Differential Induction of Pax Genes by NGF and BDNF in Cerebellar Primary Cultures

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Abstract. The Pax genes encode sequence-specific DNA binding transcription factors that are expressed in embryonic development of the nervous system. Primary neuronal cell cultures derived from the cerebellar cortex of embryonic day 14, newborn and 7-d old mice, were used to investigate the cell-type specific expression patterns of three members of the murine paired box containing gene family (Pax gene family), in vitro. Cell types which express Pax-2, Pax-3, and Pax-6 RNA in primary cultures correspond to those found in regions of the cerebellum which show RNA signals in sections of the developing mouse brain. To find mechanisms regulating Pax gene expression during cerebellar development, the differential regulation of Pax-2, Pax-3, and Pax-6 by NGF and BDNF, two structurally related neurotrophins, was studied in such primary cultures. Pax-2 and Pax-6 RNA increased slightly by 1 h and remained elevated throughout a 24-h treatment with BDNF and NGF. Pax-3 RNA was not detected in newborn cultures, but underwent a rapid (1 h) and transient (2 h) induction upon treatment with either BDNF or NGF. No response was seen with EGF or FGF. Cycloheximide treatment amplified Pax-3 induction and prolonged the signal. Thus, Pax-3 induction resembles that of the immediate-early gene c-fos, which transduces growth factor signals during the development of particular neuronal/glial cell types. The changes in Pax expression were inductive rather than trophic.

THE murine Pax proteins are sequence specific DNA-binding proteins which contain paired and homeo domains. The nuclear localization of, and transactivation by, these proteins is also consistent with a function as transcriptional modulators regulating developmental processes by altering target gene expression (Chalepakis et al., 1991; Goulding et al., 1991; Dressler and Douglas, 1992; Walther et al., 1991; Stapleton et al., 1993; Adams et al., 1992; Treisman et al., 1991). All Pax genes described to date exhibit a temporally and spatially restricted expression pattern during embryogenesis. Expression is detected along the entire anteroposterior axis of the neural tube and the hindbrain. Generally, Pax genes containing a homeobox are expressed earlier and in mitotically active tissues, whereas those lacking a homeobox are expressed later, in differentiating tissue. Pax-2, which lacks a homeobox, is first expressed in the neural tube at embryonic day 10 (E10), when neuronal differentiation begins (Dressler et al., 1990). In contrast, Pax-3 and Pax-6, which contain homeodomains, are expressed in the neural tube at embryonic day 8.5 (E8.5), and their expression patterns are complementary with respect to the dorsal-ventral axis (Walther and Gruss, 1991; Goulding et al., 1991). Notochord transplantation studies in chicken are consistent with the hypothesis that Pax-3 and Pax-6 genes are involved in dorsoventral polarization of the spinal cord, via inductive signals from the underlying notochord (Goulding et al., 1993).

The cerebellum provides a model for studies on mammalian CNS development because of its relatively simple structure, the small number of cell types and the well established pattern of histogenesis. Also, it is a source of primary cells because neurogenesis in this region is postnatal, allowing easy access to large numbers of cells in the early stages of development (Ramon y Cajal, 1929; Miale and Sidman, 1961). Although cerebellar cells are proliferative in the mouse at this stage, primary cultures prepared from this stage have less than 2% proliferative cells.

In situ analysis of embryonic cerebellar cortex showed cell-type specific expression of Pax RNAs in neurons and glia. Pax-3 is expressed only in the ventricular germinal zone of the developing cerebellum of the midgestation embryo. This zone differentiates into the Granular, Purkinje, and Molecular layers of the adult cerebellar mantle layer. Only Granular and Purkinje layers of the adult cerebellar cortex express Pax-3. Pax-6 is present in both ventricular and external Granular layers of the embryonic cerebellum, but only in the Granular layer of the adult cerebellum. Pax-2 appears in Golgi neurons, scattered through the Granular layer (Stoykova and Gruss, 1993).

Vertebrate neuronal terminal differentiation is triggered by

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cell migration, exposure to local growth factors, cell contact, and/or synaptogenesis (Jacobson, 1991). Effector molecules mediate changes in gene expression that are thought to be important for cellular differentiation. In embryos, cellular differentiation of innervating neuronal precursors is influenced by target cells, which produce limiting amounts of effector molecules required for growth, survival, and differentiation of neurons. Postnatal nervous system development also involves myelination and dendritic elaboration (Gould et al., 1989; Schafer and Friede, 1988).

Two effector molecules, NGF and brain derived neurotrophic factor (BDNF)[1], are known to affect cellular differentiation in the nervous system. In addition they aid neuronal survival and are produced in limited quantities by the target cells of neuroblasts (Barde, 1988; Levi-Montalcini, 1987; Knusel et al., 1991; Hoffer and Barde, 1988). NGF regulates neurite outgrowth, neurotransmitter production, rapid gene regulation, and survival of neuronal populations in the peripheral and central nervous system (Levi-Montalcini, 1987; Black et al., 1990; Hempestead et al., 1992). BDNF has extensive structural homologies with NGF (Thoenen et al., 1987), unique and overlapping temporal and spacial expression patterns, and functions similarly (Barde et al., 1982). Both factors bind transmembrane receptors (Ullrich and Schlessinger, 1990) and activate a kinase cascade that links protein-tyrosine to serine/threonine kinases which in turn lead to activation of transcription factors localized in the nucleus (Schlessinger and Ullrich, 1992; Chao, 1992). These receptors are expressed almost exclusively in neurons, in a developmentally regulated pattern suggesting a role in neuronal development. In cerebellum, trk transcripts have been observed in the Granular layer (Klein et al., 1989, 1990, 1991).

To gain an understanding of molecular events leading to and deriving from cell-type specific expression of Pax-genes we sought to recapitulate their cell-type specific expression in tissue culture. A primary cell culture system from cerebellum reproduced faithfully the cell-type specific expression of Pax genes. Subsequently, we tested the effect of various known growth factors on Pax gene expression. Immunofluorescence studies showed that neurotrophic factors can alter Pax expression in primary cultures. Rapid induction of Pax-3 was caused by either NGF or BDNF, but not by EGF or FGF. Alterations of Pax gene expression by neurotrophins is likely to involve a molecular signaling cascade. Primary cerebellar culture will provide an experimental system to examine the molecular cascade between NGF and BDNF, and Pax genes.

