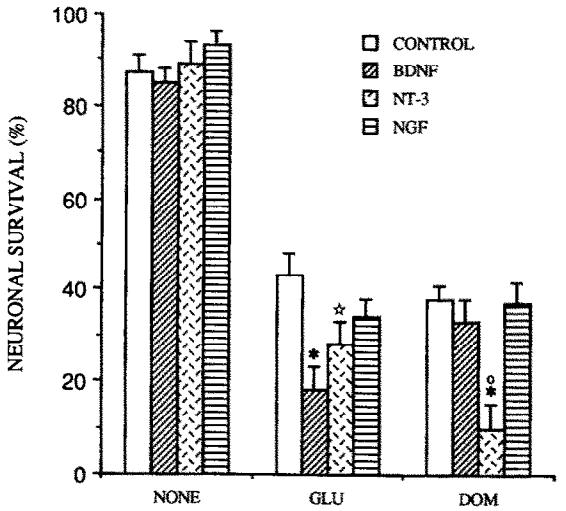

Fig. 5. Brain-derived neurotrophic factor, neurotrophin-3 and nerve growth factor do not protect cultured cerebellar neurons from excitatory amino acid toxicity. Neurotrophins were added to the cultures twice, at 3 and 10 DIC, and excitatory amino acids were added at 12 DIC. Neuronal survival was determined after 24 h exposure to glutamic acid (GLU) or domoic acid (DOM). Concentrations were, BDNF and NT-3, 12 ng/ml; NGF, 1-50 ng/ml; GLU, 20 μM; DOM, 10 μM. Values represent the mean ± S.D. (n = 3–4). *P < 0.005 and ☆P < 0.03 vs. CONTROL within the same treatment. °P < 0.02 vs. same trophin in GLU.

