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The Role of Astroglia on the Survival of Dopamine Neurons

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Abstract

Glial cells play a key role in the function of dopamine (DA) neurons and regulate their differentiation, morphology, physiological and pharmacological properties, survival, and resistance to different models of DA lesion. Several studies suggest that glial cells may be important in the pathogenesis of Parkinson's disease (PD), a common neurodegenerative disorder characterized by degeneration of the nigrostriatal DA system. In this disease the role of glia could be due to the excessive production of toxic products such as nitric oxide (NO) or cytokines characteristic of inflammatory process, or related to a defective release of neuroprotective agents, such as small antioxidants with free radical scavenging properties or peptidic neurotrophic factors.

Index Entries: Dopamine neurons; glial cells; tyrosine hydroxylase; L-DOPA; MPP+; nitric oxide; antioxidants; growth factors; apoptosis; Bcl-2 family proteins; MAP kinases; Parkinson's disease.

The role of glia in DA function could be best investigated in studies performed in vitro with DA cells in culture media where the experimental conditions are easily controlled. In this setting it has been demonstrated that glia produce multiple agents, secreted into the glia-conditioned medium (GCM), that have the property of diminishing or even reversing the effects of neurotoxic agents for DA neurons in pure neuronal cultures, such as L-3,4-dihydroxyphenylalanine (L-DOPA), MPTP, or NO. The analysis of the different glia-derived products suggested than in these effects there were more important the small molecular size antioxidants than the larger peptidic neurotrophic factors. GCM has also the property of reducing apoptosis and increasing the survival of DA neurons in vitro.

All these data suggest that GCM could be used as a powerful neuroprotective agent for DA neurons in animal models of PD. Regarding clinical applications it is too early to consider

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the use of GCM in patients with PD but it could be useful for the preparation of fetal DA neurons for transplantation and for the differentiation to DA neurons from stem cells.

Introduction: Glia and Neurons Interactions

Glial cells, the vast majority of cells in the central nervous system (CNS) which outnumber neurons by a ratio of 10 to 50, have been neglected up to recently in spite of the fact that the interactions between glial cells and neurons are crucial for the development, differentiation, maintenance, and repair of the nervous system *(1)*. The abundance of glial cells, their strategic organization, and their close proximity to neurons provide good opportunities for intercellular exchanges of information and molecules that compensate for metabolic or physiological deficiencies and stress. Glial cells have the ability to maintain appropriate neuronal electrical signaling by regulating extracellular ions *(2,3)*, pH *(4)*, and glutamate levels *(5)*. Through these mechanisms and others glial cells play important role in neuronal plasticity *(6)*; synaptic function *(7,8)*; resistance to chemical, toxic, or ischemic brain injury *(9,10)*, and recovery.

The role of glia on dopamine (DA) neuron function is particularly interesting for the following reasons. The proportion of glial cells surrounding DA neurons in the substantia nigra, the target nucleus for neurodegeneration in Parkinson's disease (PD) is the lowest for any brain area *(11–13)*, suggesting that DA neurons are more vulnerable in terms of glial support than any neuron in other brain area. Furthermore, in brains of patients with PD there is evidence for glial production of cytokines and other substances that could be harmful to neurons *(14–16)*, suggesting that glia of the substantia nigra could be actively involved, primarily or secondarily, in the neurodegeneration process. There is, however, evidence in favor of the role of diffusible signals from neurons and glia that change microglia from neurotoxic to neuroprotective with respect to embryonic DA neurons *(17)*.

In terms of the role of glia in the survival, differentiation, pharmacological activity, and resistance to lesion of DA neurons two major lines of work should be considered: 1) Glia plays a major role in the control of oxidants and 2) glial cells are a key source of neurotrophic factors for DA neurons.

The first function may be critical for DA neurons, that produce themselves a great number of free radicals in the oxidative metabolism of its own metabolite, DA, and that are known to be extremely sensitive to exogenous products that increase free radicals, such as 6-hydroxydopamine (6-OHDA), or that interfere with energy production, such as 1-methyl-4-phenylpyridinium (MPP+). A major inducible antioxidant pathway within cells is largely restricted to the astrocytes within brain and transcriptional control of target genes by antioxidant/electrophile response elements has been described in these cells *(18)*. Genes that are regulated by this mechanism include the antioxidant enzymes NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthetase, and glutathione (GSH)-S-transferase. Astrocytic metabolic processes that normally aid and/or protect neurons may be controlled via this inducible system *(18)*.

GSH is an important small antioxidant, produced by glia, with neurotrophic properties on midbrain DA neurons. GSH plays a major role in protection against oxidative stress *(12,19)* and functions to remove the H_2O_2 generated by monoamine oxidase (MAO) *(20)*. GSH peroxidase, is mainly a mitochondrial enzyme *(21)* and in the human midbrain it is exclusively located in glia *(22,23)*. GSH levels decrease with age in neurons but remain stable in astrocytes in culture *(13)*. However, neurons can maintain an intracellular GSH pool by taking up cysteine provided by glial cells *(13)*.

Another mechanism through which glia could protect DA neurons is by regulation of excitatory neurotransmission. Several studies have shown that 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) toxicity could be diminished by drugs that interfere with excitatory transmission. Glial cells could reduce the effects of excitatory amino acids by selective uptake *(24)* or by release of endogenous compounds that act as competitive inhibitors of excitatory aminoacids. One of these compounds, kinurenic acid, is the product of the enzymatic activity of kinurenic acid transaminase; one enzyme selectively localized in astrocytes *(25,26)*.

A number of experiments in vitro and in vivo have shown that DA neurons respond to a number of neurotrophic factors including GDNF, FGF, and others *(27–32)*. These compounds are glial products so it is likely that glia plays a role in the pharmacological activity, resistance to injury, and plasticity of DA neurons in health and disease *(27,28,30,33,34)*.

