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Neurotrophic activity of platelet-derived growth factor (PDGF): Rat neuronal cells possess functional PDGF β-type receptors and respond to PDGF

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ABSTRACT **The different isoforms of PDGF bind with different affinities to two different receptor types. Previously, these receptors have been demonstrated mainly on mesenchymal and glial cells. We show here that PDGF β-type receptors are present also on rat brain neurons *in vivo* as well as *in vitro*. Immunohistochemical analysis of a sequentially sectioned brain of a newborn rat revealed the presence of PDGF β receptors in neurons throughout the central nervous system. These β-receptor-bearing cells were shown to contain neurofilaments by double immunofluorescence staining. Furthermore, the PDGF-BB isoform bound to cells in a neuron-enriched culture from newborn rat brains and induced c-Fos protein in these cells. Continuous PDGF-BB treatment of primary rat brain cell cultures resulted in outgrowth of neurites and prolonged survival. These results indicate a neurotrophic effect of PDGF-BB.**

PDGF is a mitogen for mesenchyme- and glia-derived cells. It consists of two disulfide-bonded polypeptide chains, A and B, and occurs as three isoforms, PDGF-AA, PDGF-AB, and PDGF-BB (for reviews see refs. 1 and 2). The three isoforms bind with different affinities to two receptor types, α and β, which are structurally related and endowed with protein-tyrosine kinase domains (3–7). Ligand binding induces activation of the receptor kinases by formation of receptor dimers; the A subunit of PDGF binds only to α receptors with high affinity, whereas the B subunit can bind to both α and β receptors (8–10).

The *in vivo* function of PDGF is not well known. It has been suggested that the factor has a role in early development (11) and in wound healing (12). PDGF has also been found to have a role in the development of glial cells in the central nervous system (reviewed in ref. 13). PDGF-AA is produced by type 1 astrocytes, and it stimulates division and motility and prevents premature differentiation of the O-2A progenitor cells (14–16), which possess PDGF α receptors but no β receptors (17).

We have further explored the possibility that PDGF has a functional role in the brain. We show by immunohistochemistry, receptor binding and functional assays that β receptors are present on neurons in the brains of young rats.

MATERIALS AND METHODS

Immunohistochemistry of Rat Brain Sections. The brains of rats aged 1 day, 3 days, 1 week, 3 weeks, and 6 weeks (one rat of each age) were immediately frozen and sectioned coronally at three different levels. The brains of two newborn rats were cut coronally into ≈500 sections. Sections were fixed for 10 min in cold 100% acetone and stored at −20°C. PDGFR-3, a rabbit antiserum raised against a synthetic peptide corresponding to amino acids 981–994 in the murine β receptor (18), was affinity-purified using a column of immobilized peptide. The affinity-purified antiserum was used for immunoperoxidase staining (Vectastain ABC Elite kit, Vector Laboratories), as described (19). Immunoperoxidase staining with a rabbit anti-glial fibrillary acidic protein (GFAP), antiserum (Dakopatts, Glostrup, Denmark), a mouse monoclonal anti-neurofilament (NF) antibody (NN18, Sigma; ref. 20), a mouse monoclonal anti-muscle actin antibody (gift from R. Ross, Dept. Pathology, Univ. Washington, Seattle), and a rabbit antiserum against von Willebrand factor (Dakopatts, Glostrup, Denmark) was performed using the Vectastain ABC kit according to the vendor's description.

Double Immunofluorescence Staining. The newborn rat brain sections were incubated with normal goat serum for 30 min and then with the first primary antibody (PDGFR-3) overnight at 4°C. The sections were washed in phosphate-buffered saline (PBS) and incubated with biotinylated goat anti-rabbit IgG for 30 min. Bound antibody was detected by fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories) for 30 min. Sections were then incubated with normal horse serum for 30 min and with second primary antibodies [mouse anti-NF (NN18) or anti-GFAP antibody (Labsystems, Chicago)] for 1 hr, washed in PBS, and finally incubated with a Texas Red-conjugated anti-mouse IgG (Amersham) for 30 min. The sections were rinsed in PBS, mounted in gelatin/glycerol, viewed in an epifluorescence microscope with excitation at 560 nm (Texas Red) and 495 nm (FITC), and photographed.

