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Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells

(development/cerebellum/ataxia/neurodegeneration/type β transforming growth factor)

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ABSTRACT **Glial cell line-derived neurotrophic factor (GDNF) promotes survival of midbrain dopaminergic neurons and motoneurons. Expression of GDNF mRNA in cerebellum raises the possibility that cells within this structure might also respond to GDNF. To examine potential trophic activities of GDNF, dissociated cultures of gestational day 18 rat cerebellum were grown for ≤21 days in the presence of factor. GDNF increased Purkinje cell number without affecting the overall number of neurons or glial cells. A maximal response (50% above control) was elicited with GDNF at 1 pg/ml. Effects of GDNF on Purkinje cell differentiation were examined by scoring the morphologic maturation of cells in treated and control cultures. GDNF increased the proportion of Purkinje cells that displayed relatively mature morphologies, characterized by dendritic thickening and the development of spines and filopodial extensions. Morphologic maturation of the overall neuronal population was unaffected. In sum, our data indicate that GDNF is a potent survival and differentiation factor for Purkinje cells, the efferent neurons of cerebellar cortex. Together with its other actions, these findings raise the possibility that GDNF might be a critical trophic factor at multiple loci in neuronal circuits that control motor function.**

Growth factors profoundly influence the survival and differentiation of neurons during development. They may also play key roles in protection and recovery from injury during adulthood (see ref. 1). Glial cell line-derived neurotrophic factor (GDNF), a recently identified member of the type β transforming growth factor superfamily (2), was found initially to improve the survival and morphological differentiation of dopaminergic neurons of the ventral mesencephalon (3). GDNF transcripts were subsequently found in multiple brain regions (4–7). This suggests that GDNF may have pleiotropic survival effects. Recent studies have substantiated this notion. In particular, GDNF is now known to support the survival of embryonic motoneurons *in vitro* and to markedly attenuate motoneuron degeneration after transection of the facial nerve (8–10). GDNF also stimulates survival and fiber formation of peripheral autonomic ganglia (11).

Several groups have detected GDNF mRNA within cerebellum (7, 12, 13), and recent findings suggest that expression may persist from embryonic time points into adulthood (D.O.D. and J.A., unpublished data). However, nothing is known about the potential role of GDNF in this structure. To address this issue, we have studied effects of GDNF on the survival and differentiation of developing cerebellar neurons in dissociated cell culture.

We report that GDNF is the most potent survival and differentiation factor yet described for cultured Purkinje cells. GDNF did not alter glial cell number or the number and morphology of the overall neuronal population. This suggests that GDNF acts specifically on output neurons of the cerebellar cortex.

MATERIALS AND METHODS

Preparation of Cell Cultures. Dissociated cell cultures of whole cerebellum were prepared from cerebella of gestational day 18 Sprague–Dawley rats as described (14, 15). Except where otherwise noted, the culture medium consisted of minimum essential medium (GIBCO) containing Earle's salts and 2 mM glutamine. It was supplemented with 10% heat-inactivated horse serum, glucose (6 mg/ml), penicillin (0.5 unit/ml), and streptomycin (0.5 μg/ml). Cells were grown in poly(D-lysine)-coated multiwell culture plates (12 wells per plate) (23 mm) at a density of 1×10^5 cells per cm^2.

In some experiments, cells were maintained for 14 or 21 days *in vitro* under serum-free conditions (16). These cultures were prepared in Eagle's basal medium (BME; GIBCO) containing 2 mM glutamine, 10% heat-inactivated horse serum (GIBCO), glucose (6 mg/ml), penicillin (0.5 unit/ml), and streptomycin (0.5 μg/ml). Cells were plated at a density of 1×10^5 cells per cm^2 or 2×10^5 cells per cm^2 in multiwell culture plates. After cell attachment (4–5 h), the medium was replaced with serum-free BME, supplemented with insulin (10 μg/ml), sodium selenite (30 nM), progesterone (20 nM), putrescine (100 μM), transferrin (100 μg/ml), and bovine serum albumin (10 mg/ml).

Immunocytochemistry. The Purkinje population was identified by immunostaining with antiserum to calbindin (CaBP polyclonal antibody, 1:10,000; SWant, Bellinzona, Switzerland). In some experiments, sister cultures were stained with rabbit antiserum to neuron-specific enolase (NSE polyclonal antibody, 1:2000; Polyscience), a marker for the total neuron population. Other cultures were stained with antibodies to glial fibrillary acidic protein (GFAP polyclonal antibody, 1:500, Dako). Immunopositive cells were visualized by the ABC diaminobenzidine technique.