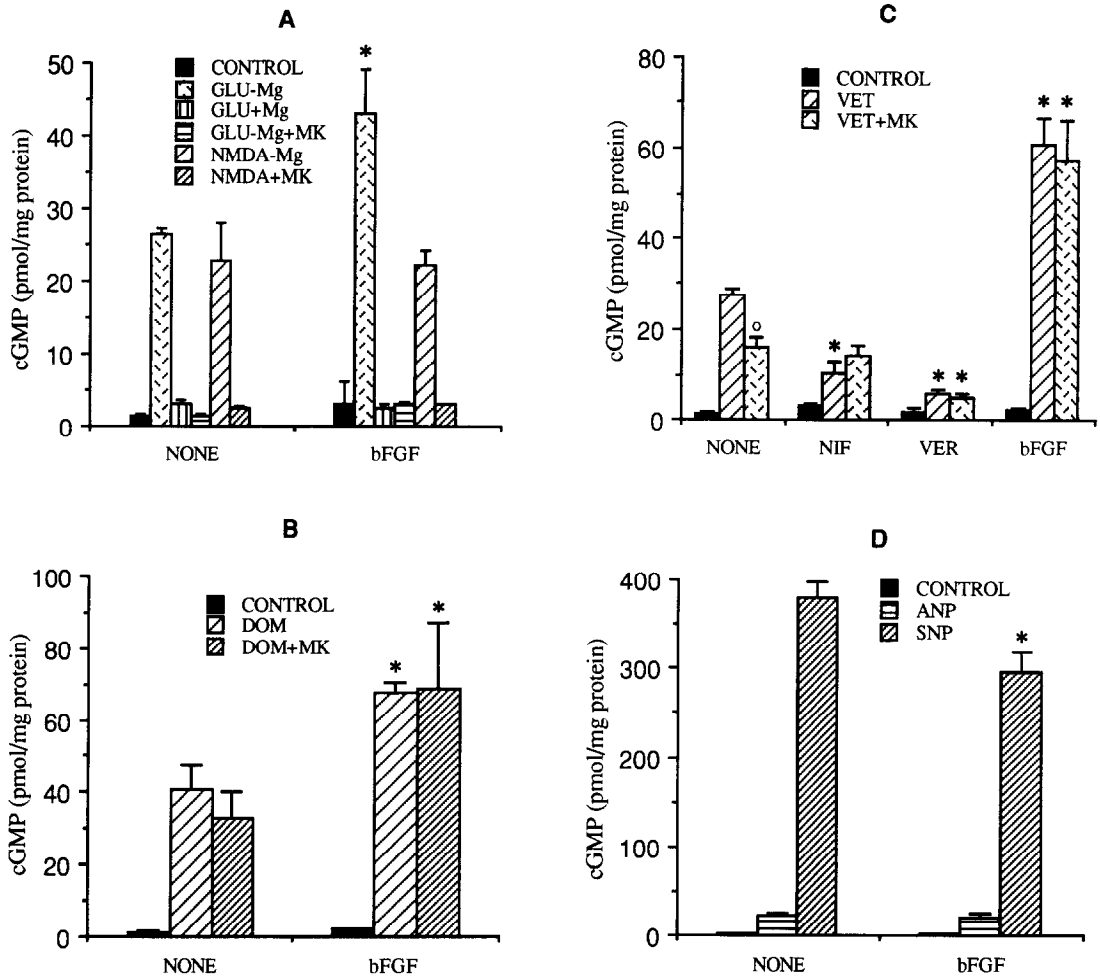

Fig. 6. bFGF increases excitatory amino acid and depolarization-induced stimulation of cGMP formation. Cerebellar cultures were either untreated (NONE) or exposed to bFGF (2.5 nM) for 6 DIC (from 5 to 11 DIC). At 11 DIC, neurons were exposed for 1 min to the indicated drugs (except in panel D). Antagonists were added 1 min before stimulation. cGMP levels in the absence (A) or in the presence (B–D) of 1 mM Mg^{2+} in unstimulated cultures were defined as CONTROL. Data are reported as the mean ± S.D. (n = 3). In panel A, stimulation of cGMP was performed both in the absence (−Mg) and in the presence (+Mg) of Mg^{2+}. Concentrations were, GLU and NMDA, 100 μM; MK-801 (MK), 1 μM. *P < 0.01 vs. GLU-Mg in the absence of bFGF. (B) Stimulation of cGMP by DOM (20 μM). MK-801 (MK), 1 μM. *P < 0.04 vs. same treatment in the absence of bFGF (NONE). (C) Depolarization-induced stimulation of cGMP formation by 30 μg/ml veratrine (VET). Nifedipine (NIF, 10 μM); Verapamil (VER; 10 μM). *P < 0.003 vs. same treatment in NONE. °P < 0.001 vs. VET in NONE. In panel D, cGMP formation by atrial natriuretic peptide (ANP, 0.1 μM × 2 min) or sodium nitroprusside (SNP; 1 mM × 2 min). *P < 0.05 vs. SNP in NONE.

DOM stimulation of cGMP both in the presence or in the absence of bFGF was not reduced by MK-801 (Fig. 6B), and DOM stimulation of cGMP was completely antagonized by CNQX (data not shown). In order to verify whether bFGF pretreatment could also increase cGMP formation following stimulation of VSCC, cerebellar neurons were depolarized using veratrine (VET; 30 μg/ml). Stimulation of cGMP by VET was significantly antagonized (63%) by the dihydropiridine nifedipine (NIF; 10 μM), and to a higher extent (78%) by the phenylalkylamine verapamil (VER; 10 μM). MK-801 (1 μM) partially antagonized (40%) VET stimulation of cGMP formation. Neither NIF nor VER antagonism of VET stimulation of cGMP was enhanced in the presence of MK-801 (Fig. 6C). VET stimulation of cGMP formation was significantly increased (226%) by bFGF pretreatment, and this enhancement was not reduced in the presence of MK-801 (Fig. 6C).

bFGF treatment selectively increased the stimulation of cGMP formation via EAA receptors and VSCC, since cGMP formation by atrial natriuretic peptide (10^{-7} M) [20] was not different in bFGF treated vs untreated cultures, and cGMP formation by the direct guanylate cyclase activator sodium nitroprusside (10^{-3} M) [20] was slightly (20%) although significantly reduced in the presence of bFGF.