The Effects of Astroglia on Dopamine Function

Several studies have shown that glial cells increase the survival and phenotype expression of DA neurons in culture. The percentage of tyrosine hydroxylase positive (TH+) cells in neuronal-enriched fetal midbrain cultures after 7 d in vitro is 2–5% of total cells *(35–37)*, but in midbrain neuron/cortical astrocytes co-cultures it increases to 30–40% of total cells *(38,39)*. Without excluding the possibility of the mediation of some of these effects by physical glial-neuronal contacts, there is now evidence in favor of the role of diffusible agents secreted by glia. For instance, midbrain glia-conditioned medium (GCM) protects DA neurons from apoptosis, increasing TH+ cells survival. GCM increases TH expression and activity as well as 3H-DA uptake and induces *de novo* synthesis of TH+ cells in fetal midbrain cultures. The protein synthesis inhibitor, cycloheximide, at 0.01 µg/mL, inhibits the GCM-induced increase of TH+ cells (Fig. 1). The diffusible agents secreted by astroglia are mainly small antioxidants with free radicals scavenging properties, such as GSH and ascorbic acid (AA), and peptidic neurotrophic factors *(40)*.

The role of GSH in PD is difficult to assess since this tripeptide does not cross the blood-brain barrier (BBB) and it is difficult to administer exogenously. The results of treatment with its precursor N-acetylcysteine (L-NAC) in experimental models of DA lesions are promising *(41)*. GSH was decreased in the substantia nigra of patients with PD *(42,43)* and that reduction is considered relevant to the pathophysiology of this disease. In PD the density of glia immunoreactive to GSH peroxidase surrounding the surviving DA neurons is increased, suggesting a compensatory mechanism that protects surviving neurons against pathological death *(22)*. MAO-B, an enzyme involved in the catabolism of DA, is present in glia in several human brain regions *(44)*. DA neurons surrounded by numerous MAO-B glial cells are better protected against the degenerative process than those in glia-poor environment *(11)*. Because absence of DA vesicle storage in the dendrites of nigral DAergic neurons may favor autooxidation of DA and formation of oxygen free radicals, the degradation of DA by glial cells may prevent this deleterious phenomenon.

Dopamine modulates glial activity. It increases GDNF in human fetal astrocytes *(45)* as well as the accumulation of intracellular cAMP in primary cortical and striatal glial cells of rats, monkeys, and humans *(46,47)*. The presence of D_1 receptor on a human fetal type I astrocyte cell line was demonstrated by Kinor et al. *(45)*; DA and the D_1 selective agonist SKF-38393 increase GDNF mRNA and this effect was blocked by a selective D_1-antagonist *(45)*. Since in vitro and in vivo GDNF is an important neuroprotective factor for PD *(32,48)*, genetically modified fetal astrocytes may serve as a drug-delivery system. Furthermore, such astrocytes can supply growth factors that may prevent or retard further degeneration, as well as initiate sprouting of residual neurons.

Altered glial function causes neuronal death and increases neuronal susceptibility to MPP+- and 6-OHDA-induced toxicity in astrocytic/