Neuronal Cell Cultures. Neuron-enriched cultures were obtained from 1- to 2-day-old Sprague–Dawley rats (21), with two rats for each experiment. The brains were dissected and the cells were dispersed by treatment with trypsin and DNase I and washed free of enzymes. Cultures were incubated for 3 days at 37°C in humidified 5% CO_2/95% air. The cells were then treated with 10 μM 1-β-D-arabinofuranosylcytosine (araC) in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 10% horse serum. This treatment was repeated after 3 and 6 days. For comparison, untreated cells were kept in DMEM with 10% fetal bovine serum in parallel cultures.

To determine the percentage of neuronal cells, we combined bisbenzimide (Sigma) staining of nuclei with immunofluorescence staining using the anti-NF antibody. Cells grown on chamber slides were washed with prewarmed medium containing 1% fetal bovine serum and then were

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Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; NF, neurofilament; NGF, nerve growth factor; PDGF, platelet-derived growth factor.

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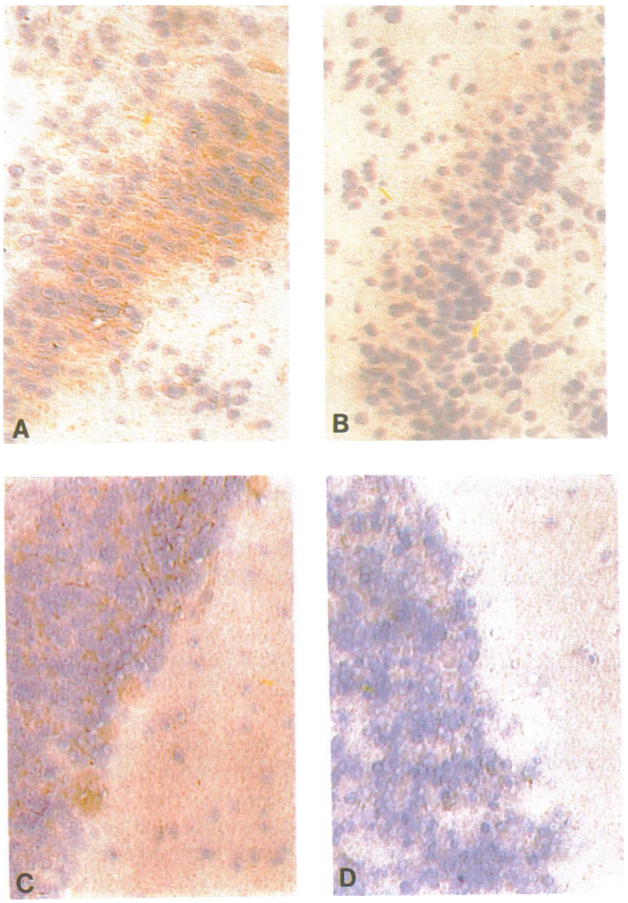
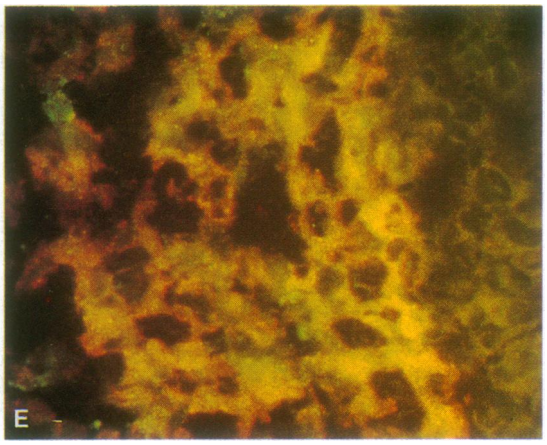
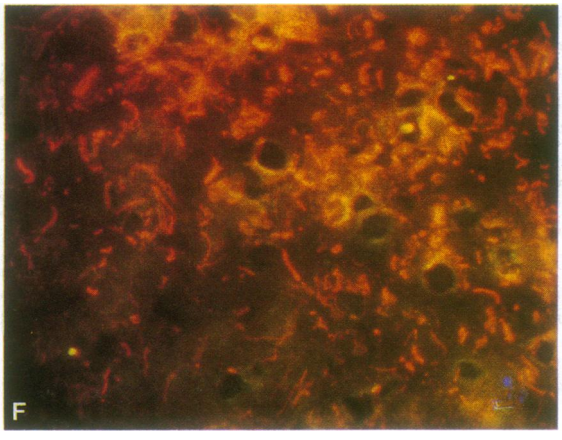
incubated with the antibody at 37°C for 30 min. The cells were washed with prewarmed medium and fixed for 10 min in methanol at −20°C. After fixation, the cells were incubated with Texas Red-conjugated anti-mouse IgG for 1 hr at room temperature. The cells were then washed in PBS, mounted, viewed in an epifluorescence microscope with excitation at 560 nm (Texas Red) and in the UV (bisbenzimide), and photographed. Immunoperoxidase staining with PDGFR-3 on cells grown on chamber slides was performed as described earlier.