Materials and Methods

Culture Conditions

Primary cerebellar cultures were prepared by modification of the method described by Schnitzer and Schachner (1981). Cerebellum from embryonic day 14, newborn and 7-d old NMRI mice was treated with 0.1% trypsin (GIBCO BRL, Gaithersburg, MD) in Ca^{2+}/Mg^{2+} free HBSS (Sigma Chem. Co., St. Louis, MO) containing 6% BSA and 3.8% $MgSO_4$ for 30 min at 37°C. Cells were washed with culture medium containing 10% FCS, dissociated by passage through a fine mesh and rinsed with culture medium. Cells were plated at density of 25×10^4 cells/cm^2 on 10-mm diam poly-L-lysine (Sigma Chem. Co.) and laminin-(Sigma Chem. Co.) coated glass coverslips in 48-well (25 μl/well) culture dishes in serum free (Costar Corp., Cambridge, MA), defined medium composed of a 1:1 mixture of DMEM and Ham F-12 nutrient, including Hepes buffer (15 mM), insulin (5 μg/ml), transferin (100 μg/ml), progesterone (20 nM), putrescine (10 μM), sodium selenite (30 nM), L-thyroxine (400 ng/ml), 3',3',5'triiodo-L-thyronine (10 ng/ml), dexamethasone (400 ng/ml), and antibiotics. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 37°C. Three h after plating, cells were treated with NGF (7s-NGF, Sigma) or BDNF, (a kind gift from Dr. Y. A. Barde, Max Planck Institute for Psychiatry, Planegg-Martinsried, Germany) at a final concentration 50 ng/ml for 15, 30, 60 min, 3 h and 24 h. Cycloheximide (CHX) (10 μg/ml Sigma) treatments were for 30 min before the addition of growth factors.

BrdU labeling was performed by the method of Gratzner (1982) using the Amersham cell proliferation kit.

Antibodies

Rabbit polyclonal antibodies against neurofilament protein-200 (Sigma Chem. Co.), a neuronal marker, and to glial fibrillary acidic protein (GFAP), glial marker, (Dakopatts, Denmark) and monoclonal antibody against calbindin D-28, Purkinje neurons marker (Jande et al., 1977) (Sigma Chem. Co.), were used at 1:200 dilution. Rabbit polyclonal antibody against trkB was a kind gift from Luis F. Parada, Frederick Cancer Research and Development Center, Frederick, MD. Detection antibodies such as 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FITC) conjugated to anti-digoxigenin sheep F(ab)' fragments (Boehringer Mannheim Corp., Indianapolis, IN), TRITC-conjugated to goat antibody against rabbit immunoglobulin G (Sigma Chem. Co.) were used at 1:100 dilution. Cy3 conjugated to goat anti-mouse IgG ($F(ab)_2$ fragments) (Dianova) was used at 1:500 dilution.

In Situ Hydridization–Immunocytochemistry

Cells grown on coverslips were fixed for 20 min with 4% paraformaldehyde, 4% sucrose in PBS. Coverslips were rinsed in PBS, transferred to 70% ethanol and stored at −20°C until use. Cells were prehybridized (200 μl per coverslip) with 5 × SSC, 5 × Denhardt's, 50% deionized formamide, 250 μg/ml yeast tRNA, 250 μg/ml denaturated salmon sperm DNA and 4 mM EDTA, for 3 h in a humid 45°C chamber, and washed at room temperature for 2 min in 70, 90, and 100% ethanol. Digoxigenin-labeled RNA probes were synthesized, using T7 or T3-polymerase according to the suppliers directions (Boehringer Mannheim Corp.) from linearized templates as described for: Pax-2 (Dressler et al., 1990), Pax-3 (Goulding et al., 1991), and Pax-6 (Walther and Gruss, 1991). Cells were incubated as above with probes (200 ng) in prehydridization buffer lacking salmon sperm DNA (2.5 ng/μl overnight at 45°C), and washed two times for 15 min with 2 × SSC, 15 min with 0.2 × SSC and two times for 15 min with 0.1 × SSC at 45°C. For double-labeling fluorescence, cells were incubated with primary antibodies for 2 h at room temperature, rinsed, and both secondary antibodies applied for 2 h (Kioussi et al., 1992). Controls were: (*a*) using sense probe; (*b*) omitting the probe, first antibody, second antibody, or all of the above. Immunofluorescence stained preparations were viewed with a Zeiss Axiophot photomicroscope. Unless otherwise stated, photographic prints referring to comparative immunostaining were prepared under identical conditions.

RNA Isolation

Total RNA was isolated from cultured cerebellar cells of newborn mice by the method of Auffray and Rougeon (1980). Briefly, 10^8 cells were cultured 3 h in vitro, harvested and homogenized in 3 M LiCl with 6 M urea and 0.1% SDS, and stored in the cold room at least overnight. Contaminants were removed by centrifugation for 30 min at 10,000 rpm and two resuspensions in 3 M LiCl. Pellet was solubilized in 10 mM Tris-HCl pH 8.0, 0.5% SDS. RNA was isolated by phenol-chloroform extraction and ethanol precipitation, and RNA was checked on agarose gels using ethidium bromide staining.

PCR-Southern Blot

Total RNA was reverse transcribed into cDNA (Pharmacia, first-strand cDNA Synthesis Kit), and amplified using Taq-DNA polymerase (Promega Corp., Madison, WI) with 35 cycles of denaturation (95°C, 3 min), primer annealing (60°C, 1.5 min) and extension (72°C, 2 min). Primers were: Pax-2, upstream

1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; CHX, cycloheximide; CNS, central nervous system; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GFAP, glial fibrillary acidic protein; NF, neurofilament protein.

5′-AGGATCATCCGGACCAAAGTT-3,′ downstream 5′-TTCCTCGGC-TCCAACGGTGAGA-3′; Pax-3, upstream 5′-AAGGCTAAACACAGCAT-CG-3′, downstream 5′-TGGTTTAGCAACCGCCGTGC AAGA-3′; Pax-6, upstream 5′-GAGTTCTTCGCAACCTGG-3′, downstream 5′-GGACTTCAGT-CACCAGGGCAAC-3′; GAPDH, upstream 5′-GGCCGTATTGGGC GCC-TGGTC-3′, downstream 5′-GAAGGGCAACTACTGTTCGAAG-3′. PCR products (190 bp for Pax-2, 230 bp for Pax-3, 142 bp for Pax-6 and 190 bp for GAPDH) were run on 2% agarose gels, blotted onto positively charged nylon membranes (Boehringer Mannheim Corp.), and hybridized with mouse Pax-2 (Dressler et al., 1990), Pax-3 (Goulding et al., 1991), Pax-6 (Walther and Gruss, 1991) and GAPDH (Fort et al., 1985) probes, respectively.

