1991

Regulation of Cerebellar Purkinje Cell Development: Interactions Between Trophic Factors and Neurotransmitters

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REGULATION OF CEREBELLAR PURKINJE CELL DEVELOPMENT: INTERACTIONS BETWEEN TROPHIC FACTORS AND NEUROTRANSMITTERS

A thesis submitted to the Faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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January, 1991

The Rockefeller University

New York

ACKNOWLEDGEMENTS

Many people made possible the work presented in this thesis. I am thankful to Dr. Silvia Christakos for providing vitamin D-dependent calcium binding protein antibodies, Dr. Paulette Bernd for the ^{125}I-NGF, and Dr. Moses Chao for the NGF-R antibody used in this study. I also thank Peter Peirce for excellent photography. To work with two fine scientists and dear friends was always enjoyable as well as productive: Enrique Escandón helped me with the cross-linking assays, and the RNAse protection experiments were done in collaboration with Robert Elliott. I am mostly grateful to Dr. Torsten Wiesel for his guidance and support, and to Dr. Ira Black for guiding me while encouraging me to develop my own ideas. I wish to especially thank Dr. Cheryl Dreyfus, not only for her scientific support, but for sharing with me her enthusiasm towards science. My deepest gratitude is to my parents and siblings for their unlimited love, and to Ron, for always being there.

Note to the reader:

The work described here is divided into units and these units presented as chapters. This is done to facilitate the reading of the story and to ease access to specific information. The first chapter states the problem and gives general background on 3 major topics in developmental neurobiology that are concerned with this thesis, namely, Purkinje cell development, the role of neurotransmitters in neuronal development, and Nerve Growth Factor. Chapters 2, 3 and 4 describe the main findings of this work and contain specific discussion of the data. In chapter 5, the general conclusions are presented, and future experimental alternatives are discussed. The materials and methods are presented in chapter 6, as reference to the experimental procedures used in this work.

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ABSTRACT

Previous study indicates that in the cerebellum, Nerve Growth Factor (NGF) and NGF receptors are highly expressed during late embryonic and early postnatal development, suggesting that NGF may play an important role in cerebellar ontogeny. However, while receptor had been localized to some cerebellar populations, delineation of receptor subtypes, and potential physiologic function remained to be defined. The objective of this thesis was to define the role that NGF plays in cerebellar ontogeny.

Initial studies were devoted to the localization of NGF binding sites to cell types in the developing cerebellum, and to the definition of receptor subtypes. ^{125}I-NGF binding was employed to delineate low- and high-affinity receptor sites in the postnatal rat cerebellum. Both high-affinity and low-affinity receptor sites were localized to cell bodies, dendrites and axonal processes of Purkinje cells. The expression of the biologically active, high-affinity receptors was found to correlate with cerebellar development, and suggested that NGF may regulate the normal ontogeny of Purkinje cells.

The development of cerebellar Purkinje cells is subject to regulation by multiple epigenetic signals. To define mechanisms by which trophic and presynaptic stimulation may potentially regulate Purkinje cell ontogeny, I studied the effects of NGF and excitatory transmitters on Purkinje cell survival and morphological maturation in dissociated cell culture. Purkinje cells were identified by expression of Vitamin D-dependent Calcium Binding Protein (CaBP), and by their characteristic morphology. NGF receptors were selectively localized to Purkinje cells both by ligand and monoclonal antibody binding, suggesting responsivity to the trophic agent. Simultaneous exposure to the pharmacologic depolarizing agents, high-potassium or veratridine, and NGF specifically enhanced Purkinje cell survival in culture. Furthermore, NGF together with the excitatory neurotransmitters, aspartate or glutamate, promoted a 2-fold increase in survival. In addition, NGF increased Purkinje cell size and promoted neurite elaboration. These effects required simultaneous exposure to NGF and either aspartate, glutamate or pharmacologic depolarizing agents. Effects on survival or neurite elaboration were not evoked by exposure to trophic factor or transmitters alone. These results suggested a novel mechanism for regulation of development, in which trophic factors and afferent stimulation interact.

To begin to investigate potential mechanisms by which excitatory signals and NGF interact in the regulation of Purkinje cell development I studied regulation of NGF receptor expression by excitatory influences in dissociated cerebellar cell cultures. Sensitive receptor-protein cross-linking, and ribonuclease protection assays were employed to evaluate NGF receptor protein, and message levels respectively. Exposure of neurons in culture to either pharmacologic depolarizing agents, or to the normal afferent neurotransmitter aspartate, resulted in a 2 to 3 fold increase in NGF receptor expression. In addition to the NGF receptor, expression of the NGF gene was examined. Cerebellar glia were identified as the source of NGF expression, and the synthesis of the trophic factor was also subject to regulation by depolarizing signals. Together, the results presented in this thesis suggest that afferent innervation and NGF interact to promote survival and morphogenesis of developing Purkinje cells.

CHAPTER 1

INTRODUCTION

Epigenetic regulation of neuronal development has proven to be of major importance in the determination of cell fate. Appropriate temporo-spatial expression of epigenetic factors and interactions between signals regulate normal, orderly neuronal development and function. A number of models based on evidence from both the peripheral and central nervous systems suggests that trophic support may be regulated by neural activity (Purves, 1988).

The concept that nerve growth factor (NGF) regulates brain neuron development has gained considerable support over the last few years (Large et al, 1986; Escandón and Chao, 1989; Lu et al., 1989; Allendoerfer et al., 1990; and for review see Whittemore and Seiger, 1987 and Dreyfus, 1989). During cerebellar development, the expression of NGF message (Large et al., 1986; Lu et al, 1989) and the expression of NGF receptors (Eckenstein, 1988; Schatteman et al., 1988; Yan and Johnson, 1988) coincide with the period of active differentiation and synaptogenesis, suggesting that NGF may play an important role during the ontogeny of this brain region.

In particular, previous studies indicating that NGF receptor protein was localized to Purkinje cells suggested that this neuronal population may be responsive to NGF during early development (Eckenstein, 1988; Schatteman et al., 1988; Yan and Johnson, 1988). Purkinje cells, the major efferent neurons of the cerebellum, play a central role in the formation of precise cerebellar cortical networks, and are critical for integration of motor function. The selective expression of NGF receptors by Purkinje cells suggested that NGF may be critical for cerebellar ontogeny. One major objective of my thesis work was to define the potential role that NGF plays during cerebellar development, and in particular, in

the regulation of Purkinje cell ontogeny.