4. DISCUSSION

In the present study, we have investigated the ability

of bFGF to influence cerebellar neurons sensitivity to neurotoxicity by EAAs occurring either via the NMDA receptor as for Glu, or via the ionotropic non-NMDA receptor as for DOM. We have found that only one time addition of 1–2.5 nM bFGF was required to significantly protect cerebellar neurons from neurotoxicity by Glu as well as from toxicity by DOM. Neuroprotection was maximal at approximately 1 nM bFGF (~ 20 ng/ml), and then it leveled off. This concentration is consistent with previously reported values for peak effectiveness of bFGF in supporting the survival of cerebellar granule neurons in culture [8] as well as of other culture systems [14]. However, it is 4–10-fold greater than the reported value for bFGF maximal effects in hippocampal neurons [10,11,24] and other cultures [5,12].

The biological response of cells to bFGF is mediated through specific receptors which have been shown to be members of the tyrosine kinase receptor family and to be phosphorylated upon binding of bFGF via an intermolecular transphosphorylation mechanism [25,26]. bFGF mRNA and FGF receptor mRNA are widely expressed in the adult rat central nervous system [27,28]. High levels of FGF receptor mRNA have been detected in the cerebellar granule cell layer, whereas the Purkinje cell and molecular layers were found to express little FGF receptor mRNA [28]. Very little or none FGF mRNA was detected in total RNA from rat cerebellum [27]. These findings suggest that cerebellum granule cells are responsive to bFGF synthesized in vivo either by glial cells or by other type of neurons. We have observed no neuronal protection from Glu or DOM in mixed neuronal-astrocyte cultures (data not shown), suggesting that bFGF may be synthesized in vivo by neuronal or non-neuronal populations not present in these cultures. Since FGF mRNA expression increases in lesioned rat brain [29], exogenously administered bFGF protection could be mimicking an in vivo situation where the factor is released following lesion.

bFGF-mediated protection appeared to be specific, since no protection was observed against Glu or DOM toxicity in cultures pretreated with BDNF or NT-3. Both neurotrophins have been suggested to play an important role in the development of the cerebellum [30,31] and BDNF mRNA levels were increased upon stimulation of Glu receptors in hippocampus and cortex [32,33]. Although BDNF and NT-3 may have critical functions in the development of the cerebellum as previously suggested [30], our results here indicate that increased levels of these factors do not result in any protective effect from excitotoxic damage of cerebellar neurons in vitro, and therefore do not seem to support a general protective role for BDNF or NT-3 during excitotoxic brain damage.

It has been suggested that some of the neurotrophic effects of FGF could be mediated by the enhancement of the neurotrophic activities of astrocytes. Thus, FGF has been shown to regulate nerve growth factor synthesis and secretion by astrocytes and fibroblasts [34]. Such an effect is unlikely to account for the neuroprotective effect of bFGF documented in the present study, since non-neuronal cell proliferation in these cultures was prevented with mitotic inhibitors, and they constitute only about 3% of the cells. Moreover, addition of up to 50 ng/ml NFG had no effect on the number of neurons surviving to neurotoxic concentrations of Glu or DOM, and exogenously added BDNF or NT-3 did not increase the neuronal survival after exposure to the toxins either. Thus, we suggest that bFGF neuroprotection in these cultures may be mediated through a direct interaction between bFGF and bFGF receptors on the neuronal surface, and not through an indirect stimulation and release of neurotrophins by non-neuronal cells. Previous studies support the view that bFGF acts directly on neurons [10,35]. However, we cannot rule out that growth factors other than NGF, BDNF or NT-3 could be released by the few glia cells present in these cultures and played an intermediary role in the neuroprotective action of bFGF.