Results

Cell-Type Specific Expression of Pax RNA

A primary cell culture system that preserves and elaborates some of the differentiated cell types found in vivo, was used. Cell cultures were prepared from the cerebellar cortex of a E14, P0, and P7 mice. Serum free conditions were used to select for neuronal cells. Various differentiated cell types were morphologically observed in the cultures after 3 h. Immunofluorescence staining of cultures with cell-type specific markers demonstrated the selection of neuronal cell types. Staining with antibodies specific for neurofilament (NF) and GFAP demonstrated that these cultures consisted of 95% neurons (Granule, Golgi, and Purkinje cells). Stellate and Basket cells could not be detected by morphological criteria in these cultures. Less than 5% of cells were astroglia, either astrocytes or Bergman glial cells. Small, round, 6–8 μm diam (Hatten, 1985) NF positive cells were identified as granule neurons and accounted for most of the cultures (Figs. 1, *A* and *C*, *G* and *I*, 2, *A* and *C*). Large NF positive cell bodies, 8–10 μm diam (Hatten, 1985), and calbindin negative, were identified as Golgi neurons. Bergmann glia cells were GFAP positive cells with large cell bodies, 10–12 μm diam (Hatten, 1985) and well differentiated processes (Fig. 2 *J*), smaller GFAP positive cells were astrocytes. The cell-type specific expression of Pax-2, Pax-3, and Pax-6 RNA was studied by in situ hybridization of these primary cultures (summarized in Fig. 3). RNA probes made with digoxigenin-modified UTP were visualized by a secondary antibody conjugated to fluorescein.

The Pax-2 probe gave a strong signal in the cell bodies of the majority of the neuronal population, which consisted of granule (Fig. 1, *A–C*) and Golgi neurons. No signal was seen in Purkinje neurons. Of the glial cells in these cultures, only astrocytes were positive for Pax-2 (Fig. 1, *D–F, arrow* in *E*). No signal was observed with the sense probe (data not shown).

Pax-6 was expressed in NF positive, granule neurons (in low levels) (Fig. 1, *G–I*), and in astrocytes (Fig. 1, *J–L*). No signal was seen in Purkinje and Golgi neurons. Sense or non-related probes gave no staining (data not shown).

The Pax-3 probe stained the cell bodies of granule neurons (Fig. 2, *A–C*) and the calbindin positive Purkinje cells (Fig. 2, *D–F*). A strong signal was also observed in astrocytes (Fig. 2, *G–I*) and in Bergman glia (Fig. 2, *J–L*).

In glial cells, all three Pax genes were expressed in astrocytes but only Pax-3 was seen in Bergman glia (Fig. 3). In astrocytes expression was seen in P7, in P0, and in E14 cerebellar primary cultures. Pax-2 and Pax-6 maintain their cell type specific expression in E14, P0 and P7-derived cultures, and show no temporal regulation during culturing. In contrast, Pax-3 is absent in short term P0 cultures while it is present in short term E14 and P7 cultures. In addition, Pax-3 in E14 and P7 cultures shuts down after 3 d of culturing and only returns after 2 more wk of culturing. The temporal changes of Pax-3 expression may be related to the transient reduction of Pax-3 signal in sections (Goulding et al., 1991).

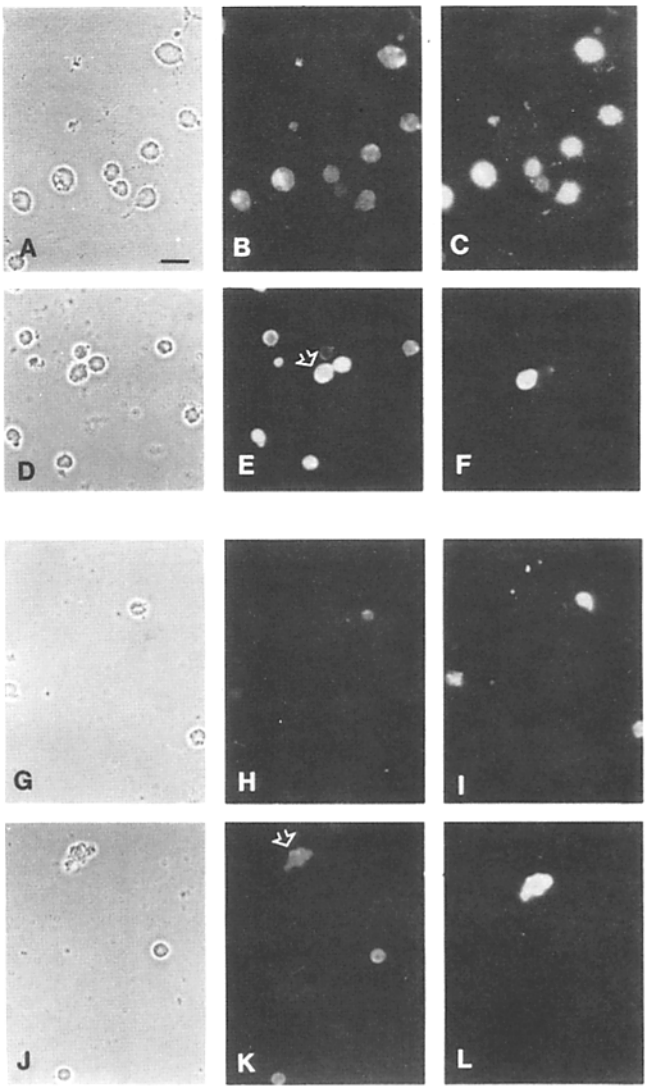

Figure 1. Pax RNA (*central panels*) and cell type marker detection (*right panels*) in cultured cells from P7 mouse cerebellum, 3 h after plating. Cells were hybridized with digoxigenin-labeled antisense probe for Pax-2 and Pax-6 and visualized with the second antibody FITC. (*A, D, G,* and *J*) Show phase contrast micrographs; (*B* and *E, H* and *K*) fluorescein optics to visualize Pax-2 and Pax-6, respectively; (*C* and *I, F* and *L*) rhodamine optics to visualize NF and GFAP, respectively. The arrows in *E* and *K* indicate the Pax-2 and Pax-6 positive astrocytes, respectively. Bar, 10 μm.