Analysis of Survival. $CaBP^+$ cells were counted in 30 fields, covering ≈24% of the culture well surface. Raw data from GDNF-treated wells, expressed as percentages of cell numbers in sister control cultures, were collected from six cultures per experiment. GDNF dose–response curves were generated from three independent experiments. Statistical analyses are described in figure and table legends.

Effects of GDNF on overall numbers of neurons and glia were assessed in some experiments. Cells stained with antibodies to NSE or GFAP were counted in 15 fields per culture and six cultures per experiment.

Analysis of Morphology. For analysis of Purkinje cell differentiation, cultures were fixed and stained with antibodies to

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Abbreviations: CaBP, calbindin; GDNF, glial cell line-derived neurotrophic factor; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein.

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CaBP. Immunopositive cells were assigned to one of five categories, based on neuritic morphology and cell shape. These categories have been shown to correspond to sequential stages in the normal development of Purkinje cells both *in vitro* (16, 17) and *in vivo* (refs. 18–24; reviewed in ref. 16). However, *in vitro* maturation lags behind *in vivo* progress through these stages by 1–3 days (16). One hundred CaBP$^+$ cells from control and GDNF-treated cultures were classified in each of three independent experiments ($n = 3$). Sampled fields were chosen randomly from three culture wells per experiment. Cell counts were subjected to ANOVA, with morphological category and GDNF treatment as grouping variables.

NSE$^+$ cells were also examined. Three culture wells were scanned at high magnification (×320) across the center of each well. The first 70 immunopositive cells observed were analyzed (23–24 cells per well). Numbers of neurites and branch points per cell were counted. Cell diameter and length of the longest neurite per cell were measured with an eyepiece micrometer. Morphological analyses were performed "blind," by two experimenters.

Chemicals. Human recombinant GDNF was purchased from PeproTech (Rocky Hill, NJ). The product was produced by expressing the gene in *Escherichia coli.* It was >98% pure by SDS/PAGE analysis.

RESULTS

To determine whether GDNF would alter survival of cerebellar cells, dissociated cultures of gestational day 18 rat cerebellum were grown in serum-containing medium for 6 days in the presence of GDNF (0.01 pg/ml to 1 ng/ml). Cultures were then fixed and stained with antibodies to CaBP, a marker of Purkinje cells. GDNF produced a dose-dependent improvement in Purkinje cell number. A 1.5-fold increase in cell number was observed with GDNF at 1 pg/ml (Fig. 1). At concentrations ≥ 100 pg/ml, GDNF elicited submaximal increases in CaBP$^+$ cell number. The GDNF-elicited increase in CaBP$^+$ cell number was not due to an effect of the factor on plating efficiency. Equal numbers of CaBP$^+$ cells were detected in control and treated cultures fixed after 1 day *in vitro* (Table 1).

Purkinje cells constitute a small subpopulation of the cells in these cultures. To test the cellular specificity of the GDNF response, sister cultures were stained with antibodies to GFAP, a marker of glia, and with antibodies to NSE, a marker of the total neuronal population. The number of glial cells was not affected by GDNF treatment (Table 1). In addition, GDNF did not alter the overall number (Table 1) or morphology (Table 2) of neurons in the cultures. As the majority of these neurons were granule neurons, it would appear that cultured embryonic granule neurons do not respond to GDNF.

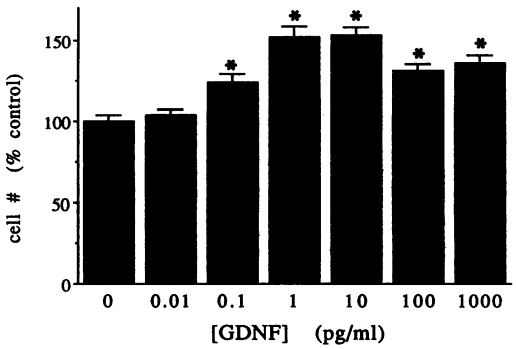

FIG. 1. Purkinje cell survival (CaBP$^+$ cell number) after 6 days of exposure to GDNF (0.01 pg/ml to 1 ng/ml). Values are mean survival data (±SEM) from three experiments ($n = 18$ wells per dose) expressed as percentages of cell numbers in respective control cultures. Data were analyzed by single factor ANOVA and post-hoc Fisher's protected least significant difference comparisons. *, $P < 0.05$ relative to control.