Purkinje cells constitute a particularly advantageous model to characterize the role of NGF in brain neuron development: The cellular events that lead to the morphological and functional differentiation of Purkinje cells (Altman, 1972; Altman and Bayer, 1985b), and the role that several environmental signals play in cerebellar ontogeny have been extensively studied (Ito, 1984). For example, a number of studies have indicated that differentiation of Purkinje cells is influenced by two distinct afferent systems, the parallel and climbing fibers (Ramón y Cajal, 1911; Rakic and Sidman, 1973; Bradley and Berry 1976a, 1976b; Berry and Bradley 1976a; Sotelo and Arsenio-Nunes 1976). In the cerebellum, normal afferent innervation may be necessary for trophic regulation of neuronal development. Consequently, the possibility that NGF and putative afferent neurotransmitters interact in the regulation of Purkinje cell development remained to be examined.

As indicated above, the main objective of my doctoral research was to define the potential role that NGF plays during cerebellar development. For this purpose, it was essential to define whether cerebellar neurons are directly receptive to NGF during development. Therefore, I initially examined the expression of biologically active, high-affinity NGF receptors by Purkinje cells. Once it became evident that Purkinje cells may have the capability to respond to NGF, the next question was to determine the role that NGF plays during Purkinje cell development, and whether interactions between trophic and other epigenetic signals are required for the regulation of neuronal development. This proved to be the case, and the potential mechanism by which these epigenetic signals interact in the regulation of development was also examined.

A brief general review of the knowledge on the three major topics of developmental neurobiology that are concerned with this doctoral dissertation will now follow.

Purkinje cell development

The cerebellar circuitry

The basic circuitry of the cerebellum has been known since the classical studies of Ramón y Cajal (1911). The cerebellar cortex consists of several classes of neurons distributed in an elaborate but stereotyped pattern. Purkinje cells are the pivotal elements of the circuit, since their axons provide the sole output from the cerebellar cortex. They receive synaptic information from the two main afferent systems to the cerebellum, the climbing and mossy fibers. Climbing fibers originate in the inferior olive, and contact Purkinje cells in a one to one relationship. Mossy fibers, originating from numerous extracerebellar sources, synapse on granule cells that act as a relay and direct the information to Purkinje cells through their axons, the parallel fibers. Three major classes of inhibitory interneurons are present in the circuit whose role it is to modulate the excitatory responses triggered by the two afferent systems. This include the stellate, basket and Golgi cells. A less abundant neuronal population, the Lugaro cells, may also participate in the modulation of incoming information (Ito, 1984).

Purkinje cell development and differentiation

The cerebellum arises from the metencephalic portion of the neural tube as a bilateral thickening of the alar plate (Altman and Bayer, 1985a). The two cerebellar plates gradually fuse at the dorsal midline along a rostral to caudal gradient. The cerebellar cortical cells originate from two separate germinal zones. Purkinje cells, Golgi cells and some glial cell types emerge from the ventricular neuroepithelium, while basket, stellate, granule and glial cells arise from the external granular layer, a secondary germinative epithelium in the pial surface of the cerebellar plates. Therefore, to reach their final position within the cerebellar cortex, neurons arising from the two distinct proliferative areas migrate in opposite directions.

In the rat cerebellum, Purkinje cell birth takes place during embryonic days 14 to 17

(Altman and Bayer, 1978; 1985b). Beginning at embryonic day 16, these neurons start to migrate and to settle under the dorsal surface of the cerebellar primordium. At birth, Purkinje cells have reached the cortex forming a layer several cells thick, and with time they disperse and reorganize to form a monolayer (Altman, 1972). Coincident with the migratory process, their morphological differentiation begins.

The development of Purkinje cells was first described by Ramón y Cajal (1911), and his initial description has now been corroborated and extended by several other laboratories (Altman, 1972; Berry and Bradley 1976b; Altman and Bayer, 1985b; Morris et al., 1985). The differentiation of Purkinje cells can be divided into three chronological stages: During the initial stage of differentiation, Purkinje cells are small and fusiform in shape, and are distributed in multiple layers. In the second stage, neurons align into a single row and assume a multipolar shape that is characterized by the presence of numerous somatic processes. This stage was named by Ramón y Cajal as "phase of initial disorientation of dendrites". During the final stage of differentiation, or "stage of orientation and of regularization of dendrites", somatic processes withdraw gradually and dendrites are rearranged in a plane perpendicular to the axis of the folia.

With the development of specific biochemical and anatomical markers, it has been shown that the Purkinje cell population possesses some degree of heterogeneity. During early development, biochemical heterogeneity is seen among clusters of Purkinje cells, however, with time it disappears (Wassef and Sotelo 1984; Wassef et al., 1985). This early heterogeneity may be due to differences in timing of initial development among Purkinje cells from diverse cerebellar lobules (Altman 1978). Conversely, expression of other cellular markers is evenly distributed during early development, and is gradually restricted to subsets of Purkinje cells (Leclerc et al., 1988; Dore et al., 1990). Thus, later in development, biochemically and physiologically distinct Purkinje cells are organized within the cerebellar cortex in parasagittal, band-like compartments of two different phenotypes

(reviewed by Ito, 1984; and Wassef et al., 1990). Although phenotypic expression seems to be independent of afferent regulation, compartmentalization appears to correlate with the establishment of normal olivocerebellar topography (Arsenio-Nunes et al., 1988; Wassef et al., 1990).

The importance of both genetic and epigenetic information during Purkinje cell development has been suggested by two lines of evidence: Purkinje cells have the capability to develop dendritic trees in culture if neurons are exposed to extrinsic influences prior to explantation (Calvet and Calvet, 1988 a,b). In addition, embryonic Purkinje cells have the ability to migrate to their correct place within the cerebellar cortex, to develop dendritic trees, and to establish normal synaptic contacts after transplantation into adult hosts (Sotelo and Alvarado-Mallart, 1987).

Several intrinsic and extrinsic signals are known to regulate the development of Purkinje cells. For example, a vast number of genetic mutations are known to alter Purkinje cell development and survival (Sotelo, 1990). These mutations may be directly linked to the Purkinje cell genetic program, or might affect other cell types that in turn modify the normal development of the Purkinje cell population. In addition, among some of the epigenetic signals also known to affect Purkinje cell differentiation are thyroid hormone levels, and undernutrition (Legrand et al., 1982; Ito, 1984). Perhaps the most important extrinsic influence affecting Purkinje cell differentiation is that provided by afferent innervation.