Neuronal cell types also showed differential Pax expression with respect to cell type. Granule neurons showed expression of all three Pax genes. In contrast, Golgi neurons expressed only Pax-2 (Fig. 3) and Purkinje neurons expressed only Pax-3 (Figs. 2, *D–F*, 3). In E14 cerebellum, the immature Purkinje neurons are the first neurons to appear (Miale and Sidman, 1961) and may have a role in the generation of effector molecules (Feddersen et al., 1992).

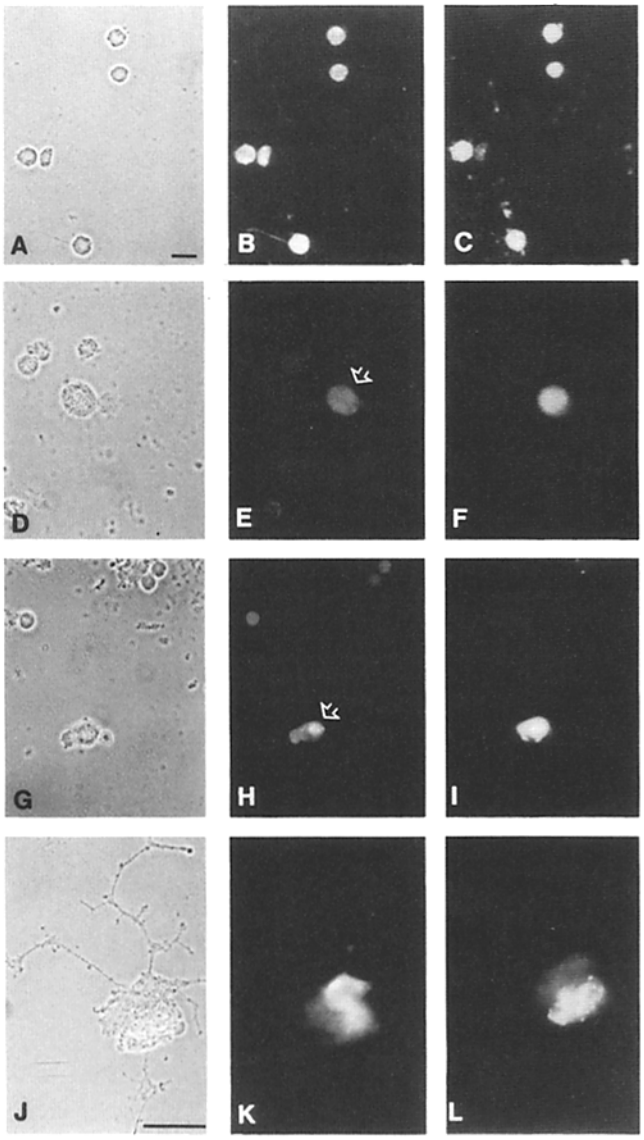

Figure 2. Pax-3 RNA (*central panels*) and NF, calbindin and GFAP proteins (*right panels*) in cultured cells from P7 mouse cerebellum, 3 h after plating. (*A*, *D*, *G*, and *J*) Show phase contrast micrographs; (*B*, *E*, *H*, and *K*) fluorescein optics to visualize Pax-3; (*C*, *F*, *I*, and *L*) rhodamine optics to visualize NF (*C*), calbindin (*F*), and GFAP (*I* and *L*). The arrow in *D* indicates the Pax-3 positive Purkinje neuron which is surrounded by smaller cells. The arrow in *H* indicate the Pax-3 positive astrocyte. The large GFAP positive cell body in L indicates a Bergmann glia which is Pax-3 positive. Bar, 10 μm for *A–I* and Bar, 10 μm for *J–L*.

Pax RNA Is Regulated by Growth Factors

The primary tissue cultures described above were used to test if and which known effector molecules can modulate Pax gene expression, and to observe cell-type specificities of each type of regulation. After 3 h in vitro, in serum free medium, 95% of the cells in cerebellar cultures (P0) are neurons. At this time stimulation with 50 ng/ml NGF or BDNF induced the neuronal cells to develop neurite processes. Activation of Pax gene activity was assessed by in situ hybridization (Figs. 4, 5, 6) at various times after factor addition.

		Developmental Stage								
		E 14			P0			P7		
	Pax	2	3	6	2	3	6	2	3	6
neurons	Granule	+	+	+	+	-	+	+	+	+
	Golgi	+	-	-	+	-	-	+	-	-
	Purkinje	-	+	-	-	-	-	-	+	-
glia	Astrocytes	+	+	+	+	-	+	+	+	+
	Bergmann	-	+	-	-	-	-	-	+	-

Figure 3. Pax phenotype of cultured cerebellar cells. Granule neurons are among the smallest cells in the brain and are by far the most numerous of cerebellar neurons (outnumbering Purkinje neurons 28 to 1). Purkinje and Golgi neurons have both large bodies, easily detectable in phase contrast microscopy. Basket cells and stellate cells were not detectable in these cultures. Astroglia are also far less numerous than granule neurons, constituting roughly 10–12% of the total cell population dissociated from early postnatal mouse cerebellar tissue (Hatten and Liem, 1981). At P7, only 1% of the cells seen in cultures of dissociated cerebellum are oligodendroglia (Hatten, 1985).

Pax-2 RNA is present in 30% of granule neurons of untreated P0 and P7 cultures. In presence of NGF the percentage of cells expressing Pax-2 tripled within 1 h and remained elevated for at least 24 h (Fig. 4). In presence of BDNF, the percentage of expressing cells decreased to 10% by 1 h and recovered to 50% by 24 h (Fig. 4).

A slight change was observed for Pax-6 RNA levels after treatment with either neurotrophin, although NGF slightly raised levels after 24 h. In untreated cultures only 10% of the total cell population expressed Pax-6. After 24 h treatment with NGF or BDNF, 40% and 30% of cells express Pax-6, respectively (Fig. 4).