Receptor Binding Studies. Cells were cultured for ≈10 days in 12-well plates and treated with araC as described above. The binding assay was as described (19).

Determination of c-Fos Induction. Neuron-enriched cell cultures were rendered quiescent by serum starvation for 48 hr and treated for 0.5, 1, 2, 4, and 24 hr at 37°C with PDGF-BB (10 ng/ml) (22). Cells were then washed in PBS, fixed in methanol at −20°C for 10 min, and incubated overnight at 4°C with a polyclonal sheep antibody against a synthetic peptide of the c-Fos protein (Cambridge Research Biochemicals, Harston, U.K.). Biotinylated anti-sheep immunoglobulin (Amersham) and Vectastain Elite complex were used to visualize the reaction. Controls included unstimulated cells, as well as both stimulated and unstimulated cells incubated with normal sheep serum instead of primary antiserum.

Primary Cultures of Newborn Rat Brain Cells with PDGF. Primary cultures were obtained from two or three newborn rat cerebelli for each experiment as described above. They were grown on chamber slides, originally with equal numbers of cells per slide, in DMEM with 10% fetal bovine serum for 2 days. Culture medium was then replaced with serum-free medium. On the following day (day 0), the medium was replaced with serum-free medium with or without PDGF-BB (10 ng/ml). This treatment was repeated every other day. Cells were fixed with prewarmed acetone at day 0 (before addition of PDGF-BB) and on days 1, 3, 4, and 5 and then incubated with a mouse anti-NF antibody and a biotinylated horse anti-mouse IgG. Bound antibody was detected with FITC-avidin as described above. NF-positive cells and neurite-bearing NF-positive cells were counted.

RESULTS

Rat Brain Neurons Possess PDGF β Receptors. Immunohistochemical staining of rat brain sections with PDGFR-3, an antiserum specifically recognizing the β receptor (18), revealed a large number of intensely stained cells in very young brains (up to 1 week). Comparison of the immunoreactivity of neuronal cells (in structures such as cortex, hippocampus, and basal ganglia) in brain sections of rats of different ages revealed a gradual decrease of both the number of positively stained cells and the intensity of the staining in sections of older rat brains. In all rats at different ages, however, PDGFR-3 stained two characteristic cerebellar cell types: the Purkinje cells and cells in the granular cell layer (Fig. 1*C*). The staining with PDGFR-3 was specific, as the

FIG. 1. Immunohistochemical staining and double immunofluorescence staining of brain sections. Affinity-purified PDGF β-receptor antiserum produced distinct staining of neuronal cells in the pyramidal cell layer of hippocampus of a newborn rat (*A*) and of Purkinje cells and granular cells of the cerebellum of a 6-week-old rat (*C*). Immunoreaction was greatly reduced when the antibody was incubated with the peptide before analysis (*B* and *D*). The nuclei of the trigeminal nerve were stained with PDGF β-receptor antiserum (PDGFR-3) visualized by FITC (green) and with anti-NF antibody visualized by Texas Red (red); the two antigens colocalize and are seen as a yellow staining (*E*). For comparison, cells in the brainstem were stained with PDGFR-3 (green) and with anti-GFAP antibody (red), showing different antigen localizations (*F*). (*A–D*, ×380; *E* and *F*, ×600.)

immunoreaction was blocked by the synthetic peptide against which the antiserum was raised (Fig. 1*D*).