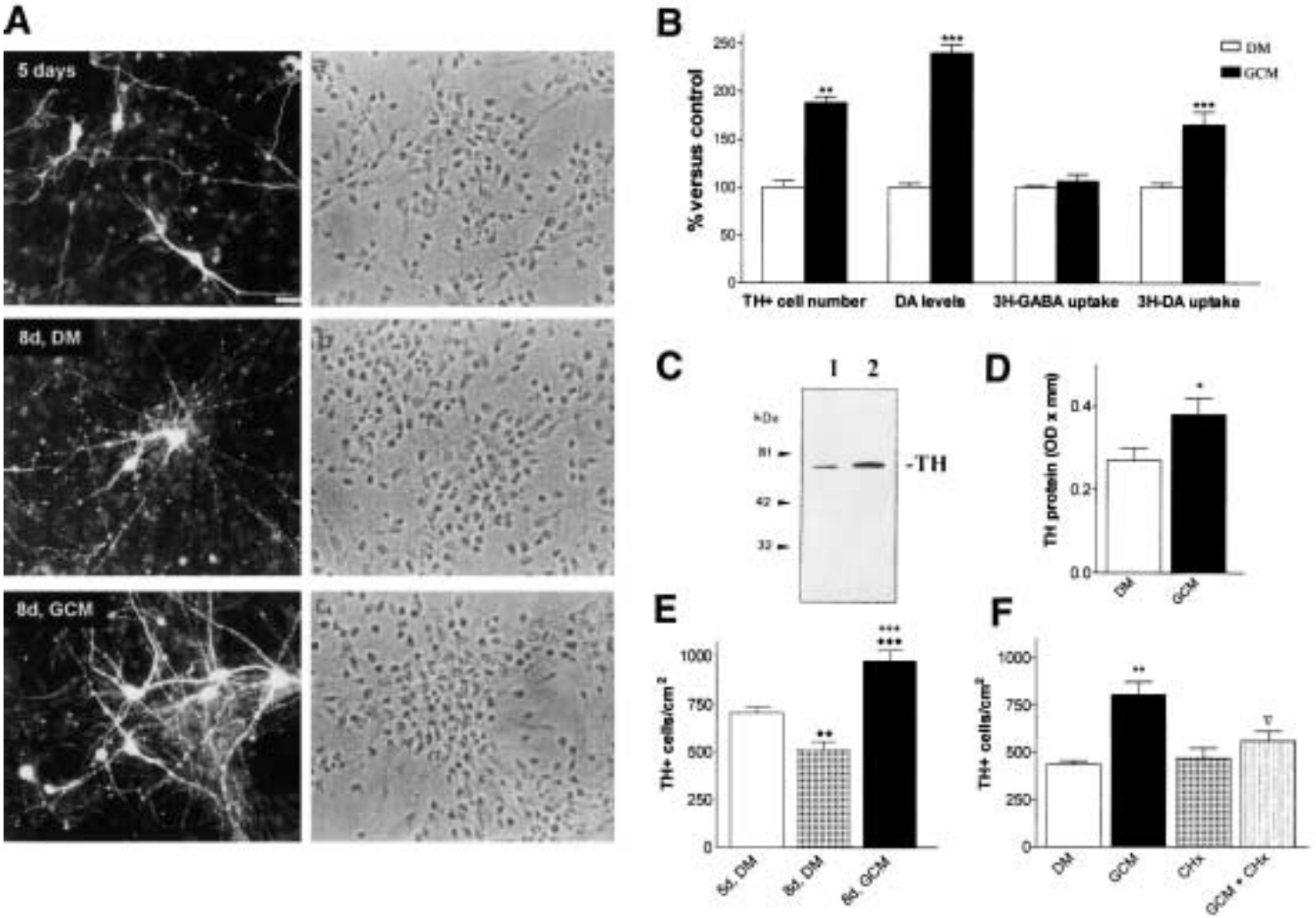

Fig. 1. Effect of glia-conditioned medium (GCM) on *de novo* expression of tyrosine hydroxylase (TH) in fetal rat midbrain neuronal cultures. **(A)** TH immunofluorescence (left panels) and their respective phase contrast micrographs (right panels) at 5 and 8 d in vitro. Cultures were treated with serum free-defined medium (DM) for 5 or 8 d and with GCM for the last day. Scale bar = 25 μm. **(B)** Effects of GCM on: TH+ cell number, DA levels, 3H-GABA and 3H-DA uptake from cultures treated with DM or GCM for 24 h. Control values are: TH+ cells/cm^2, 752 ± 25; DA levels, 335 ± 12 pg/well; 3H-GABA uptake, 65033 ± 729 cpm/well; 3H-DA uptake, 33274 ± 1347 cpm/well (n = 9). **(C)** Western blot for TH protein. Lane 1, fetal mesencephalic cultures treated, at 6 d in vitro, with DM for 24 h. Lane 2, GCM-treated cultures for 24 h. **(D)** Values are expressed as the mean of OD × mm ± S.E.M. (n = 6) of three experiments. **(E)** Histogram corresponding to TH immunofluorescence showed in (A). Control values are 707 ± 28.5 TH+ cells/cm^2 (n = 5). **(F)** Effects of protein synthesis inhibitor cycloheximide (CHx) on TH expression. Cultures were treated, at 5 d in vitro, with DM, GCM, and/or cycloheximide 0.01 μg/mL for 24 h. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *"t"* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ GCM vs controls, ◆◆$p < 0.01$, ◆◆◆$p < 0.001$ 8d DM and 8d GCM vs 5d DM; +++$p < 0.001$ 8d GCM vs 8d DM-treated group, Δ$p < 0.05$ GCM plus cycloheximide vs GCM-treated group.

ventral mesencephalic co-cultures *(49)*. Glial activation causes neuronal death via a mechanism that appears to involve the release of reactive oxygen / nitrogen species and glutamate from astrocytes. Glial GSH depletion or complex I inhibition did not cause neuronal death, but potentiated the toxicity of MPP+ and 6-OHDA, possibly via the release of glutamate and reactive oxygen species (ROS) from astrocytes *(49)*. Neuronal death caused by 6-OHDA was increased in ventral mesencephalic cultures previously cultured with lipopolysaccharide-activated and GSH-depleted, but not complex I-inhibited astrocytes, compared with co-cultures containing normal astrocytes. Treatment of co-cultures with GSH prevented the increased neuronal susceptibility to 6-OHDA *(49)*.

In addition, astrocytes inhibit the expression of p53, a tumor-suppressor gene proposed as the "guardian of the genome" *(50)*, and increase the survival in neuroblastoma cells treated with 6-OHDA *(51)*. p53 induces apoptosis by upregulating Bax and down regulating Bcl-2 *(52,53)*. Therefore, astrocytes can support, via cell-cell interactions, the viability of neurons. Perturbation of this relationship may put the neurons at risk of toxic insults.

Though the cause of DA cell death in PD is unknown, there is evidence to suggest that altered glial function may play a partial role in the neurodegenerative process *(14–16,54–56)*.

The Critical Factor for L-DOPA Neurotrophism or Toxicity on DA Neurons is Glia

During the last 20 years there has been much debate about the role of L-3,4-dihydroxyphenylalanine (L-DOPA), the most effective compound for the symptomatic treatment of PD, in the progression of the disease, and in the survival of the remaining nigrostriatal DA neurons. This debate was promote by the conflicting evidence that L-DOPA increases life expectancy in patients with PD *(57)*, but also the prevalence of dyskinesia and fluctuations in patients receiving this treatment *(58)*. Recently, this issue has been partially solved with refinements in techniques for experiments in culture.

L-DOPA is toxic for DA neurons in pure neuronal culture *(35,59–62)*. The toxic effect is not restricted to DA cultures but also affect to other cells such as pheochromocitoma and melanoma *(63,64);* based in these experiments, L-DOPA was, at certain point, considered as a putative treatment for melanoma. L-DOPA toxicity has not been proven in animals with partial DA lesions, where it could even improve recovery *(65)*, or in patients with other disorders of the basal ganglia such as dystonia or without neurological disease *(66,67)*.

Since most experiments in vitro showing L-DOPA toxicity were performed in neurons cultured in the absence of glia, we hypothesized that the discrepancy between the effects of L-DOPA in vivo and in vitro may be related to the presence or absence of glia, respectively *(36)*. Fetal midbrain neuronal cultures were treated with L-DOPA, 200 μ*M*, in the presence or absence of mesencephalic GCM. In the absence of GCM, L-DOPA greatly reduced the number of TH-immunoreactive neurons and increased the levels of quinones in the medium; GCM prevented these effects of L-DOPA and increased the length and arborization of neurites of the TH+ cells, as well as 3H-DA uptake (*see* Table 1). Furthermore, L-DOPA and GCM have sinergistic effects on TH expression in human catecholamine-rich cells *(68)*. These studies reveal that the toxicity of L-DOPA in DA neurons in vitro is prevented by soluble factors secreted by midbrain glial cells. Therefore, these experiments provide an explanation for the discrepancies between studies on L-DOPA toxicity in vitro and results obtained in experiments in vivo and clinical data in patients with PD.