Pax-3 expression was not detected in granule and Purkinje neurons in P0 cultures. However, it was rapidly induced by treating P0 primary cells with either NGF or BDNF (Fig. 5). After 30 min, strong Pax-3 signals were observed in 20% or 40% of the cells after NGF or BDNF treatment, respectively. At 1 h, signals reached maxima of 60% and 70% positive cells. Two h later the signals were less than 5%. At 24 h, signals had returned to undetectable levels. This rapid induction is similar to that observed for the NGF mediated induction of c-fos RNA, which returns to its low basal level within 2 h after NGF treatment (Mildbrandt, 1987).

Cycloheximide, in combination with NGF causes superinduction of c-fos RNA, by increasing the c-fos mRNA half-life, presumably by stalling regeneration of a component of the RNA degradation pathways or by decreasing the level of a repressor that inhibits c-fos transcription (Greenberg et al., 1986). CHX in combination with either NGF or BDNF also superinduced Pax-3 RNA after 30 min. The percentage of Pax-3 positive cells at 30 min increased from 60 to 80%. As with c-fos superinduction, the Pax-3 RNA fluorescence remained elevated until 24 h (Fig. 6). Thus, in several respects, Pax-3 behaves like the immediate early gene c-fos. The transient increase in Pax-3 RNA occurred in newborn granule cells and Purkinje cells of P0 primary cultures, both of which have NGF receptors in vitro.

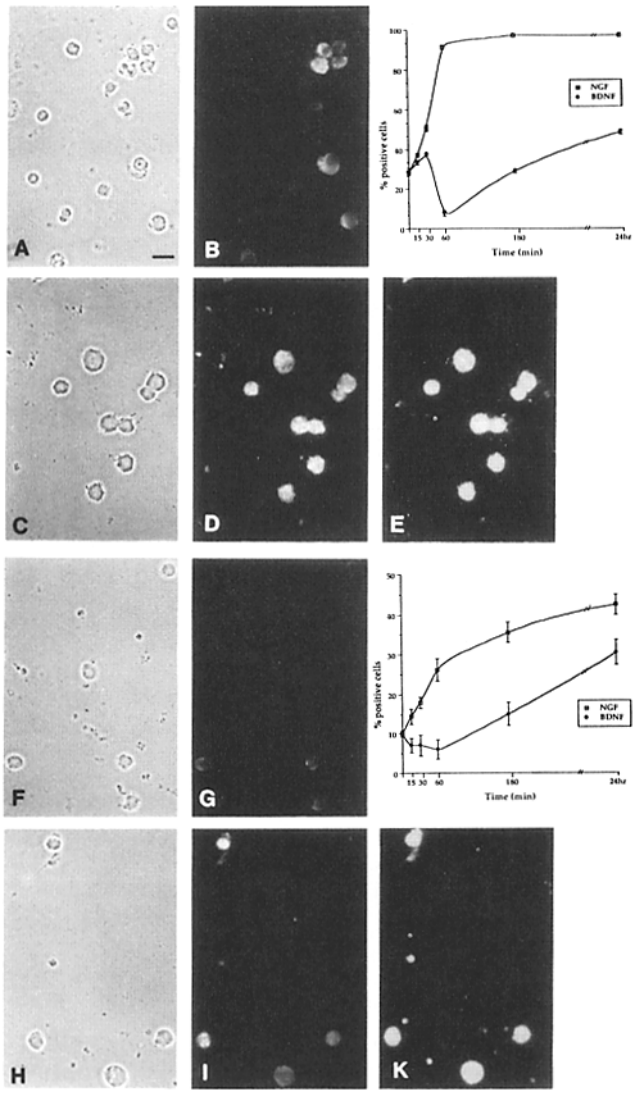

Figure 4. Alterations of Pax-2 (*A–E*) and Pax-6 (*F–K*) RNAs in the presence of NGF. Cultured cells from newborn mouse cerebellum, 3 h after plating and treated with NGF for 0 (*A* and *B*, and *F* and *G*) and 24 h (*C–E* and *H–K*), and hydridized with digoxigenin-labeled antisense probe for Pax-2 (*B* and *D*) and Pax-6 (*G* and *I*). (*A*, *C*, *F*, and *H*) Show phase contrast micrographs; (*B* and *D*) fluorescein optics to visualize Pax-2 and (*G* and *I*) fluorescein optics to visualize Pax-6. (*E* and *K*) Rhodamine optics to visualize NF. Bar, 10 μm. Quantitation of Pax-2 and Pax-6 labeled cells in vitro by counting. Random sets of 100 cells ($n = 4$) were counted in defined optical planes after 15, 30, 60, and 180 min and 24 h after treatment with NGF or BDNF. No change was observed in the % of positive cells in untreated cultures after 15, 30, 60, and 180 min and 24 h.

NGF and BDNF only resulted in changes of levels of Pax-gene expression. No switch in the cellular specificity of Pax-gene expression was observed with either factor.

To determine if the effect of NGF and BDNF was mediated by a trophic or inductive mechanism, immunocytochemical experiments were carried out. The mitotic activity of each cell of a culture is reflected by the amount of bromodeoxyuridine (BrdU) incorporation. In cerebellar primary cultures, 3 h in vitro, only 0.5% of the cells incorporated BrdU. The percentage of proliferative cells increased to 2–4% after 24 h of NGF or BDNF treatment (data not shown). Time points at 30, 60, and 180 min gave values between 0.5 and 2%. The increase in Pax-3 expression occurred within 1 h, and in the presence of CHX, within 30 min. Thus, the increase in Pax-3 RNA cannot be explained by a trophic mechanism.

NGF Receptors in Cerebellar Neurons

Immunocytochemistry was used to determine if NGF receptors are present in neuronal cells of our cerebellar primary cultures. Cerebellar P0 cultures, 3 h in vitro, were treated with a polyclonal antibody against trkB. The Purkinje (Cohen-Cory et al., 1991) and granule neurons (data not shown) were labeled by the trkB antibody, which recognizes trkA, B, and C (Parada, L. F., personal communication).