A detailed histological study on the spatial distribution of the PDGF β-receptor is summarized in Table 1. Several identifiable neuronal structures were stained with PDGFR-3. Intense staining was observed in pyramidal cells of the hippocampus (Fig. 1*A*), in the granular layer of the cerebellum, in basal ganglia, in brainstem nuclei, and in the subventricular zone. In contrast, neurons in the cerebral cortex were stained moderately and neurons in the thalamic region only weakly. Capillary endothelium, perivascular connective tissues, and meninges were also stained, as reported previously (19, 23). Staining of the serial sections with anti-NF, anti-GFAP, anti-muscle actin, and anti-von Willebrand factor was used for histological orientation (data not shown).

Double immunofluorescence staining of newborn rat brain sections with PDGFR-3 and anti-NF antibodies confirmed that these antigens resided in the same cells (Fig. 1*E*). This indicates that neuronal cells possess PDGF β receptors. In contrast, glial cells were stained with anti-GFAP but did not react with PDGFR-3 (Fig. 1*F*).

Cultured Neuronal Cells Have PDGF β Receptors. Cultures of brain cells were established from 1- to 2-day-old rats and treated with araC to enrich the neuronal population. In such cultures 80–90% of the total number of cells, as determined by bisbenzimide staining of nuclei, stained with anti-NF (Fig. 2*A*) and were therefore identified as neuronal. The non-neuronal population consisted of either astrocytes, stained with anti-GFAP, or endothelial cells, recognized by antibodies against von Willebrand factor (data not shown). The PDGFR-3 antiserum stained positively only a part of the untreated cell population but almost all cells that were treated with araC (Fig. 2 *B* and *C*).

The neuron-enriched cultures were used for analysis of the binding of radiolabeled PDGF. Fig. 3 shows the results of one such assay, representative of several assays performed on different neuron-enriched cultures. ^{125}I-PDGF-BB bound to the cells and the binding was blocked by unlabeled PDGF-BB competitor, but not by PDGF-AA. In contrast, ^{125}I-PDGF-AA did not bind at all. The relatively low cell density in the cultures probably accounts for the incomplete blocking of ^{125}I-PDGF-BB binding by unlabeled PDGF-BB, since experiments with equally sparse fibroblast cultures gave similar results (not shown). The binding data indicate that neuron-enriched cultures contain PDGF β receptors but no α receptors, since the α receptors bind all isoforms of PDGF, whereas the β receptors bind only PDGF-BB with high affinity (1).

PDGF β Receptors on Rat Brain Neurons Are Functional. The neuron-enriched cultures stimulated with PDGF-BB

Table 1. Distribution of PDGF β-receptor immunoreactivity on neuronal cells in newborn rat brain

Cerebrum	
Cortex (general)	+
Piriform cortex	++
Insular cortex	++
Hippocampus	
CA1–4	+
Dentate gyrus	++
Neuroepithelium	+++
Anterior olfactory nucleus	++
Caudate putamen	+
Accumbens nucleus	+
Lateral septal nucleus	+
Median septal nucleus	+
Diencephalon	
Thalamic nuclei	+
Hypothalamic nuclei	+
Neuroepithelium	+++
Mesencephalon	+
Brainstem	
Vestibular nuclei	+
Dorsal cochlear nucleus	+
Ventral cochlear nucleus	+
Spinal trigeminal nucleus	+++
Cerebellum	
External granule cells	+++
Granule cells	++

+++, All neurons expressed strong immunoreactivity; ++, most neurons expressed moderate immunoreactivity; +, most neurons expressed weak immunoreactivity.