Purkinje cell differentiation coincides with the formation of initial afferent contacts. Climbing fibers establish early synaptic contacts on Purkinje cells bodies before arborization begins (Mason and Blasezki, 1989). These contacts continue through their development, and by postnatal day 3 in the rat, these synapses are already functional (Crepel, 1971; Shimono et al., 1976; Puro and Woodward 1977a). During development of the dendritic tree, there is a transient state of redundancy of connectivity, since most Purkinje cells are multi-innervated by climbing fibers (Crepel et al., 1976), and not until

later in development is the one-to-one relationship achieved. The regression of redundant contacts appears to be the result of a process of competition between climbing and parallel fibers (Sotelo, 1990), and is caused by a reduction in olivary axonal branching (Delhaye-Bouchaud, 1985). Following the establishment of climbing fiber contacts and the initial development of the dendritic tree, the soma of Purkinje cells begins to receive inhibitory synaptic contacts from basket cells. After this stage, the soma of Purkinje cells is covered by a sheath of glial cells.

The importance of early climbing fiber contacts for the normal development of Purkinje cells was first suggested by Ramón y Cajal (1911). Climbing fibers induce the formation of somatic processes which then develop into the elaborate dendritic tree. Arborization of climbing fibers and development of Purkinje cell dendrites proceed simultaneously during differentiation. In the absence of climbing fiber contacts, the size of the dendritic tree is dramatically reduced. This is due to a decrease in both the total number of dendritic segments and of dendritic branching (Bradley and Berry, 1976; Sotelo and Arsenio-Nunes, 1976).

During the second and third postnatal weeks of development, granule cells migrate to the internal granular layer and the first synaptic contacts between parallel fibers and Purkinje cell dendrites begin (Puro and Woodward, 1977b). Contacts with parallel fibers seem necessary for the induction of the terminal dendritic branches or "spiny branchlets" (Sotelo, 1981).

Work done over several years with mutant mice and agranular cerebella has suggested that the presence of granule cells is not a prerequisite for the survival of Purkinje cells. However, contacts with parallel fibers play an important role in the development of the dendritic arbor (Rakic and Sidman, 1973, Sotelo 1975, Berry and Bradley 1976b; Crepel et al., 1980; Sadler and Berry, 1989). In the absence of parallel fibers, the development of spiny branchlets is impaired, and primary and secondary branches are

randomly oriented. In the absence of parallel fibers, Purkinje cells will form few dendritic spines that are partly innervated by mossy fibers, thus allowing the formation of heterologous synapses that will compensate for the missing excitatory input (Sotelo, 1990). Together, these models have demonstrated the importance of early excitatory synaptic contacts for the development of a functional synaptic circuitry.

The excitatory amino acids L-glutamate and L-aspartate are considered the major excitatory neurotransmitters in the cerebellum. While aspartate has been claimed to mediate the excitatory action of climbing fibers, glutamate mediates the action of parallel fibers (Ito, 1984). These amino acids act on specific receptors on the cell membrane to elicit specific responses to excitatory stimulation. The receptors for excitatory amino acids have been divided into 3 major groups based on electrophysiologic and binding properties of agonists. These are the N-methyl-D-aspartic acid (NMDA), quisqualate and kainate receptor types. Extensive study has been aimed at determining the role that each receptor class plays in the development and function of Purkinje cells (Crepel et al., 1983; Dupont et al, 1987; Garthwaite et al., 1987; Lee et al., 1988; Hicks et al., 1989; Cambray-Deakin et al., 1990). Functional quisqualate and kainate receptors are already present in developing Purkinje cells, while expression of NMDA receptors seems to be transient and developmentally regulated. Although this transient expression of NMDA receptors coincides with the establishment of excitatory contacts, the role that this receptor type plays in development is still controversial. Recent evidence suggests that early responses to excitatory stimulation may be mediated by non-NMDA receptors (Audinat et al., 1990).

Purkinje cells and growth factors

Although multiple genetic and epigenetic factors have been shown to regulate cerebellar ontogeny, little is known about the potential role that classical neurotrophic factors play during development. Over the last few years, more effort has been devoted to

understanding trophic regulation of cerebellar development (Hatten et al, 1988). To date, it has been suggested that cerebellar neurons may be the target of trophic regulation by several known growth factors. Expression of specific receptors to epidermal growth factor or EGF (Gómez-Pinilla et al., 1988), fibroblast growth factor or FGF (Heuer et al., 1990), and nerve growth factor or NGF (Eckenstein, 1988, Schatteman et al., 1988; Yan and Johnson, 1988) by Purkinje cells has been determined. However, the role that these trophic agents play in the development of this neuronal population remained to be defined. One of the objectives during my graduate studies was to define the role that NGF plays during cerebellar development, and this work is now presented on this thesis.

Neurotransmitters in the regulation of neuronal development

During the development of the nervous system, cell fates are determined in large part by interactions between intrinsic properties of the neurons and the microenvironment. A number of studies done over the last few years indicate that afferent neurotransmitters and neural activity may regulate neuronal differentiation and synaptogenesis (reviewed by Black, 1978; Mattson, 1988; Purves, 1988; and McDonald and Johnston, 1990). Neurotransmitters may modulate neuronal development by regulating neuronal survival, dendritic and axonal structure, synaptogenesis and activity-dependent synaptic plasticity. Both excitatory and inhibitory neurotransmitters may serve neurotrophic functions during development (Hansen et al., 1988; McDonald and Johnston, 1990).

Recent evidence suggests that excitatory neurotransmitters have trophic influences on differentiating neurons and regulate development of neuronal cytoarchitecture in the central nervous system (Mattson 1988, McDonald and Johnston, 1990). The two major excitatory neurotransmitters known to modulate neuronal function are L-glutamate and L-aspartate, and their actions have been characterized by the role that distinct receptor agonists play during development and plasticity (Tremblay et al., 1986; Pearce et al., 1987; Balazs

et al., 1988; Mattson et al., 1988; Bode-Greuel and Singer, 1989; Represa et al., 1989; Cline and Constantine-Patton, 1989). As previously indicated, excitatory amino acid receptors can be divided into three major classes, the NMDA, kainaite and quisqualate receptors. Although kainate and quisqualate may have multiple receptor sites, for convenience, these receptors have been grouped as of the non-NMDA type.

It has been suggested that NMDA receptors may be directly involved in the regulation of neuronal development in several brain structures. This excitatory amino acid receptor system appears to be very active during restricted developmental periods and contributes to the regulation of several forms of synaptic plasticity and neuroarchitecture. For example, in the visual system of mammals, high expression of NMDA receptors parallels the time course of the critical period when the visual system is suceptible to experience-dependent modifications (Bode-Greuel and Singer, 1989). In addition, in lower vertebrates, NMDA receptors have been involved in the experimental formation of occular dominance columns, suggesting that neural activity acting through specific excitatory amino acid receptors mediates segregation of synaptic inputs (Cline et al., 1987; Cline and Constantine-Patton, 1989). In the cerebellum, sensitivity to NMDA application is significantly higher during development than in adult life (Garthwaite et al., 1987), suggesting that excitatory amino acids acting through NMDA receptors may regulate cerebellar ontogeny. Indeed, it has been shown that exogenously applied NMDA promotes the survival of granule cells in culture (Pearce et al., 1987; Balazs et al., 1988; 1989).