The protective effect of GCM on L-DOPA toxicity was associated to a reduction of quinones in the medium. Several neurotrophic factors increase the activity of important enzymes of the free radical scavenging system. NGF induces superoxide dismutase (SOD) *(69)*. Therefore, GCM may decrease L-DOPA

Table 1
Effects of Glia-Conditioned Medium (GCM) on L-DOPA, MPP^+, and Nitric Oxide-Induced Toxicity in Fetal Midbrain Neurons

Treatments	TH^+ cells (% vs C)		^{3}H-DA uptake (% vs C)		% Apoptotic cells	
Control	100 ± 10		100 ± 12		13.8 ± 1.0	
L-DOPA	27 ± 9	***	42 ± 3	***	27.5 ± 2.9	***
GCM	207 ± 30	***	216 ± 19	***	7.3 ± 0.5	***
L-DOPA + GCM	171 ± 12	+++ ***	124 ± 12	+++	15.5 ± 1.7	++
Control	100 ± 5		100 ± 10		13.1 ± 1.6	
MPP^+	49 ± 6	***	25 ± 4	***	21.5 ± 1.7	***
GCM	180 ± 8	***	134 ± 12	*	8.3 ± 0.6	*
MPP^+ + GCM	162 ± 7	+++ ***	51 ± 10	+ ***	10.3 ± 0.8	+++
Control	100 ± 7		100 ± 7		13.4 ± 2.8	
NO	68 ± 6	*	56 ± 6	**	24.6 ± 2.6	*
GCM	203 ± 5	***	347 ± 22	***	9.7 ± 1.4	
NO + GCM	182 ± 17	+++ **	360 ± 15	+++ ***	9.2 ± 1.1	+++

Cells were treated with GCM or 200 µ*M* L-DOPA / 10 µ*M* MPP^+ / 200 µ*M* DEA / NO alone or in combination, for 24 h after 5 d in culture. The cultures treated with the neurotoxins and GCM were exposed to both agents simultaneously. Control values for TH^+ cells were from 1000–1600 cells / cm^2 and for ^{3}H-DA uptake from 80×10^3 to 120×10^3 cpm / mg of protein. Values represent mean ± SEM (n = 6–12) of two independent experiments for each experimental model. Statistical analysis was performed by one-way analysis of variance followed by Student's t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs control; + = $p < 0.05$, ++ = $p < 0.01$, +++ = $p < 0.001$ with respect to the group treated with the neurotoxin in each experimental model.

toxicity by reducing pro-oxidants in culture. NGF and BDNF are expressed at high levels in cultured glia *(70)* though they have been detected only in brain neurons in vivo. GDNF was isolated as a DA selective neurotrophic factor produced by glia *(71)* and bFGF is expressed by both glia and neurons *(72)*.

Since the neuroprotective effect of mesencephalic GCM on L-DOPA toxicity could be explained by the effect of small antioxidants, such as GSH or AA, or by the larger polipeptidic neurotrophic factors, such as GDNF, FGF, and others, we tested several filtrate fractions of GCM with different molecular weights. The most potent neuroprotective effect was mainly retained by the filtrate fraction smaller than 10 kD, meaning that the most effective neuroprotective compounds were the small antioxidants. The levels of AA were three times higher in midbrain GCM than in defined medium (Fig.2A). This difference of concentration suggested that glia accumulated AA. We have shown that AA, at concentrations of 25 µ*M*, protects midbrain DA neurons from L-DOPA toxicity *(40)*. GSH levels in the media of GCM-treated cells increase to 1218% vs controls (Fig.2A). In addition, gel electrophoresis of GCM indicate seven news majorities bands present on GCM, with relative molecular weight 181, 158, 143, 55, 42, 35, and 33 KDa, respectively (Fig.2B).

GSH induced a dose-dependent increase of TH+ cells and ^{3}H-DA uptake and a reversal of