RNA-PCR Analysis

A second method was employed to measure the changes of Pax expression in response to NGF and BDNF. Due to the low quantity of cells and RNA in such primary cultures it was necessary to use PCR technology. With the help of this method, in situ hybridization data showing that Pax-2, Pax-3, and Pax-6 gene expression is differentially induced by NGF and BDNF could be confirmed (Figs. 4 and 5). RNA was isolated from primary cultures and PCR products generated using Pax-2, 3, and 6 specific primers pairs. Products were hybridized on Southern blots with mouse Pax probes. A strong 190-bp band, representing Pax-2 RNA levels was detected (Fig. 7 *A*). After 1 h of BDNF treatment Pax-2 RNA level decreased (Fig. 7 *B*, lane *2*) and increased again after 24 h (Fig. 7 *B*, lane *3*). No change in Pax-2 RNA was observed with NGF (Fig. 7 *A*). A weak 230-bp band for Pax-3 was detected after 30 min of NGF treatment (Fig. 7 *A*, lane *3*), increased after 1 h (Fig. 7 *A*, lane *4*), and was absent after 24 h. Similar transient induction kinetics of Pax-3 were observed with BDNF treatment (Fig. 7 *B*). Pax-6 showed no obvious change of the signal of the 142-bp band after NGF (Fig. 7 *A*) or BDNF (Fig. 7 *B*) treatment. The slight increase in Pax-6 PCR signal matched the slight increase in Pax-6 RNA fluorescence observed (Fig. 4). In general, the PCR-RNA detection recapitulated the changes of Pax-expression observed with RNA fluorescence detection in situ primary cultures. Thus, the primary culture system provided a convenient means to monitor cell-type specific expression of particular genes. Because they recapitulated the cell-type expression patterns observed by in situ hybridization of embryo sections, primary cultures were useful for determining the impact of various effector molecules on Pax expression in different cell types.

Using such primary cultures, we demonstrated that NGF and BDNF cause a transient increase in Pax-3 RNA which has kinetics similar to those observed for c-fos induction in other systems. This transient induction occurred in Purkinje and granule neurons but was not observed in glial cell types.

Discussion

In this study, the late embryonic and early postnatal expression of Pax genes was investigated at the cellular level. We demonstrate that primary cultures of cerebellar cortex maintain cell-type expression specificities of Pax genes that cor-

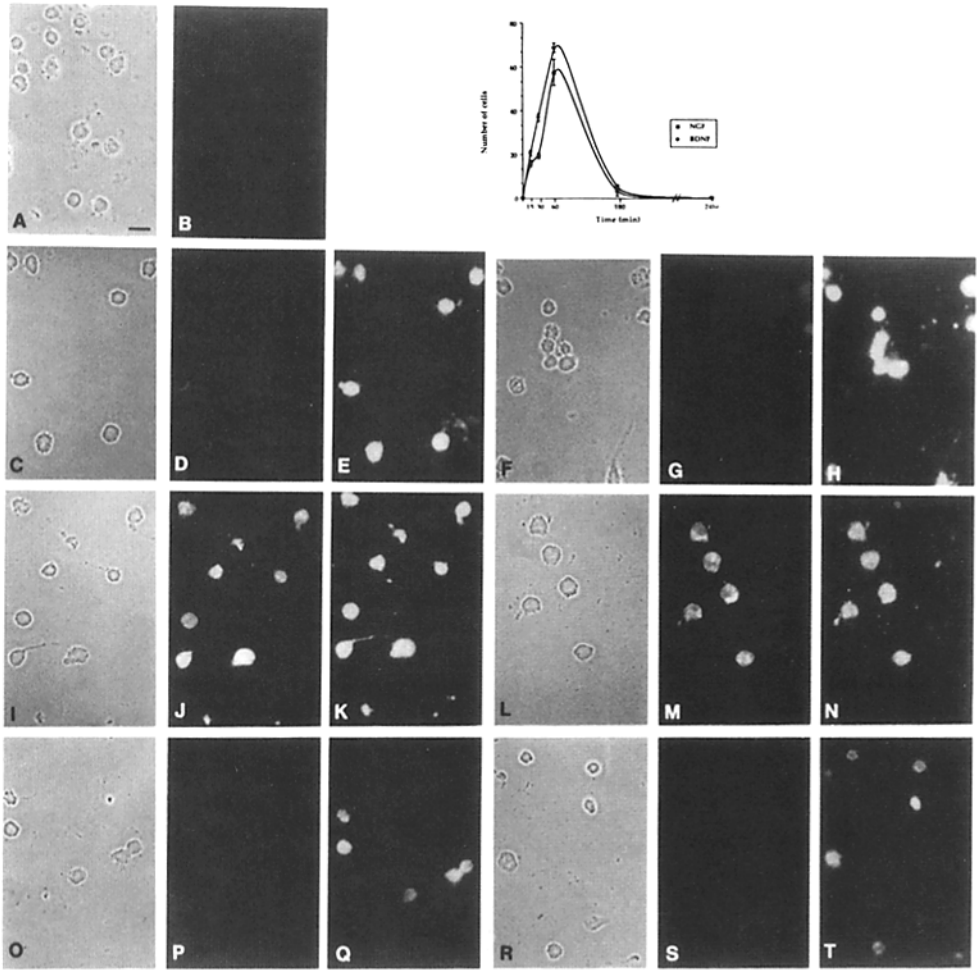

Figure 5. Transient induction of Pax-3 in presence of BDNF and NGF. Cultured cells from newborn mouse cerebellum, 3 h after plating and treated with BDNF (*B*, *D*, *J*, and *P*) or NGF (*B*, *G*, *M*, and *S*) for 0, 30 (*D* and *G*), 60 (*J* and *M*) min and 24 h (*P* and *S*), were hydridized with digoxigenin-labeled antisense probe for Pax-3. Pax-3 is absent for the untreated cells. After 15 min in presence of factors a subpopulation of cells starts to express Pax-3 until 60 min. After 24 h in culture, Pax-3 is low again. (*A*, *C*, *I*, *O*, *F*, *L*, and *R*) Show phase contrast micrographs; (*E*, *K*, and *Q*) rhodamine optics to visualize NF after BDNF; and (*H*, *N*, and *T*) after NGF treatment 30, 60 min and 24 h, respectively. Bar, 10 μm. Quantitation of Pax-3 positive cells by counting. Random sets of 100 cells (n = 4) were counted in defined optical planes after 15, 30, 60, and 180 min and 24 h after treatment with NGF or BDNF. No change was observed in the % of positive cells in untreated cultures after 15, 30, 60, and 180 min and 24 h.

respond with those previously observed by or inferred from in situ analysis of mouse sections.

The neurotrophins NGF and BDNF, altered Pax expression in these primary cultures. Pax-3, in particular, showed a rapid and transient RNA induction upon neurotrophin treatment, which cannot be explained by a trophic mechanism. This strongly suggests that Pax-3 acts along the chain of molecular events by which NGF and BDNF cause neuronal differentiation.