In addition to the role in survival and synaptic specificity, excitatory amino acids have been shown to play an important role in neurite outgrowth. In the hippocampus, glutamate modulates neurite outgrowth and the overall morphology of developing pyramidal neurons. In this case, the effects of glutamate appear to be mediated through non-NMDA receptors (Mattson et al., 1988). Therefore, regulation of neuronal development by excitatory neurotransmitters may occur at several distinct levels that include

neurite elaboration, survival and synaptogenesis. In addition, these effects may be mediated by actions at different receptor types, and may involve the activation of distinct intracellular mechanisms.

Recent studies have indicated that calcium, cyclic nucleotides and inositol phospholipid-derived second messengers may mediate neurotransmitter effects on neurite outgrowth. Calcium, in particular, appears to play a central role in communicating the neurotransmitter-initiated signals to the cytoskeletal and vesicular apparatus responsible for controlling neuronal form. A series of changes in calcium status of different regions of a growing neurite may actually be required for outgrowth (Grinvald and Faber, 1981; Connor, 1986; Kater et al., 1988). This local regulation of growth cone behavior probably allows modifications of neuroarchitecture and, in turn, proper integration of neuronal interconnections. The involvement of a calcium-mediated mechanism in the regulation of dendritic growth by glutamate has been shown for the hippocampal pyramidal neurons (Mattson et al., 1988). Moreover, recent evidence suggests that excitatory neurotransmitters and growth factors may interact to regulate the development of this neuronal population. This type of interactions may occur at the second messenger level (Mattson et al.,1989).

For many years, a trophic role for excitatory neurotransmitter in the development of Purkinje cells has been proposed (Ramón y Cajal, 1911; Rakic and Sidman, 1973; Berry and Bradley 1976a,b; Bradley and Berry 1976a,b; Sotelo and Arsenio-Nunes, 1976). However, attempts by several groups to discern how these agents may regulate their development and survival, had not been completely successful (Hockberger et al., 1989). During my thesis work I examined the possibility that excitatory neurotransmitters may interact with trophic factors in the regulation of neuron development, and the results of these efforts are presented in this doctoral dissertation.

Nerve growth factor and neuronal development

Nerve growth factor: a trophic factor for both peripheral and central neurons

Neuronal development, neuritic outgrowth, transmitter specificity and regulation of programmed cell death have all been postulated to be mediated in part by the release of target-derived trophic factors. Nerve growth factor (NGF) was the first neurotrophic factor to be discovered (for review see Levi-Montalcini, 1987), and for over several decades NGF provided the only basis for the concept that trophic molecules are necessary for survival and neuronal development. Initial work in the peripheral nervous system demonstrated that NGF promotes the survival and maintenance of sympathetic (Levi-Montalcini and Angeletti, 1968) and sensory neurons (Johnson et al., 1986). The role of NGF as a target derived trophic factor has been confirmed by several lines of evidence. The degeneration of neurons after anti-NGF antibody treatment (Cohen, 1960; Levi-Montalcini and Booker, 1960) and the reduction of neuronal cell death after NGF administration (Oppenheim et al., 1982), indicate in vivo requirement of the trophic agent. Moreover, the specific localization of NGF and its expression in limiting amounts in target tissues (Shelton and Reichardt, 1984), and the expression of specific receptors by responsive cells (Herrup and Shooter, 1973, Frazier et al, 1974, Yankner and Shooter, 1982) support these findings. In addition to its trophic effect, NGF has been proposed to act as a tropic molecule for both sensory and sympathetic neurons (for review see Levi-Montalcini, 1987). However, to date, the role of NGF as chemotropic agent during development remains controversial (Davies et al, 1987).

Increasing evidence suggests that Nerve Growth Factor (NGF) plays an important role in the central nervous system (CNS) as well (for review see Thoenen et al., 1987; Whittemore and Seiger, 1987; Dreyfus, 1989). Initial studies indicated that NGF regulates the development of central cholinergic neurons. NGF elevates choline acetyltransferase (ChAT) activity (the enzyme involved in the synthesis of acetyl choline) in basal forebrain and striatal neurons (Hefti et al., 1984; Martinez et al. 1985; Mobley et al., 1986; Martinez

et al., 1987; Vantini et al., 1989), and increases survival of septal cholinergic neurons both in culture and in vivo (Hefti, 1986; Hartikka and Hefti, 1988; Hatanaka et al., 1988). In addition to the role in survival and transmitter determination, a role for NGF in the regulation of neuroblast proliferation has now been proposed (Cattaneo and McKay, 1990).

The localization of NGF and its receptors to brain areas other than the basal forebrain/striatum-hippocampal system suggested that NGF actions during brain development are not restricted to the central cholinergic neurons. The transient expression of NGF receptors in multiple brain areas including retina, cerebral cortex, brainstem, olfactory bulb and cerebellum (Large et al, 1986; Raivich et al., 1987; Eckenstein 1988; Schatteman et al., 1988; Yan and Johnson, 1988; Escandón and Chao, 1989; Lu et al. 1989; Allendoerfer et al., 1990), supports the contention that NGF regulates the ontogeny of diverse neuronal populations. Moreover, the distinct developmental patterns of NGF receptor expression in different brain areas, suggests that NGF may play distinct roles in individual brain structures (Buck et al., 1988; Lu et al., 1989).

The traditional model for neurotrophic support suggests that the trophic agent is synthesized by target cells, binds to specific receptors in responsive cells and is then transported retrogradely from the target source to the cell bodies of the long projecting neurons. Support that a target-derived trophic factor model may operate in the basal forebrain-cholinergic system comes from the distribution of NGF protein and its mRNA in target tissues (Seiler and Schwab, 1984; Ayer-LeLièvre et al., 1988, Whittemore et al., 1988).

Recent evidence on the transient co-localization of NGF and NGF-R messages in particular brain areas strongly supports the concept that NGF may function as a local factor during neuronal development as well (Lu et al., 1989). NGF, acting in the immediate microenvironment may regulate the development of local circuit neurons, and specificity

may be conferred by selective expression of receptors by responsive cells. In turn, receptors may be localized to cell bodies, dendrites, and axonal terminals.