Our data indicate that the mechanism by which bFGF protects neurons from toxicity may involve changes in calcium-dependent second messengers. A greater rise in cGMP formation, which depends on extracellular calcium influx, was observed in bFGF-pretreated neurons after exposure to EAAs or after depolarization of the neurons with veratrine. These observations are consistent with the possibility that bFGF might modify calcium influx via modulation of VSCC number or function, as already suggested for growth factors in other cell types [36]. No increase in cGMP formation was observed in unstimulated cultures pretreated with bFGF in comparison with untreated cultures, consistent with previous data showing that FGF alone did not significantly alter basal levels of intracellular calcium in hippocampal neurons [11]. It is worth noting that cGMP formation by NMDA was not enhanced in bFGF pretreated neurons, suggesting a role for calcium conductances other than NMDA receptor-associated channels in bFGF neuroprotection. Studies are currently in progress in order to test this hypothesis. Our preliminary results suggest a selective involvement of VSCC in bFGF action.

bFGF has been suggested to reduce Glu toxicity in cultured hippocampal neurons by preventing Glu-induced rises in intracellular calcium [11]. Our findings that cGMP formation was increased in bFGF-treated neurons upon stimulation either by Glu or DOM, or upon depolarization, suggest that bFGF protection in cultured cerebellar granule neurons may involve an elevation rather than a decrease in intracellular calcium. Several lines of evidence support the view that cultured hippocampal neurons and cerebellar granule cells may follow different regulation patterns. Thus, a depolarization-induced calcium influx is essential in securing the maintenance of cerebellar granule cells in culture, and

a K^+ concentration of 25 mM is required in the growth medium [37], while hippocampal neurons are usually maintained in culture in as low as 5 mM K^+. Neuronal sensitivity to excitotoxicity is also different in both types of cultures, so that only 20 μM Glu is needed to produce 60% reduction in cerebellar neuron survival [18], compared with 200 μM Glu for hippocampal neurons [11].

As mentioned above, cerebellar granule cells are maintained in culture under depolarizing conditions (25 mM KCl), allowing a throphic influx of calcium inside the cell [37]. It has been recently shown that rises in intracellular calcium may lead to inactivation of NMDA channels, and inactivation could be triggered by opening of either NMDA or VSCC [38]. However, neither ligand binding nor channel opening was absolutely required to elicit this calcium-dependent inactivation [38]. It could be conceivable that by enhancing an existing calcium current resulting from spontaneous activity, bFGF lowered the number or function of NMDA-receptors available on the neuronal surface. NMDA channel inactivation was suggested to occur by calcium binding to either the channel itself or a nearby regulatory protein to alter channel gating [38]. Since bFGF protection from EAA toxicity in granule cells required rather long exposure times, it would be conceivable to suggest an action on a calcium-dependent regulatory protein. This sort of mechanism could explain bFGF protection from Glu toxicity involving the NMDA receptor. However, since neurotoxicity by DOM was not prevented by the NMDA-channel blocker MK-801, other mechanisms for bFGF protection from DOM toxicity must be involved. bFGF has been shown to affect gene expression in a variety of cell types, including neurons [39,40], and the protective effects of bFGF against EAA toxicity may be also mediated by actions on protein expression. The observations that bFGF protection required pretreatment prior to the insult would be consistent with this possibility. Experiments are currently carried out in order to clarify this mechanism.

In conclusion, we have shown here that long exposures of cultured cerebellar granule cells to bFGF, protected neurons against excitotoxicity by Glu via the NMDA receptor and by DOM, specifically acting at the non-NMDA receptor. Our data indicate that bFGF may be regulating intracellular second messenger levels depending on calcium influx, thus suggesting that calcium influx may play a key role in different signalling pathways associated to neuronal degeneration and neuronal death.

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