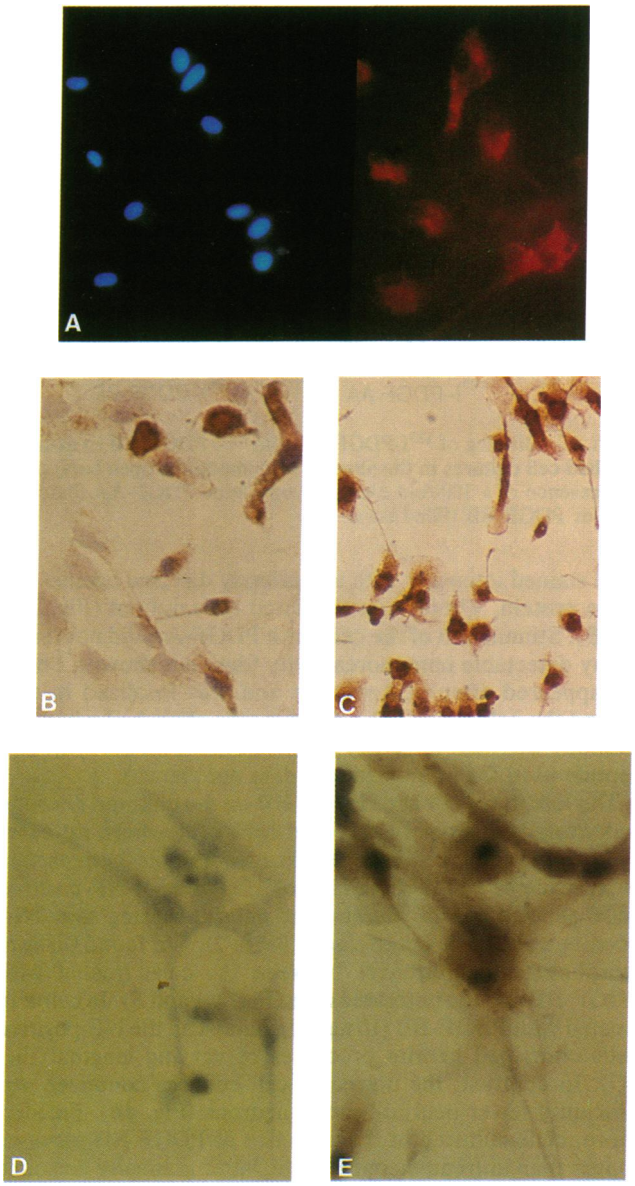


FIG. 2. Phenotypic characterization of araC-treated cultured rat brain cells and immunohistochemical examination of the c-Fos protein in neuron-enriched cultures. About 80–90% of the total araC-treated cells stained positively with the anti-NF antibody visualized by Texas Red (*A Right*). The number of neuronal cells was determined by counterstaining the nuclei with bisbenzimide (*A Left*). Immunoperoxidase staining with PDGFR-3 showed that only some cells of the untreated culture (*B*), but almost all cells of the culture treated with araC (*C*), stained positively. c-Fos induction was examined in cells fixed before (*D*) or after (*E*) treatment with PDGF-BB for 30 min, by staining with a sheep antiserum raised against a synthetic c-Fos peptide. (*A–C*, ×400; *D–E*, ×600.)

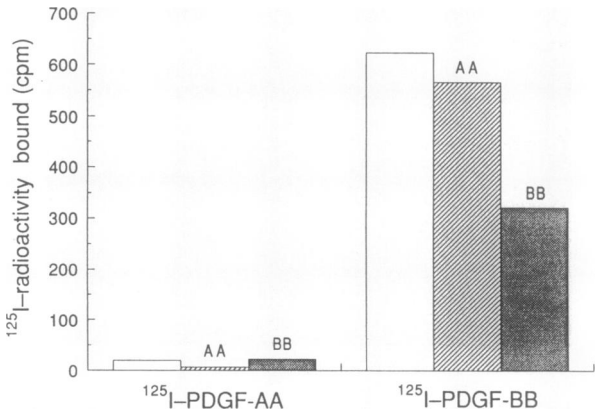


FIG. 3. Binding of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB to neuron-enriched cell cultures in the absence of competitor (open bars) or in the presence of a 100-fold excess of unlabeled PDGF-AA (hatched bars) or PDGF-BB (filled bars).