Nerve growth factor: Biosynthesis

The structure of biologically active NGF is now well characterized. The β-NGF molecule consist of two identical chains of 118 amino acids each, with 6 cysteines and 3 disulfide bridges each. This β-NGF dimer is part of a larger protein complex with sedimentation coefficient of 7S. This complex is composed, in addition to the β subunit, of two molecules of the α-subunit, a protease inhibitor, and two molecules of the γ-subunit, a proteolytic enzyme thought to be involved in the processing of the pro-NGF. The α- and γ-peptides are members of the kallikrein family of serine proteases. The multisubunit complex therefore has the composition $\alpha_2\beta\gamma_2$ and is stabilized by zinc ions (Greene and Shooter, 1980). The primary structure of NGF, deduced from cDNA clones is now known for the mouse, human, bovine, rat, chick and snake, and shows a high degree of conservation among the different species (Whittemore et al., 1988). To date, only limited information is available on the residues participating in the binding of NGF to its receptor (Greene and Shooter, 1980; Ibáñez et al., 1990).

In addition to NGF, two other neurotrophic molecules with strong homology to NGF have now been identified. These are brain-derived neurotrophic factor or BDNF (Leibrock et al., 1989) and neurotrophin-3 or NT-3 (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990). It is now suggested that these 3 members of the NGF family may bind to the same receptors, and the difference in their action may reside in specific modifications to the receptor sites (Rodriguez-Tébar et al., 1990). However, this remains to be proven.

Nerve growth factor: Receptors and mechanism of action

Selective responsiveness to NGF among different neuronal populations is conferred by expression of specific cell surface receptors. NGF binds to its receptor, forming a receptor/NGF complex, which is internalized and transported back to the cell body where some of the physiologic actions take place. Specific binding of NGF can be mediated by either of two distinct receptors that differ in their association and dissociation constants (Landreth and Shooter, 1980; Schechter and Bothwell, 1981). These receptors are known as the high-affinity or type I, and the low-affinity or type II receptors ($K_d = 10^{-11}$ M and $K_d = 10^{-9}$ M, respectively). While the functional role of low-affinity receptors is still unresolved, it has been proposed that NGF binding to high-affinity receptors is required for internalization and function (Bernd and Greene, 1984; Green et al., 1986). Recent evidence indicates that both receptor subtypes are encoded by the same gene. Transfer of the human low-affinity NGF receptor gene into mutant PC12 cells lacking NGF receptors restores low- and high-affinity binding (Hempstead et al., 1989). The final elucidation of the mechanisms involved in the transition from the low to high-affinity state will prove to be key in the understanding of NGF function. Current theories propose that an accessory membrane protein, which may associate with the receptor binding moiety, might be responsible for this transition.

Several different signal transduction pathways have been proposed to be involved in mediation of cellular responses to NGF. Recent evidence suggests that *ras* or *ras*-like proteins are involved in at least two different pathways of signal transduction (Bar-Sagi and Feramisco, 1985; Borasio et al., 1989; Szeberenyi et al., 1990). The *ras* oncogene protein is a G protein-related product and possesses GTPase activity, suggesting the involvement of G proteins in the mediation of NGF effects. In addition to G proteins, multiple kinase pathways have also been involved in the NGF-mediated increase in early gene expression.

Among these, protein kinase N, a serine kinase (Volonte et al., 1989, Greene et al., 1990) has been shown to mediate some of NGF effects.

In the course of differentiation, a cascade of intracellular events are activated by NGF. These early and late events have been defined mainly from studies in NGF-responsive, rat pheochromocytoma cells or PC12 cells, and some have been corroborated in sensory and sympathetic neurons. Early responses to NGF occur within minutes of exposure and do not involve transcription. Among these events are the hydrolysis of phosphoinositides, activation of N kinase, changes in protein phosphorylation and increase uptake of nutrients, and are associated with regulation of growth cone shape and surface morphology (Greene, 1984; Connolly et al., 1987; Rowland et al., 1987; Aletta et al 1988a). In addition, early events seem to be associated with NGF effects on neuronal survival (Greene, 1984).

Delayed responses to NGF are frequently transcriptionally associated, and some examples are the induction of neurite outgrowth, formation of synaptic varicosities, development of electrical excitability and induction of several neuron-specific proteins (Greene, 1984; Aletta et al 1988 a, b; Mandel et al., 1988; Aletta et al., 1989). Delayed events may be associated with neurite outgrowth as indicated by the regulation of microtubule associated (Aletta et al., 1988a) and intermediate filament proteins (Aletta et al., 1988, 1989), or with neuronal survival as exemplified by NGF's ability to inhibit the expression of genes for programmed cell death (Chang et al., 1990; Oppenheim et al., 1990). The distinction between pathways by which NGF may promote either neurite outgrowth or neuronal survival are now beginning to be elucidated (Greene et al., 1990).

DEVELOPING PURKINJE CELLS EXPRESS LOW- AND HIGH-AFFINITY NERVE GROWTH FACTOR RECEPTORS.

As indicated in the Introduction, early studies identifying receptors suggested that NGF may act on Purkinje cells, particularly during neonatal development. However functional NGF binding sites and NGF actions were yet to be defined.

The objective of the experiments presented in this chapter was to localize NGF binding sites to cell types in the developing cerebellum and to define receptor subtypes. Initial studies were aimed at determining whether these receptors may be functional. For this purpose, a specific ^{125}I-NGF binding assay was employed to delineate both low- and high-affinity receptor sites. Particular attention was given to the expression of NGF receptors by developing Purkinje cells.

A cell marker to visualize developing Purkinje cells

Purkinje cells, the largest neurons in the cerebellar cortex, can be easily identified by their characteristic morphology. However, during early development, when Purkinje cells assume a variety of cell shapes and sizes, identification solely by morphology is unreliable. To identify developing Purkinje cells and to visualize individual cells and processes, Purkinje cells were immunostained with vitamin D-dependent calcium binding protein (CaBP) antibodies. CaBP is a cell specific antigen exclusively expressed by Purkinje cells within the cerebellum (Jande et al., 1981; Christakos et al., 1987).

Sagittal cerebellar sections from embryonic day 18 (E18), P2 and P10 were immunostained with CaBP antibodies, to visualize developing Purkinje cells *in vivo* during

3 distinct developmental stages. At E18, immunopositive cells display simple morphological features; a round cell body and elongated process typical of migrating neurons are seen (Fig 1A). At P2, cells have migrated almost to their final position in the Purkinje cell layer, and have started to develop an immature dendritic tree (Fig 1B). At P10, cells are organized in a single layer and immunoreactivity can be detected on cell bodies, axons and dendrites (Fig 1C). Figure 2 shows a sagittal section of a folium of the P10 cerebellum stained with the avidin-biotin peroxidase technique. With this technique, axons and distal dendrites become more apparent.