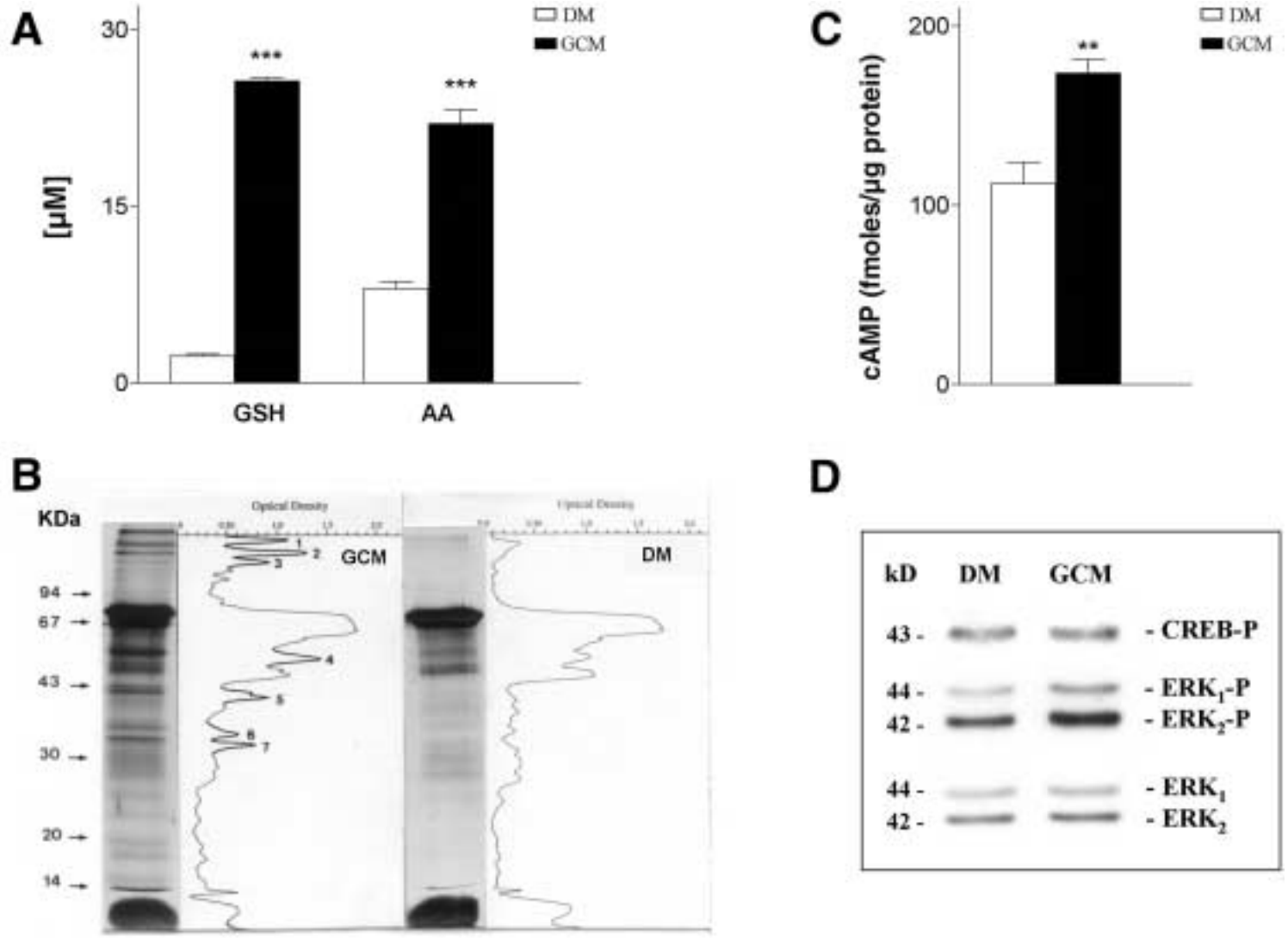

Fig. 2. Mechanisms of fetal midbrain glia-conditioned medium (GCM) on dopamine phenotype expression. **(A)** Effects of GCM on glutathione (GSH) and ascorbic acid (AA) levels in the medium of fetal midbrain cultures. Cells were treated, at 5 d in vitro, with DM or GCM for 24 h. **(B)** Gel electrophoresis of GCM and defined medium (DM) and their corresponding densitometric scanning. Numbers 1 to 7 on left panel indicate the seven news and majorities bands present on GCM with relative molecular weight 181, 158, 143, 55, 42, 35, and 33 KDa, respectively. **(C)** Effects of GCM treatment for 24 h on cAMP levels, determined by the method of Gilman *(122).* Control values: 112 ± 11.8 fmoles cAMP/µg of protein. Results are expressed as the mean ± S.E.M. (n = 6). Statistical analysis was performed by ANOVA followed by the Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$ GCM vs DM. **(D)** Effects of 30 min treatment with GCM on ERK-1/2 MAP kinases and CREB-P expression. Western blot analysis of MAPK-P and CREB-P proteins from control (DM) and GCM-treated groups. Control of charge with total MAPK.

L-DOPA-induced elevation of quinones. In control cultures, 400 μ*M* GSH increased the number of TH immunoreactive cells to 179% of the control value. Since GSH did not significantly modify the total number of cells in the culture, this increase in TH immunoreactive cells suggested that GSH had a selective neurotrophic effect on DA neurons. In cultures treated with L-DOPA, GSH, 400 μ*M* totally prevented the elevation of quinones and the disappearance of TH immunoreactive cells, but not the reduction of ^{3}H-DA uptake induced by L-DOPA. Since ^{3}H-DA uptake is considered an index of arborization, GSH is more effective for the prevention of cell death than for the damage to the terminals of the DA neurons.

The effect of GCM was mediated, at least in part, by the elevation of GSH on DA cultures. The levels of GSH, which was 2.25 ± 0.22 μ*M* in the medium of control cultures, was below control levels, 1.23 ± 0.18 μ*M* in cultures treated with L-DOPA, and rose to 25.6 ± 0.28 μ*M* in cultures treated with GCM, and to 27.4 ± 0.36 μ*M* in cultures treated with L-DOPA+GCM. In clinical studies, GSH is decreased in the substantia nigra of patients with idiopathic PD *(42,43,73)* and L-DOPA treatment reverses GSH depletion *(74)*.

Regarding the putative neuroprotective effects in this model of conventional neurotrophic factors, none of the peptidic neurotrophic factors had the properties observed with GCM. Overall the compounds tested, only NGF significantly reduced the elevation of quinones induced by L-DOPA. The effect of NGF, however, on TH immunoreactive cells was modest. NGF partially restored the number of TH immunoreactive cells from 27% of the control, in the cultures trested with L-DOPA, to 61% of controls in cultures treated with L-DOPA+NGF. The protective effect of bFGF, BDNF, and GDNF was somehow larger since these compounds, when given with L-DOPA restored the number of TH+ cells to 80, 124, and 111% of controls, respectively, and without modification of quinone levels. This dissociation between quinone quenching effect and preservation of TH immunoreactive cells suggests that the protection of midbrain DA neurons by mesencephalic GCM may be related to several independent mechanisms. Recent studies have shown that astrocyte-derived trophic factors increase survival of developing DA neurons through the inhibition of apoptotic cell death *(34,75–77)*.

GCM not only protected from L-DOPA toxicity but increased *de novo* synthesis of TH+ cells. Tyrosine hydroxylase gene promoter activity is regulated by both, cyclic AMP-responsive element (CREB) and AP1 sites *(78–80)*. GCM increases AMPc levels (Fig. 2C) and phosphorylation of extracellular signal-regulated protein kinases (ERK1 and ERK2) elements of MAPK signaling pathway, but fails to change CREB-P expression (Fig. 2D). MAPK signaling pathway promotes cell survival by a dual mechanism comprising the post-translational modification and inactivation of a component of cell death machinery and the transcription of pro-survival genes *(81)*. Furthermore, MAPK is an essential pathway mediator of TH induction *(80)*. During differentiation, signals may be transmitted via MAPK to the TH-AP-1 site to increase activators and reduce repressors, helping to shift the balance in favor of TH gene expression at this and possible other important regulatory sites on the gene *(80)*. Our results indicate that in the induction of TH expression by GCM may be implicated, at least in part, MAPK signaling pathway.

Catecholamines are required for the development of the nervous system and survival. Inactivation of the TH gene produced the death of 90% of mutant mice fetuses whereas administration of L-DOPA to pregnant females resulted in complete rescue of mutant mice *(82)*. Transgenic animals with mutations of the TH gene have a poorer prognosis than those with mutations of the DA-β-hydroxylase gene *(83)*, suggesting that DA is essential for development of the nervous system. It is likely that L-DOPA and catecholamines are critical factors for promoting the survival and differentiation of DA neurons and that the effects of L-DOPA on DA cells in vivo might be neurotrophic or neurotoxic depending on the local environment produced by glial cells. Another variable that could

explain the different effects is the age of the DA neurons used in each experiment since the effect of L-DOPA could be developmentally related.