were stained intensely with an antibody directed against the c-Fos protein, whereas unstimulated cells did not (Fig. 2 *D* and *E*). Stimulation of the cells with PDGF-AA did not result in any detectable immunoreactivity (data not shown). Staining appeared after ≈30 minutes and was localized in and around the cell nuclei. The staining was abolished when the antibody was neutralized by preincubation with the synthetic peptide used for immunization (data not shown).

The long-term effect of PDGF-BB on the cultured neurons was examined. Freshly prepared cells of newborn rat cerebellum were cultured in serum-free medium in the presence or absence of PDGF-BB. The total number of NF-positive cells as well as the number of neurite-bearing and NF-positive cells were determined (Table 2). We found distinct morphological differences between the cells treated with PDGF-BB and the untreated cells (Fig. 4 *A* and *B*). In cultures treated with PDGF-BB (10 ng/ml), almost all the NF-positive cells contained neurite processes of varying lengths (Fig. 4*C*). In contrast, the untreated cell cultures contained significantly fewer neurite-bearing neurons (Fig. 4*B*). Furthermore, cells cultured in the presence of PDGF-BB survived longer than untreated controls; the latter gradually detached from the slides and then vanished during the observation period (Table 2).

Table 2. Influence of PDGF-BB on neuronal survival and process outgrowth

Treatment	Cells scored	Percent				
		Day 0	Day 1	Day 3	Day 4	Day 5
PDGF-BB	NF+	100	74.1	57.4	28.1	32.9
	NF+ with neurites	6.0	8.6	26.4	37.6	24.2
No PDGF	NF+	100	91.6	19.1	16.7	13.9
	NF+ with neurites	6.0	10.7	15.6	21.4	15.7

Rat brain cells were isolated and maintained as described in *Materials and Methods*. After 1 day in serum-free medium, cells received medium containing PDGF-BB (10 ng/ml) or no PDGF (day 0). The cells were fixed on day 0 before the addition of PDGF-BB and on days 1, 3, 4 and 5, and then stained with FITC-coupled anti-NF. The number of NF-positive (NF+) cells in each chamber was scored by counting five fields of the same position in each chamber. The total number of NF-positive cells on day 0 in the counted area was 502 and was set as 100%. The number of NF-positive and neurite-bearing cells is expressed as percent of the number of the NF-positive cells in each chamber. Cells with processes clearly longer than their own cell bodies were regarded as neurite-bearing cells. Data presented are from a representative example of six similar experiments performed.