Localization of NGF receptors to developing Purkinje cells by ^{125}I-NGF binding

Previous evidence from our laboratory showed that NGF-R mRNA is transiently expressed during development of the postnatal rat cerebellum. Message levels peak at postnatal day 10 (P10) and decline immediately thereafter, reaching extremely low levels by P20. Consequently, I analyzed NGF binding at P10 in the rat to define the cerebellar cell types that express NGF receptors during development. In addition, receptor subtypes were determined. For ^{125}I-NGF binding to both receptor subtypes (low- and high-affinity), cerebellar sagittal slices were incubated with 0.2 nM ^{125}I-NGF for 1 hr at 37^{o}C, as described in the methods section (see Chapter 6). Incubation with ^{125}I-NGF specifically labeled binding sites on all Purkinje cells. Silver grains were concentrated on cell bodies, dendrites, and on axonal processes of CaBP-immunopositive Purkinje cells (Fig 3a). On some cells, a higher density of silver grains was localized to the distal dendrites, while on others, the highest density was observed at the basal pole of the cell body. The density of silver grains varied within (Fig 3a) and among folia (not shown). Grain density appeared to be correlated with the degree of maturation of Purkinje cells in different folia (Altman and Bayer, 1985b), and with different developmental stages (see below), as evidenced by the

Figure 1.- Photomicrograph of Purkinje cells in the developing cerebellum, immunostained for CaBP. Ten micron cryostat sagittal sections from E18 (A), P2 (B), and P10 (C) rat cerebellum were immunostained with anti-CaBP antiserum and fluorescein conjugated secondary antibodies. A) View of the cerebellar primordium; the pial surface is at the top, and ventral at the bottom of the figure. B) and C) View of a folium of the developing cerebellar cortex. Scale bar, 50μm.

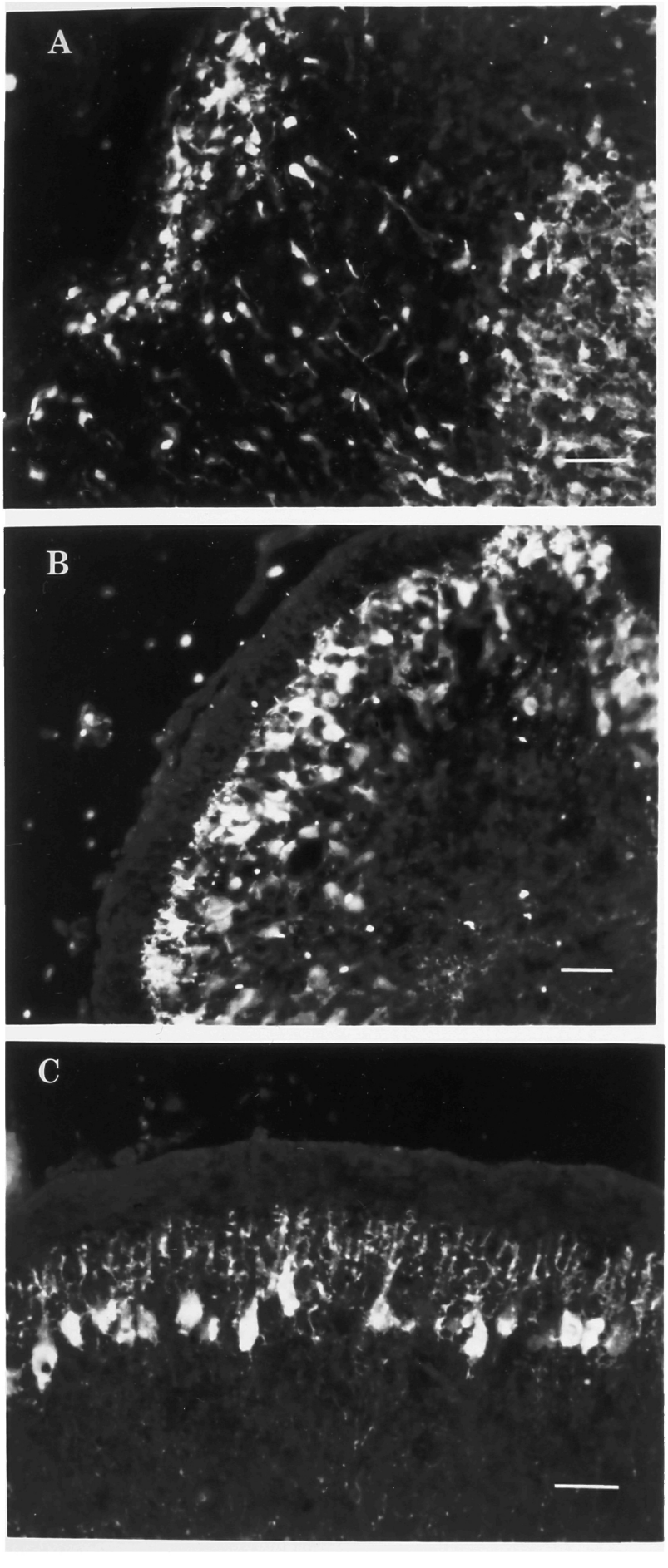
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Figure 2.- Photomicrograph of Purkinje cells in the postnatal day 10 rat cerebellum. Ten micrometer cryostat sagittal sections were immunostained with anti-CaBP antibodies. For visualization, the avidin/biotin technique (ABC kit, Vector Labs.) was used. Immunoreactivity to anti-CaBP antibodies delineates cell bodies, dendrites, and axonal processes on developing Purkinje cells. Note immunoreactivity of cell body, dendrites (arrow), and axonal processes (arrowheads). (a) Scale bar, 100 μm. (b) Scale bar, 20μm.