Table 1. NSE$^+$, CaBP$^+$, and GFAP$^+$ cell numbers in cultures grown 1 or 6 days under control conditions or in the presence of GDNF (10 pg/ml)

Treatment	Days *in vitro*	No. of NSE$^+$ cells	No. of CaBP$^+$ cells	No. of GFAP$^+$ cells
Control	1	ND	5386 ± 146	ND
GDNF	1	ND	5294 ± 96	ND
Control	6	7516 ± 292	275 ± 28	14,086 ± 302
GDNF	6	7488 ± 207	444 ± 22*	14,398 ± 374

Cultures were grown for 1 or 6 days before fixation and staining. NSE$^+$, CaBP$^+$, and GFAP$^+$ cell counts were made in sister cultures; 24% of each culture well was sampled for CaBP staining and 12% was sampled for GFAP and NSE (n = six wells per group). A significant difference between control and treated cultures was observed only for CaBP$^+$ cell counts after 6 days *in vitro.* ND, not determined. Results are expressed as means ± SEM.
*$P < 0.05$ relative to control by Student's unpaired t test.

Examination of CaBP$^+$ cells revealed four general morphologies, previously described as characteristic of Purkinje cells between days 4 and 14 in culture (16, 17). These morphologies resemble those displayed *in vivo* between embryonic day 17 and postnatal day 10. Purkinje cells progress from an early apical morphology (Fig. 2*A*), characterized as stage A, through a period of increasing neuritic extension and apical development, which we have designated stage B (Fig. 2*B*). Between 4 and 10 days *in vitro*, a regression of the primitive processes occurs and short perisomatic processes appear (stage C; Fig. 2*C*). The end of this period is marked by development of thick primary dendritic branches bearing immature spine-like and filopodial extensions (stage D; Fig. 2*D*). Between 14 and 21 days, Purkinje cells begin to exhibit thick-branched dendrites, covered with the dendritic spines characteristic of mature Purkinje cells *in vivo.* No cells in this fifth stage were found in cultures grown for 4–6 days.

In control cultures, the time course of changes in Purkinje cell morphology was consistent with previous reports (16, 17). At day 4, the distribution of cells in categories A–D was 51%, 44%, 5%, and 0%, whereas at day 6 it was 22%, 51%, 25%, and 3%. The distribution of morphologies clearly shifted from mainly embryonic to perinatal stages over this period.

GDNF treatment enhanced progress through early stages of morphological maturation in cultures fixed at 6 days *in vitro* ($F = 8.8$; df = 3, 16; $P \leq 0.005$) (Fig. 3). A majority of Purkinje cells in the treated cultures displayed perisomatic processes and/or broad dendrites with filopodial extensions (46% of cells in stage C and 12% in stage D). In contrast, control cultures displayed mainly prenatal morphologies (22% of cells in stage A and 51% in stage B).

To determine whether GDNF treatment might facilitate progress of the cell into the final stage of building, a major dendritic shaft and accumulating spines, some cultures were grown for up to 21 days *in vitro.* For these experiments,

Table 2. Morphologic differentiation of total neuronal population (NSE$^+$ cells) in cultures grown 6 days under control conditions or in the presence of GDNF (10 pg/ml)

Treatment	No. of neurites	No. of branch points	Cell diameter, μm	Neurite length, μm
Control	3.5 ± 0.2	3.2 ± 0.3	10.6 ± 0.3	89 ± 8
GDNF	4.0 ± 0.2	3.6 ± 0.3	10.8 ± 0.3	103 ± 9

Cells were grown for 6 days before fixation and staining. Three control cultures and three GDNF-treated cultures were examined. The diameter of each culture well was scanned at ×320. The first 70 NSE$^+$ cells observed were analyzed and measured with an eyepiece micrometer. No significant differences were observed between control and GDNF treated cultures. Results are expressed as means ± SEM.