All three Pax genes are expressed by granule neurons, which are the only neurons formed postnatally in the mammalian brain and whose formation is restricted to a relatively short postnatal period (Altman and Bayer, 1987). While Pax-2 and Pax-6 RNA levels change moderately, with NGF and BDNF treatment, Pax-3 RNA levels change dramatically. To initiate the differentiation program in neuronal cell types, NGF and BDNF interact with cell-surface receptors. Granule neurons have low affinity NGF receptors, after 3 h in vitro (data not shown). Pax-3 is also expressed in the Purkinje neurons, which have high affinity NGF receptors (Cohen-Cory et al., 1991). Pax-2, which was expressed in the granular layer of the cerebellum (Stoykova and Gruss, 1993), was also expressed in Golgi neurons, which are scattered throughout this layer.

Pax-3 was the only gene examined which was expressed in Bergmann glia. The cell bodies of Bergmann glia are located in the Purkinje cell layer of the postnatal cerebellum. Their processes guide the anomalous inward migration of granule neurons (Rakic, 1971; Ramon y Cajal, 1929). Two mechanisms, one based on neuron-glia contacts and the other on soluble differentiation factors, are possible for this effect. In P0 cultures, Bergman glia lack Pax-3. However, Pax-3 can be induced by the soluble factors NGF or BDNF. Astrocytes, the second glial population of this system, provide mechanical and metabolic support for neurons by guiding axon positioning of young neurons and by organizing mature neurons into compartments (Ramon y Cajal, 1929; Sidman and Rakic, 1973). They also stimulate neurite outgrowth and transfer proteins to axons. Astrocytes expressed all of the studied Pax genes. Pax may be involved in neuron-glia interactions in such a way that the end result may be influential in astroglial differentiation processes.

NGF and BDNF, both known to affect neuronal survival in vivo, were tested in our primary cultures. Pax genes were differentially induced in the presence of NGF or BDNF but not EGF and FGF. Generally, BDNF and NGF, elucided a similar response for each gene in each cell type. The exception, Pax-2 increased expression with NGF, and decreased expression with BDNF in 0.5–1 h. Thereafter, Pax-2 attained similar expression with both factors by 24 h (Fig. 4). The induction of RNA detected by in situ techniques was also measured by PCR-Southern blot (Figs. 4 and 7). No clear change in the levels of Pax-6 transcripts were observed after treatment with both factors after 24 h (Figs. 4 and 7).