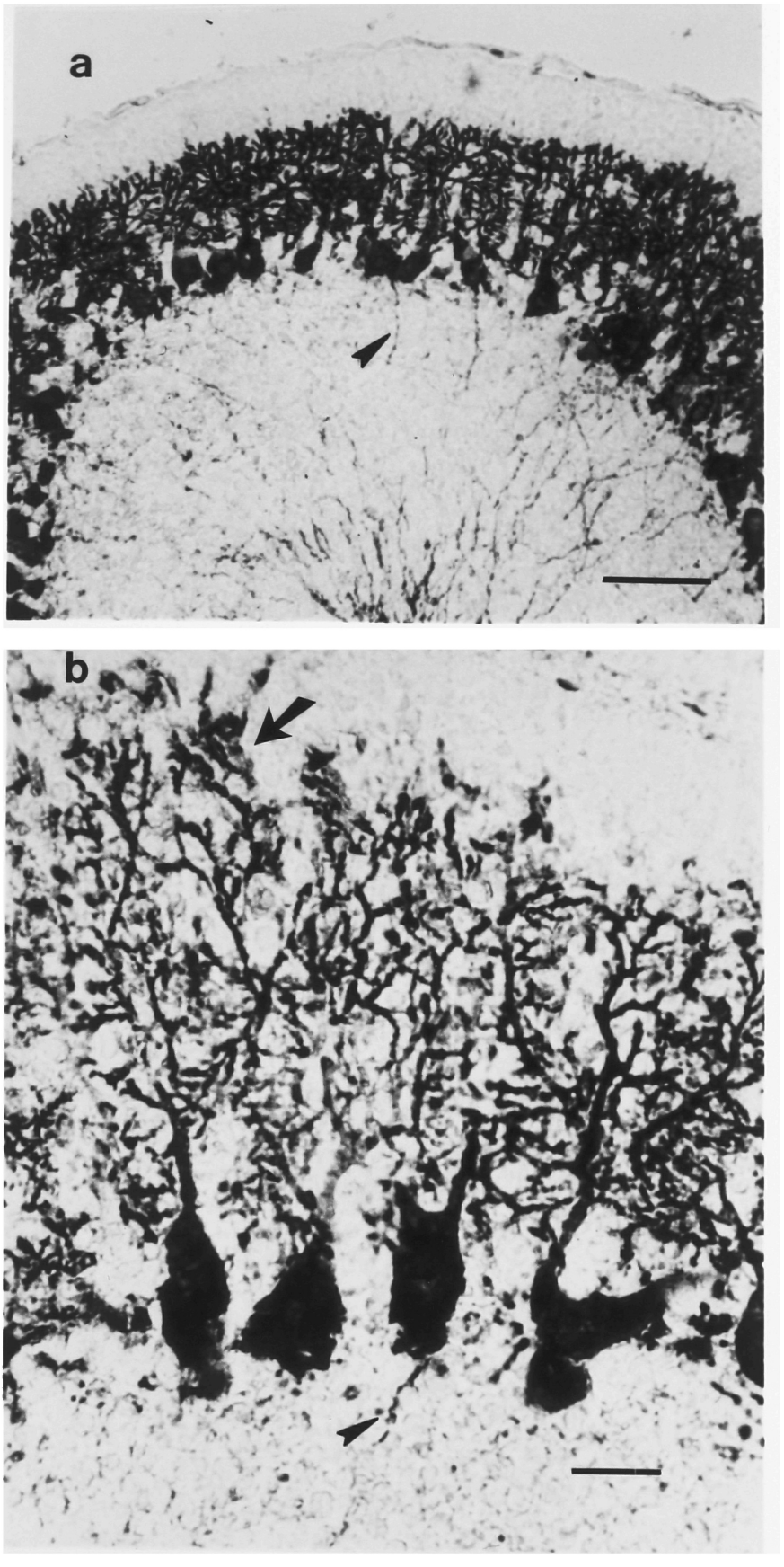
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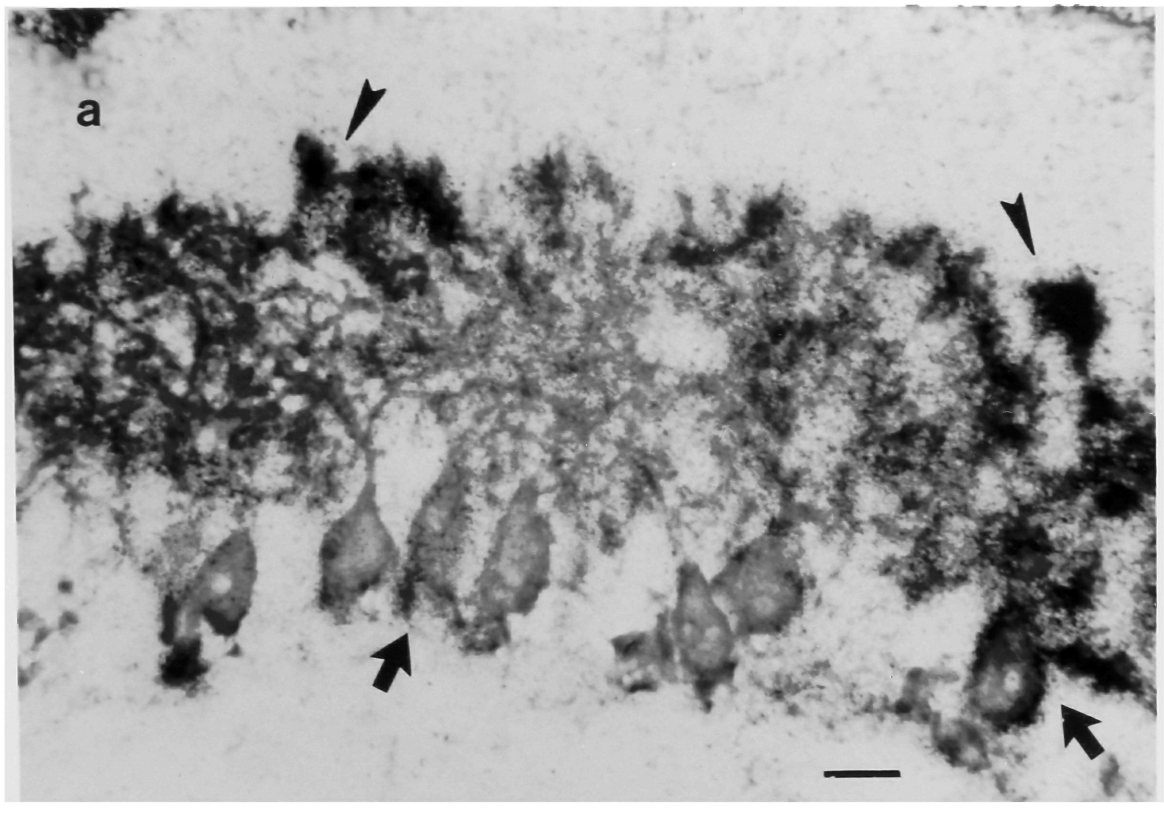
Figure 3.- ^{125}I-NGF binding to Purkinje cells in the postnatal day 10 rat cerebellum. ^{125}I-NGF binding was identified as silver grains over different cell areas. Bright field illumination reveals the presence of grains over immunoreactive Purkinje cells. (a) Silver grains are localized on the cell body, dendrites, and axonal processes of Purkinje cells. Note high density of silver grains over distal dendrites (arrowheads) and cell bodies (arrows). (b) Control slice, note only background binding. Scale bar, 20μm.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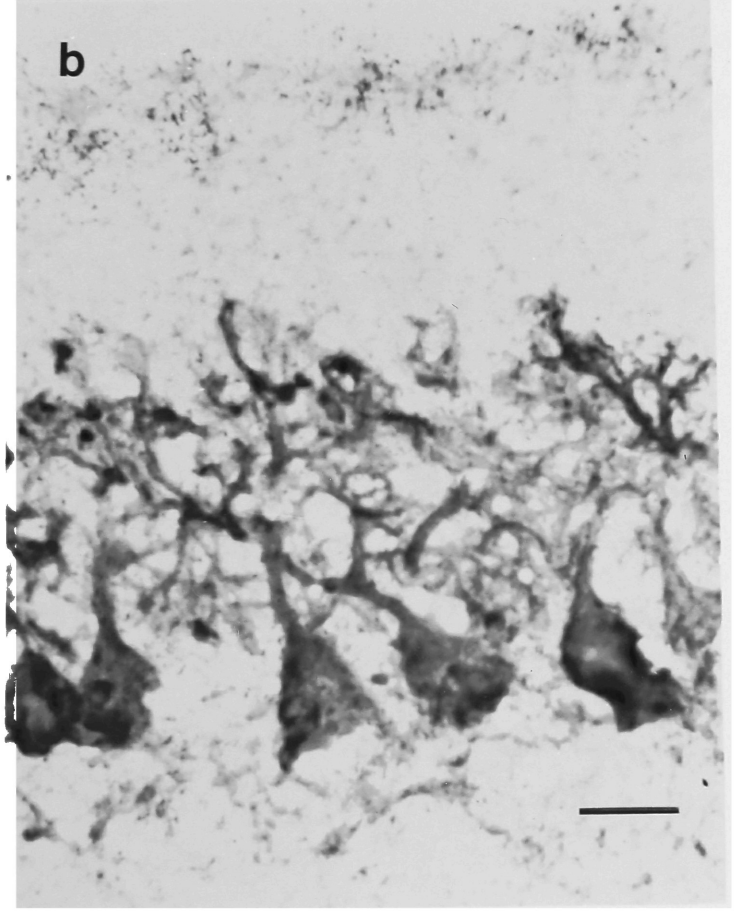
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size and ramification of the dendritic tree. Specific ^{125}I-NGF binding to Purkinje cells was completely abolished by incubation with 1000-fold excess unlabeled NGF (Fig 3b), a specific control previously described (Bernd et al, 1988).