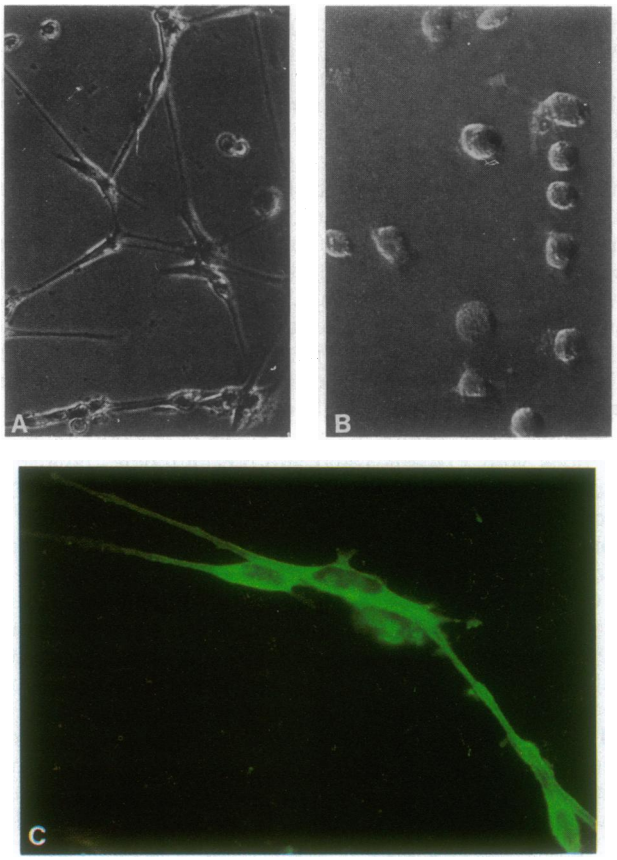


FIG. 4. Phase-contrast and immunofluorescence photomicrographs of primary cultures of rat brain cells. Cells were cultured in the presence of PDGF-BB (10 ng/ml) (*A* and *C*) or without PDGF (*B*) in serum-free medium for 5 days. Most of the cells treated with PDGF-BB contained long neurite processes (*A*), in contrast to the untreated cells, which began to detach from the slide (*B*). The neuronal nature of the cells was determined by immunofluorescence with anti-NF (*C*). (*A* and *B*, ×480; *C*, ×600.)

DISCUSSION

Our data show that neuronal cells from the central nervous system of postnatal rats possess functional PDGF β receptors and that PDGF has neurotrophic functions on these cells. The first neurotrophic factor to be identified, and still the best-characterized one, is nerve growth factor (NGF) (24). During the last decade, insulin, epidermal growth factor, and basic fibroblast growth factor were found to be active on cultured neurons (24, 25), and two neurotrophic factors with structural similarity to NGF were recently identified (26, 27). PDGF B-chain immunoreactivity has been demonstrated in several neuronal cells, and a marker gene placed under the transcriptional control of the B-chain gene promoter was expressed in neurons of transgenic mice (28). Also, the PDGF A chain was shown to be present in neuronal cells of embryonic and adult mice (29). However, those studies did not demonstrate a functional effect of PDGF on neuronal cells.

The majority of the neurons in the newborn rat brain are postmitotic and undergo differentiation processes. Known exceptions include the granule cells of the dentate gyrus in hippocampus and cerebellum, which proliferate in the immediate postnatal period (30). We found that many neurons in the young rat brain (1–7 days old) stained with the PDGF β-receptor antiserum, as did postmitotic cells such as Purkinje cells of the adult cerebellum. However, the most intense staining was observed in immature cells such as external granule cells in cerebellum and cells in the subventricular zone of the newborn rat brain. Since the receptor

levels decreased in older rats, our findings suggest that immature neurons, in particular, respond to PDGF-BB. It is possible that PDGF is a mitogen for the dividing neuroblasts and that PDGF receptors gradually decrease in the postmitotic cells; receptors on such cells do not necessarily have any function after the last cell division. Such a situation prevails in the oligodendrocyte lineage; the O-2A progenitor cells divide in response to PDGF-AA but expression of PDGF α receptors persists for some time in the postmitotic oligodendrocytes (17).

That PDGF-BB induced the c-Fos protein and caused a morphological change in cultured neurons, however, favors a functional role of PDGF in such cells. c-Fos is induced in neuronal cells by various stimuli and is thought to play a role in the regulation of the transcription of certain genes encoding factors or neurotransmitters required for neuronal functions (31, 32). Furthermore, we observed outgrowth of neurite processes in the neuronal cultures after treatment with PDGF-BB.

These findings support the possibility that PDGF is required for the differentiation of immature neurons into different neuronal subpopulations. Thus, PDGF-BB might be required for the enlargement of the cell soma as well as neurite outgrowth of the young neuron after it has completed its last mitotic division. NGF has such a neurotrophic function on postmitotic sensory and sympathetic neurons in culture (33).

In conclusion, our data imply that PDGF may function as a neurotrophic factor. The fact that PDGF α receptors are expressed in oligodendrocyte progenitor cells whereas PDGF β receptors are expressed on neurons suggests that the different isoforms of PDGF may regulate growth and differentiation of different cell types in the developing central nervous system by paracrine and autocrine routes.

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