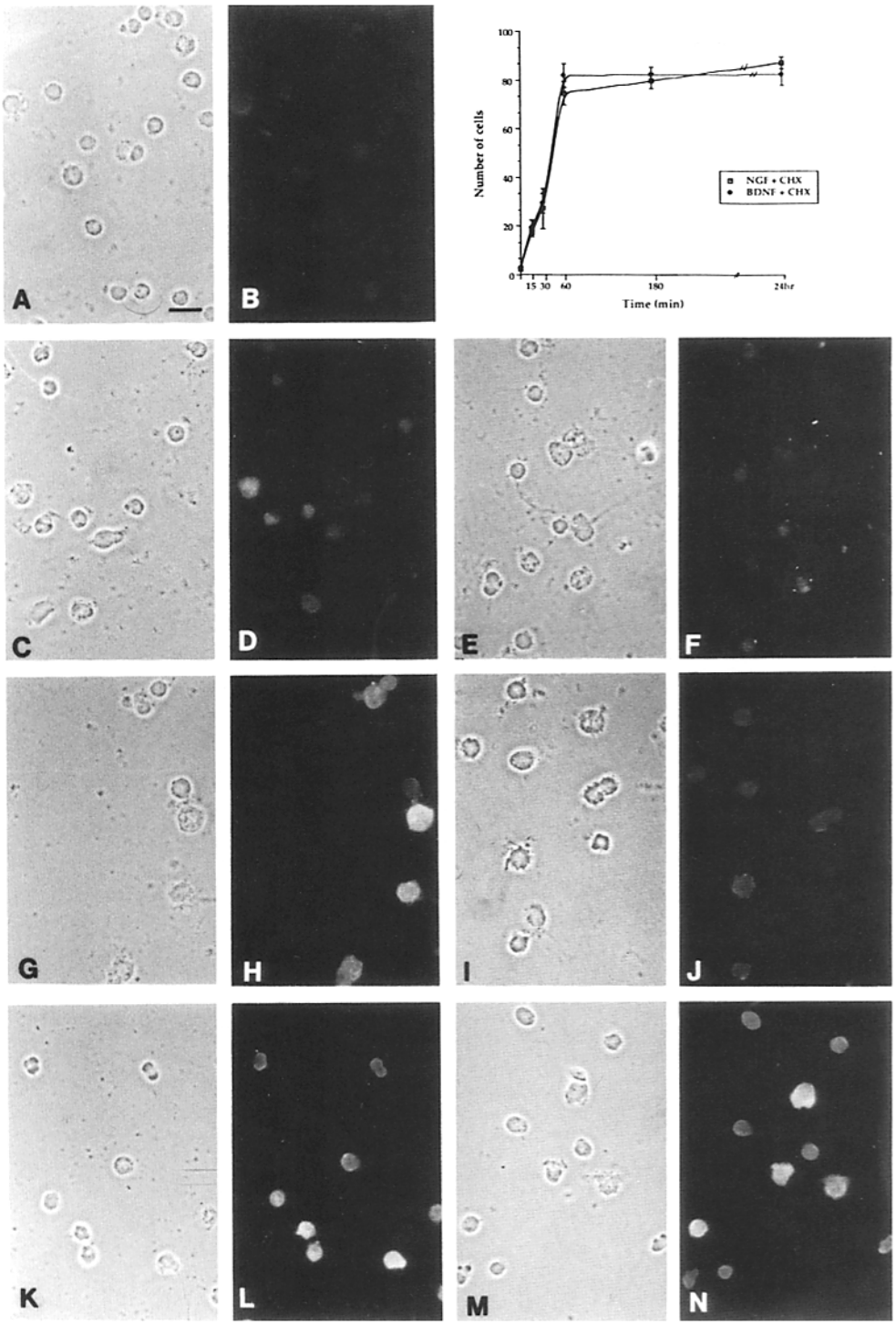

Figure 6. Pax-3 is continuously expressed in presence of BDNF or NGF after treatment with the proteinsynthesis inhibitor CHX. Cultured cells from newborn mouse cerebellum, 3 h after plating, pretreated for 30 min with CHX before the addition of BDNF (*B, D, H,* and *L*) or NGF (*B, F, J,* and *N*) for 0, 30, 60 min and 24 h, respectively, were hybridized with digoxigenin-labeled antisense probe for Pax-3. CHX in combination with NGF or BDNF caused superinduction of Pax-3 RNA after 30 min which remained elevated until 24 h. (*A, C, G, K, E, I,* and *M*) Show phase contrast micrographs; (*B, D, H, L, F, J,* and *N*) Show fluorescein optics to visualize Pax-3. Bar, 10 μm. Quantitation of Pax-3 positive cells by counting. Random sets of 100 cells ($n = 4$) were counted in defined optical planes after 15, 30, 60, and 180 min and 24 h after treatment with NGF or BDNF. No change was observed in the % of positive cells in untreated cultures after 15, 30, 60, and 180 min and 24-h.

In both Purkinje and granule cells of our cerebellar P0 primary cultures, the level of Pax-3 RNA in untreated cells was undetectable, but was rapidly induced by treating the cells with NGF or BDNF (Fig. 5). Also, in primary cultures of dorsal root ganglia (sensory neurons) Pax-3 was induced transiently after 30–60 min of neurotrophin treatment (Kioussi, C., and P. Gruss, unpublished data). This rapid, but transient induction, is very similar to that observed for the NGF-mediated induction of the c-fos RNA. As with the c-fos induction, CHX alters the response of Pax-3 RNA to NGF or BDNF by increasing the RNA levels at the induction peak and by eliminating the subsequent decline in RNA levels.

Interaction of NGF or BDNF with their low affinity receptors stimulates the intrinsic tyrosine kinase activity of trk, thereby initiating a signaling cascade, which involves the phosphorylation of intracellular proteins at tyrosine residues (Kaplan et al., 1991*a*, *b*; Klein et al., 1991) and the transcription of the numerous (immediate early) genes, including c-fos, NGFIA, and NGFIB (Greenberg et al., 1985; Milbrandt, 1987, 1988).

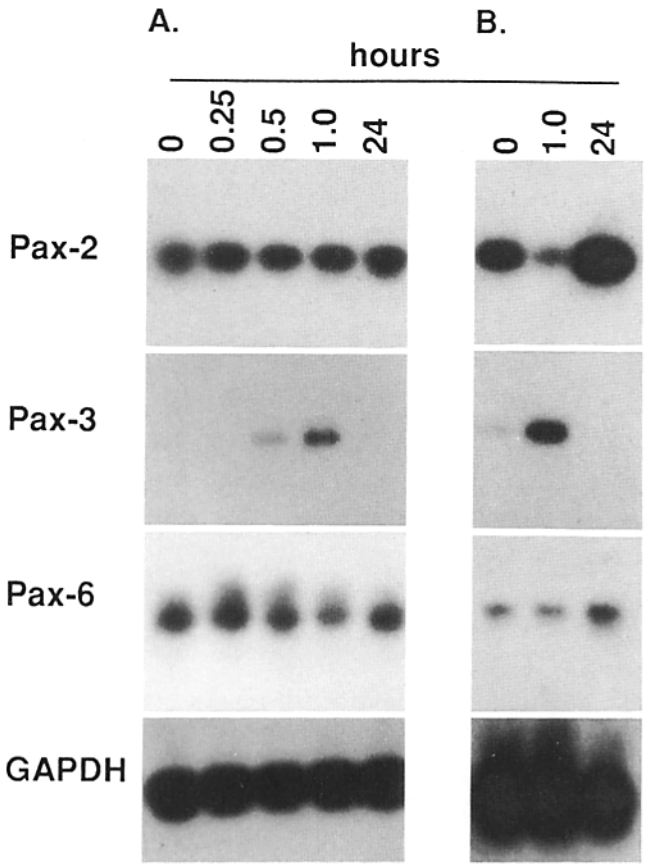

Figure 7. Time course of the NGF and BDNF-mediated induction of Pax-2, Pax-3, and Pax-6 mRNA. Cultured cells from mouse cerebellum were treated with NGF (*A*) for 0, 0.25, 0.5, 1, and 24 h and with BDNF (*B*) for 0, 1, and 24 h. Total RNA was transcribed into cDNA and amplified using Taq-DNA polymerase. The PCR products were probed with Pax-2, Pax-3, and Pax-6 cDNA. The 190-bp Pax-2, 230-bp Pax-3, and 142-bp Pax-6 band were indicated. GAPDH hydridization serves as a control for loaded RNA.

The lack of proliferation in our cultures during the Pax-3 induction interval strongly argues against a trophic mechanism and for an inductive mechanism such as described above. Neuronal cells of our primary cerebellar cultures contain antigens to both trk (low affinity) and p75 (high affinity) NGF receptors. Trk receptor antigen was detected in Purkinje, granule, and sensory neurons in our cultures. Sections of cerebellum reveal trk staining but lack enough resolution to determine whether Purkinje or granule cells (or both) express this receptor in vivo (Cohen-Cory et al., 1991). Similarly it remains unresolved if trk is expressed in vivo in sensory neurons of dorsal root ganglia (Frazier et al., 1973). p75 receptor antigen is expressed at much higher levels in Purkinje cells than in granule cells, both in vivo and in vitro (data not shown). It is also present in sensory neurons in vivo and in vitro. The lowest concentrations of NGF and BDNF which elicit the Pax-3 RNA induction (50 ng/ml) are similar to those used to elicit c-fos induction (100 ng/ml in PC12 cells). c-fos induction can be mediated either by trk or p75 NGF receptors. It remains unclear which of the two types of NGF receptors mediates the induction of Pax-3 RNA. However, a mechanism involving the low affinity receptors is more likely because the p75 antigen (high affinity) is either very low or absent in granule cells. Alternatively, both receptor types could mediate the response. Although other unknown NGF receptors cannot yet be rigorously excluded, it seems likely that neurotrophin binding to the low or either NGF receptor initiates the set of events that results in Pax-3 induction. Thus, Pax-3 could act as an immediate-early gene in the transduction of neurotrophin signals during the development of the mammalian cerebellum.

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