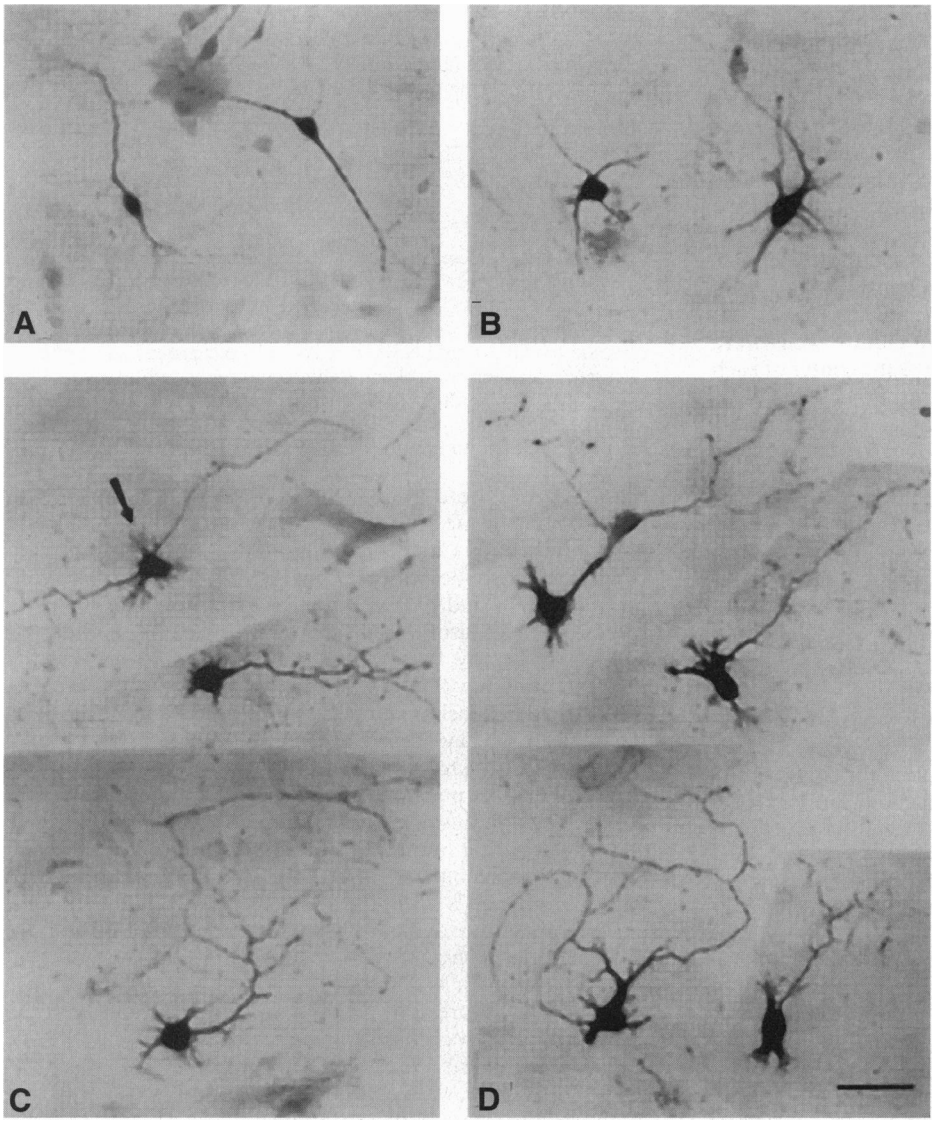

FIG. 2. Morphological differentiation of Purkinje cells in cultures of dissociated whole cerebellum. Cultures were maintained for 6 days *in vitro*. Purkinje cells were visualized by staining cultures with antibodies to CaBP. The Purkinje cells exhibit morphologies characteristic of four early developmental stages. (*A*) An embryonic stage with one or two long processes. (*B*) Late apical cone stage characterized by multiple processes and disoriented dendrites. (*C*) Emergence of short perisomatic processes and appearance of spines (arrow indicates a cell just entering the next stage). (*D*) Development of thick dendritic branches bearing filopodia and immature spine-like extensions. (Bar = 50 μm.)

serum-free culture conditions (16) were adopted, because in the presence of serum, glial proliferation prohibits Purkinje cell survival beyond 10 days (data not shown). In the absence of serum, glial cell numbers at 6 days were 95% lower than they were in the presence of serum. $GFAP^+$ cell numbers were unaffected by GDNF (806 ± 60 in control and 796 ± 65 in treated cultures in a representative experiment; means ± SEM; $n = 6$).