We therefore tested the effect of L-DOPA on the survival of postnatal DA neurons using ventral midbrain neuron/cortical astrocyte cocultures in serum-free medium. L-DOPA (50 μ*M*) protected against the cell death of DA neurons and increased the number and branching of DA processes. The stereoisomer D-DOPA (50–400 μ*M*) was not active. The L-aromatic amino acid decarboxylase inhibitor (L-AAD) carbidopa (25 μ*M*) did not block the neurotrophic effect. These data suggest that the neurotrophic effect of L-DOPA is stereo-specific but independent of the production of DA. In a different set of experiments it was shown that L-DOPA increased the level of GSH. Inhibition of the γ-glutamylcysteine synthetase by L-buthionine sulfoximine (3 μ*M* for 24 h) blocked the neurotrophic action of L-DOPA (Fig. 3). N-acetyl-l-cysteine (L-NAC, 250 μ*M* for 48 h), which promotes GSH synthesis, had a neurotrophic effect similar to L-DOPA. These data suggest that the neurotrophic effect of L-DOPA may be mediated, at least in part, by the elevation of GSH content *(38)*.

Previous studies indicate that DA added to culture medium increases the expression of TH and L-AAD in primary cultures of fetal neurons *(84)*, and the neurotransmitter itself has been postulated as a neurotrophic factor *(85)*. If conversion of L-DOPA to DA is required for its neurotrophic effects, the response should be blocked by the L-AAD inhibitor carbidopa *(63)*. Our study reveals, therefore, that the mechanism responsible for the neurotrophic effect of L-DOPA is independent of its conversion to DA and that L-DOPA neurotrophic effects may depend on the glia environment.

GCM Protects and Rescues DA Neurons from MPP+-Induced Toxicity

Exposure to MPTP produces in humans and experimental animals an akinetic rigid syndrome (ARS) similar to idiopathic PD. MPTP is converted into the active neurotoxic agent MPP+ by the action of MAO and MPP+ is selective accumulated by DA neurons through an active uptake mechanism and results in the selective loss of DA neurons *(86,87)*. ARS induced by MPTP is considered the best experimental model of PD and therefore it is of great interest to investigate the effect of GCM on cultures of DA neurons treated with MPP+.

MPP+ interferes with energy production in the mitochondria by inhibition of complex I. Apoptotic cell death, that occurs in DA neurons in culture, is increased by MPP+ *(88,89)*. There is also an increased number of apoptotic cells in the substantia nigra of patients with PD *(90)*. Therefore, the effect of GCM on apoptosis of DA neurons could provide insights into the role of glia in PD.

In fetal midbrain neuronal cultures in the absence of GCM, MPP+ reduced the number of TH immunoreactive neurons and increased apoptosis. In the cultures treated with GCM and MPP+, GCM counteracted the effects of MPP+ and increased the length and arborization of TH+ neurites. The protective effect of GCM was maximal in cultures co-treated with GCM and MPP+ simultaneously (Table 1), but it also restored DA parameters in cultures receiving GCM 1 or 3 d after MPP+. The protective effect of GCM was negligible in cultures pretreated with GCM and receiving MPP+ 24 h later.

The toxicity of MPP+ on DA neurons has two time-related components; the first one is characterized by rapid cell death due to the failure of energy production related to inhibition of respiratory activity produced by MPP+ blockade of complex I activity, and the second is related to apoptosis that takes place after the acute phase. The greatest severity of TH+ cells dropping out occurs during the first 24 h of treatment and is less pronounced from day 1–3. GCM reduces apoptotic cell death; this effect may explain the augmentation of TH+ cells by GCM. Our data, however, suggest additional mechanisms of protection since reduction of apoptosis will not be enough to

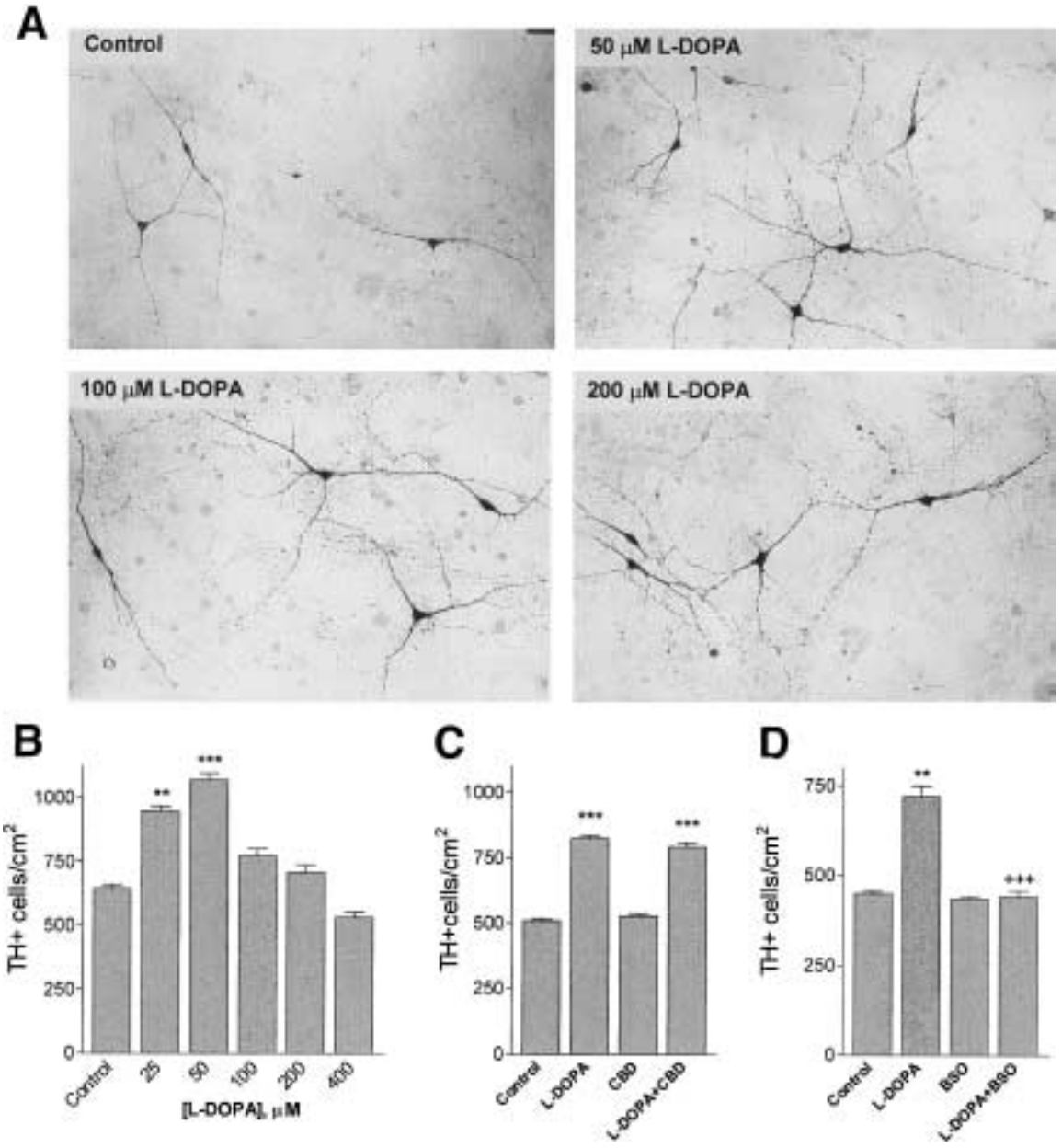

Fig. 3. Neurotrophic effects of L-DOPA in postnatal cortical astrocytes/ventral midbrain dopamine neurons co-cultures. **(A)** TH+ DAB-immunostaining of dopamine neurons. Cultures were treated, at 5 d in vitro, with L-DOPA 50 μ*M*, 100 μ*M* and 200 μ*M* for 48 h. Scale bar = 30 μm. **(B)** Effects of L-DOPA (50, 100, 200, and 400 μ*M*), L-DOPA 50 μ*M* and/or L-aromatic amino acid decarboxylase inhibitor carbidopa (CBD) 25 μ*M* **(C)**, and L-DOPA 50 μ*M* and/or GSH synthesis inhibitor L-buthionine-[S,R]-sulfoximine (BSO) 3 μ*M* **(D)** on TH+ cell number. Statistical analysis was performed by one-way ANOVA followed by the Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ L-DOPA-treated groups vs controls; $^{+++}p < 0.001$ 50 μ*M* L-DOPA plus 3 BSO μ*M* vs 50 μ*M* L-DOPA-treated group.

explain the protection against MPP+-induced TH+ cell death that occurs in the first hours of exposure to the neurotoxin.

Furthermore, in midbrain cultures maintained for 7 d in vitro, a brief exposure to GCM reversed the effect of spontaneous drop out of DA neurons and restored the number of TH+ cells to levels higher than those counted the first day after plating. Elevation of TH+ cell number above baseline after 7 d in culture and 3 d after exposure to MPP+ suggests that GCM, in addition to inhibition of apoptosis, induces *de novo* expression of TH+ cells and therefore, has neurotrophic activity *(77)*. Consequently, GCM enhances recovery of damaged neurons or expression of the DA phenotype rather than resistance to the neurotoxin.

The high levels of antioxidants present in GCM may play a very important protective role during acute exposure to MPP+ since MPTP produces free oxygen radicals and oxidative stress in vitro and in vivo *(91–93)* and vitamin E, β-carotene, AA, and L-NAC attenuate the MPTP toxicity in mice in vivo *(94,95)*. MPP+ increases the production of superoxide radical *(92);* this radical is transformed into free oxygen and hydrogen peroxide by SOD. Transgenic mice that overexpress the SOD-1 are resistant to MPTP-induced DA toxicity *(96)* and L-DOPA-induced toxicity *(38)*, suggesting that scavenging of the superoxide radical is protective in these models of toxicity.

Strain-dependent susceptibility to MPTP- and MPP+-induced parkinsonism is determined by glia. The mechanism of strain specificity, however, remains unknown and that it does not involve the MAO-B conversion of MPTP to MPP+ *(97)*. One study examining DA transporter function, necessary for MPP+ uptake, demonstrated no differences in transporter kinetics between the two strains of mice with different MPTP susceptibility *(98,99)*. Although differences in iNOS levels after MPTP or MPP+ administration have not been examined, it is possible that this pathway could underlie some of the differences in toxicity *(15)*. Another possibility is the differences of efficiency of glial cells to produce or maintain neuronal GSH levels in the different strains of mice *(100)*.

GCM Protects Fetal Midbrain Cultures from NO-Induced Toxicity

There is evidence suggesting that nitric oxide (NO) plays an important role in DA cell death in PD. Peroxynitrite may also play a role in this disease; an increase of nitrite concentration in the cerebrospinal fluid (CSF) of patients with PD has been reported *(101)* and NO radicals have been detected in PD substantia nigra *(102)*. Finally, the core of Lewy bodies in PD are immunoreactive for nitrotyrosine *(103)*. Thus, the aim of this study was to investigate the effects of NO on apoptosis and functionality of DA neurons and glial cells.

The experiments were carried out in neuronal-enriched midbrain cultures treated with the NO donor diethylamine/nitric oxide complexed sodium (DEA/NO). DEA/NO, at doses of 25 and 50 μ*M*, is neurotrophic for DA cells, increasing the number of TH^+ cells, TH^+ neurite processes, DA levels, and ^{3}H-DA uptake. A dose of 25 μ*M* DEA/NO protected DA cells from apoptosis. In addition, it induced *de novo* TH synthesis and increased intracellular reduced GSH levels, indicating a possible neuroprotective role for GSH *(75)*. However, in doses ranging from 200–400 μ*M*, DEA/NO decreased TH^+ cells, DA levels, ^{3}H-DA uptake, and the number of mature oligodendrocytes. No changes in either the amount or morphology of astrocytes and glial progenitors were detected. A dose- and time-dependent increase in apoptotic cells in the DEA/NO-treated culture was also observed, with a concomitant increase in the proapoptotic Bax protein levels and a reduction in the ratio between Bcl-xL and Bcl-xS proteins. In addition, DEA/NO induced a dose-and time-dependent increase in necrotic cells. 1H-(1,2,4) oxadiazolo (4,3a) quinoxaline-1-one, a selective guanylate cyclase inhibitor, did not revert the NO-induced effect on 3H-DA uptake *(75)*.