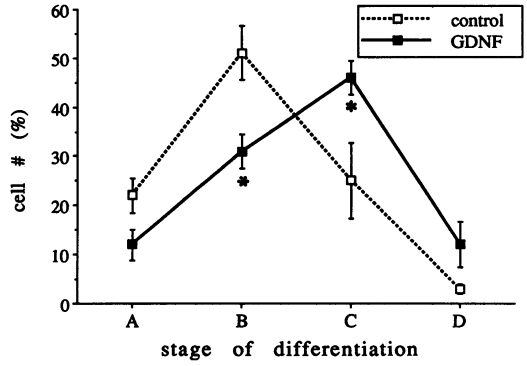

FIG. 3. Frequency distribution of Purkinje cell morphologies after 6 days *in vitro* under control conditions or in the presence of GDNF (10 pg/ml). One hundred $CaBP^+$ cells were classified in each of three separate experiments, according to the stages of maturation depicted in Fig. 2. Data represent mean cell numbers per 100 cells ($n = 3$) ± SEM. GDNF treatment promoted the appearance of later morphologies in the Purkinje cell population ($F = 8.8$; df = 3, 16; $P \leq 0.005$). *, $P < 0.05$ relative to control by post hoc Fisher's protected least significant difference test.

In serum-free cultures that were plated at double the usual density (2×10^5 cells per cm^2), the distribution of Purkinje cell morphologies on day 6 was comparable to published findings (16) and our observations in the presence of serum. By day 21, a minority of Purkinje cells progressed into the fifth and final morphological stage (stage E; Fig. 4). GDNF increased both the overall number of Purkinje cells (100% ± 8% to 155% ± 17%, means ± SEM; $n = 12$; $P < 0.05$) and the proportion of cells that displayed stage E morphologies. In a sampling of 100 Purkinje cells from each of six cultures, GDNF increased the proportion of cells in stage E from 8% ± 1% to 16% ± 2% (means ± SEM; $n = 6$; $P < 0.05$ by Student's unpaired t test).

In cultures that were plated at a lower density (1×10^5 cells per cm^2), GDNF improved Purkinje cell number (data not shown) but did not influence progress of the cells into the final stage of differentiation. Morphologic development of these cells appeared to be arrested in stages C and D on both days 14 and 21. This is consistent with previous observations that suggest cultured Purkinje cells must make contact with a dense carpet of granule neurons for cells to enter the final stage of dendritic development (16).