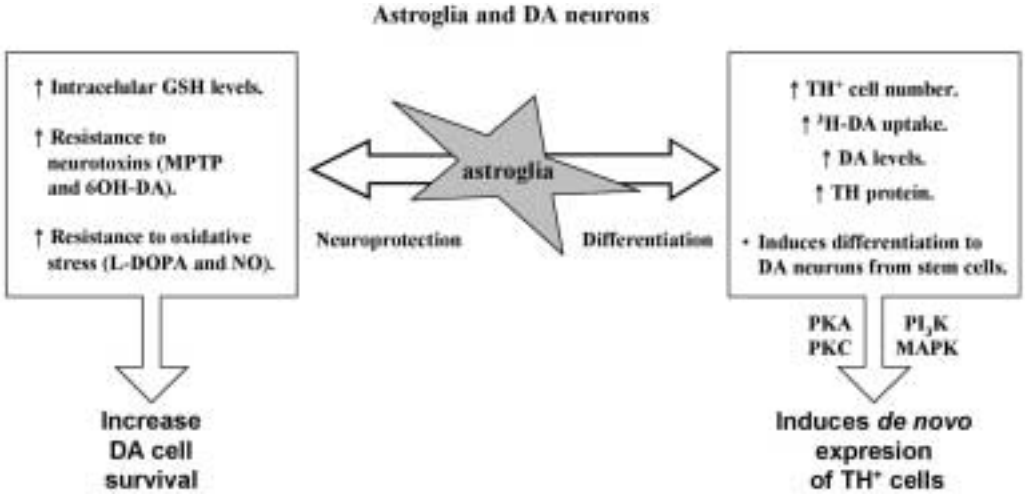

Fig. 4. The role of astroglia in the survival, differentiation, pharmacological activity, and resistance to lesion of dopamine neurons.

GCM, obtained from fetal midbrain astrocyte cultures, totally protected neuronal-enriched midbrain cultures from NO-induced toxicity. No neurite degradation was detected, as shown by phase contrast and TH immunostaining, nor was a decrease in TH^+ cell number or ^3H-DA uptake observed. In addition, we detected no increase in apoptotic cell death and GCM protected from the DEA / NO change on pro- and anti-apoptotic Bclx protein expression. GCM protects from DEA / NO-induced neurotoxic effects when given simultaneously (Table 1). GCM inhibits apoptosis but is also neurotrophic for DA neurons *(36,40,77)*. As previously discussed, these protective effects during acute exposure to DEA / NO could be related to the small molecular size antioxidants and neurotrophic factor contained in GCM (Fig. 4).

Effects of GCM on the Generation of Tyrosine Hydroxylase-Producing Neurons from Stem Cells

Several studies have underlined the importance of diffusible and contact-dependent signals from ventral mesencephalic glia in the induction *(77,104–106)*, differentiation *(107)*, and survival *(30,108–112)* of mesencephalic DAergic precursors. Furthermore, glial cells have been shown to be a useful vehicle for neuronal transplantation *(113,114)*.

The EGF-responsive stem cells self-renew; produce neurons, astrocytes, and oligodendrocytes; and may participate in repopulating the adult brain *(115–117)*. The principle phenotypes of the neurons generated by these precursors are GABA and substance-P *(116,118)*. Co-culture of these EGF-generated neuronal precursors with postnatal astrocytes yielded additional neurotransmitter phenotypes, such as neuropeptide Y, somatostatin, methionine-enkephalin, and glutamate *(119)*. Further addition of bFGF to these precursor / astrocyte co-cultures generated TH^+ neurons *(120)*. GCM from postnatal striatal astrocytes and from the B49 rat glial cell line have synergistic effects with bFGF for the generation of TH-producing neurons from precursors of the embryonic striatum and adult forebrain. The induction of TH expression occurs at the level of gene transcription. GCM promoted differentiation, at least in part by driving the striatal precursors out of cell cycle *(120)*. Astroglial cells function

as a support for migrating neuronal precursors in the developing brain and provide physiological assistance to neuronal functions in the mature brain. Recent experiments suggest that astrocytes may function in vivo as neural precursors *(121)* and glial diffusible factors may also instruct neuronal precursors to commit to a particular lineage.

Midbrain astrocytes induce the TH expression in cortical neurons and *Nurr*1-overexpressing neural stem cells *(107)*. This process requires both the overexpression of the nuclear receptor *Nurr*1, a transcription factor of the thyroid hormone/retinoic acid nuclear receptor superfamily, and factors derived from local type 1 astrocytes. *Nurr*1 regulates target genes before actual induction of a TH-positive phenotype. In such a way, *Nurr*1 may bestow competence upon multipotent cells to respond to specific factors, including those derived from ventral mesencephalic astrocytes *(107)*. In this respect, the induction of an unlimited number of midbrain DAergic neurons in vitro, that can engraft in vivo, could prove particularly useful in cell-replacement strategies to treat PD.

Conclusions

It is clear, from the evidences reviewed above that glia plays a very important function in the differentiation, survival, pharmacological properties, and resistance to injury of DA neurons, and that the role of glia is mediated, at least in part, by the secretion of chemical substances to the media. The effectiveness of the "glial cocktail" is superior to that of any of the products actually available for protection of DA neurons; therefore, we can state that in this subject "nature is better than art." It is possible that the effect of glia is mediated by several products, combined in the right proportions, some of them may not yet have been identified.

These data make GCM a very interesting candidate for neuroprotection in experimental models of PD. Glial products, however, could be deleterious under certain circumstances. Identification of the mechanisms involved in the neuroprotective and deleterious roles of glial cells is important from a therapeutic point of view. Stimulation of the former and inhibition of the latter may represent targets for therapeutic strategies aimed at slowing down or reversing the pathologic process of PD.

With regard to the therapeutic use of GCM in patients more studies are needed. There are, however, fields where the application of GCM could be immediate. These are the fields of fetal cells transplantation and differentiation of DA neurons from stem cells. Survival of embryonic DA neurons following transplantation is extremely low (5–20%). Strategies to increase this survival are critical to the future of transplantation for PD. Pretreatment of DA neurons in culture with GCM prior to cerebral implantation offers great potential for the augmentation of survival.

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