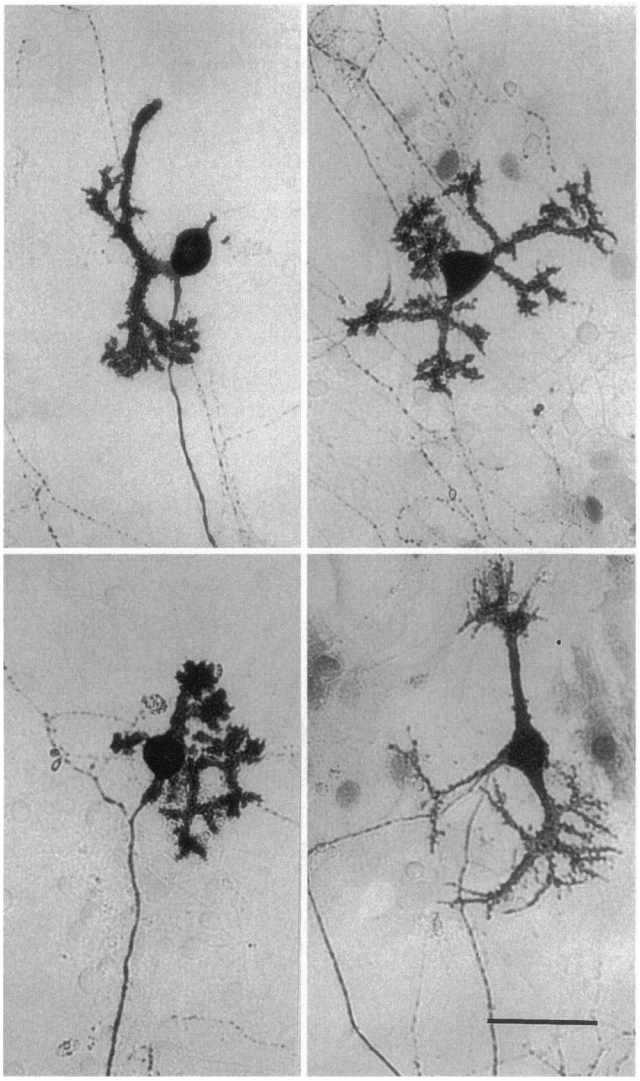

FIG. 4. Late stage Purkinje cell morphologies in cerebellar cultures plated at a density of 2×10^5 cells per cm^2 and grown for 21 days in serum-free medium that contained GDNF (10 pg/ml). Purkinje cells were visualized by staining cultures with antibodies to CaBP. At this late time point, the majority of immunopositive cells exhibited morphologies resembling stages C and D (see Fig. 2 *C* and *D*). A minority (16% ± 2%) had thick dendritic branches with spine-like extensions. The morphologies of cells in this category (stage E) approached those of mature Purkinje cells in other culture systems and *in vivo* (16). Four Purkinje cells in stage E are shown in this figure. (Bar = 50 μm.)

DISCUSSION

GDNF selectively enhances cultured Purkinje cell survival and morphologic maturation. As the overall number and morphology of neurons in the cultures was not altered by GDNF, it is likely that the effects are restricted to Purkinje cells. Granule neurons, the predominant neuronal population in these cultures, do not appear to be targets of GDNF action. Cerebellar glia were also unaffected. It remains to be investigated whether other small subpopulations, such as Golgi and stellate and basket cells, respond to GDNF.

GDNF, the type β transforming growth factor proteins, neurotrophins, and platelet-derived growth factors belong to a structural superfamily of growth factors containing a cysteine knot motif (25). In view of the structural similarity, low-affinity interactions between exogenous GDNF and receptors for other growth factors must be considered. In the present study, a direct high-affinity interaction with specific receptors would appear more likely, as very low concentrations were required to elicit a maximal response. Reverse transcriptase PCR analysis has revealed that GDNF message is present in rat cerebellum during embryonic development and remains detectable into adulthood (D.O.D. and J.A., unpublished data). These observations support the contention that GDNF is a trophic factor in cerebellum. It will be interesting to determine whether GDNF plays a role in the maintenance of Purkinje cells during adulthood as well as during development.

Although the cellular source of GDNF remains unknown, it is possible that GDNF constitutes a target-derived trophic factor for the Purkinje cell. Springer *et al.* (7) reported the successful amplification of cDNA for GDNF from rat cerebellum but not from human cerebellar cortex. As the rat mRNA was isolated from the entire cerebellum, they suggested that GDNF expression might occur in deep cerebellar nuclei rather than in cerebellar cortex. Deep cerebellar nuclei are the targets of Purkinje cell axons.

The possibility that GDNF is secreted by granule cells cannot be discounted. Enriched cultures of granule neurons express GDNF mRNA (12). Granule neurons are also a putative source of neurotrophin-3, another growth factor that selectively promotes differentiation (26) and survival (27) of Purkinje cells.

Granule neurons provide afferent innervation to the Purkinje cell and are important for its normal morphological development. It has been reported that *in vitro* production of mature dendritic shafts and spines requires that cells be grown on a dense network of granule neurons (16). In the present study, a minority of Purkinje cells displayed increased dendritic shaft and spine development at a comparatively low plating density of 2×10^5 cells per cm^2. GDNF potentiated this effect. This may be interpreted as evidence that GDNF either promotes morphologic development or preferentially improves survival of more differentiated Purkinje cells.

Purkinje cell development is likely controlled by coordinated interactions of multiple trophic stimuli. In addition to GDNF, nerve growth factor (14, 15, 28), neurotrophin-3 (26, 27), ciliary neurotrophic factor (29), and insulin-like growth factor 1 (30) regulate Purkinje cell survival and differentiation. Each of the factors elicits incremental improvements in cell number, suggesting either that distinct subpopulations of Purkinje cells have differing trophic requirements or that trophic activities interact (1). GDNF and ciliary neurotrophic factor have been found to synergistically increase choline acetyltransferase activity in cultures of facial motoneurons (9). Within cerebellum, the possibility of additive and synergistic interactions between GDNF, other neurotrophic factors, and effects of cell–cell contact remain to be explored.

GDNF is the most potent growth factor yet found to act upon the Purkinje cell population. Low concentrations of GDNF also increase the survival of motoneurons (8, 10). In addition, GDNF promotes the survival and differentiation of midbrain dopaminergic neurons (3, 31, 32). Collectively, these observations indicate that GDNF may influence development, maintenance, and regeneration at multiple loci within neuronal circuits that regulate motor function.

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