Localization of NGF binding sites was also determined at an earlier stage in development. In the P4 rat cerebellum, Purkinje cells have acquired their final position and dendritic development begins. At this time, specific ^{125}I-NGF binding sites were localized to cell bodies of Purkinje cells and to developing dendrites (Fig 4a).

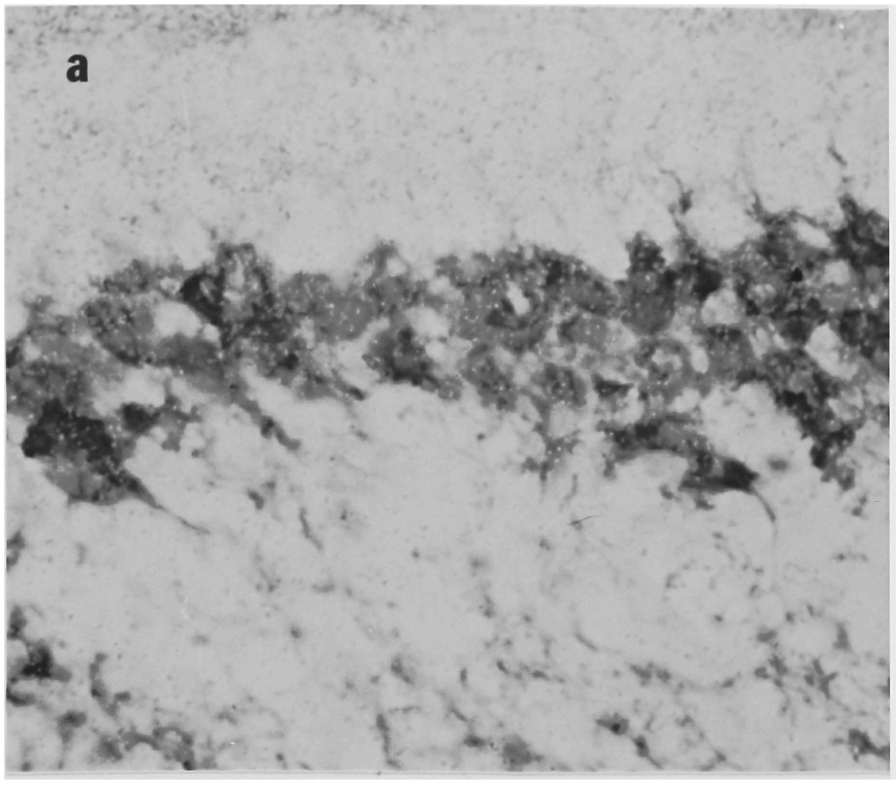
To define Purkinje cell receptor subtypes, I took advantage of the difference in dissociation constants between high- and low-affinity receptors. High-affinity NGF receptor sites ($K_d = 10^{-11}$M) exhibit no significant dissociation at 4°C, while low-affinity receptors ($K_d = 10^{-9}$M) exhibit fast dissociation (Landreth and Shooter, 1980; Schechter and Bothwell, 1981). To selectively displace low-affinity binding, cerebellar slices were exposed to a chase of 0.2 μM unlabeled NGF for 30 min at 4°C, following incubation with the iodinated ligand (Bernd et al., 1988). The protocol revealed high-affinity binding sites on dendrites, cell bodies and axonal processes of Purkinje cells (Fig 5a). Binding to high-affinity receptors was also specific, since only background grains were detected upon exposure to excess non-radioactive NGF (Fig 5b).

In addition to Purkinje cells, NGF binding sites were localized to cells in the external granular layer, both at postnatal days 4 and 10. Silver grains were observed under conditions in which both high- and low-affinity sites were selected. No attempt to further characterize the cell types expressing the receptors was made in this study.

Adult rat cerebellar slices were incubated with 0.2 nM ^{125}I-NGF to label both low- and high-affinity sites. Expression of NGF receptors by Purkinje cells appeared to be transient, since specific binding was not detected in the adult rat cerebellum (Fig 6).

In parallel studies, binding to slices was used as a method to quantify binding. Counts per minute (cpm) incorporated per milligram of wet weight were determined in

Figure 4.- ^{125}I-NGF binding to Purkinje cells in the postnatal day 4 rat cerebellum. (a)^{125}I-NGF binding is visualized as silver grains over cell bodies and developing dendrites. With the use of a "pol" cube and epifluorescent illumination, silver grains appear as green particles over immunostained Purkinje cells. Note accumulation of silver grains over cell bodies. (b) Control slices, note only background binding. Scale bar, 20μm.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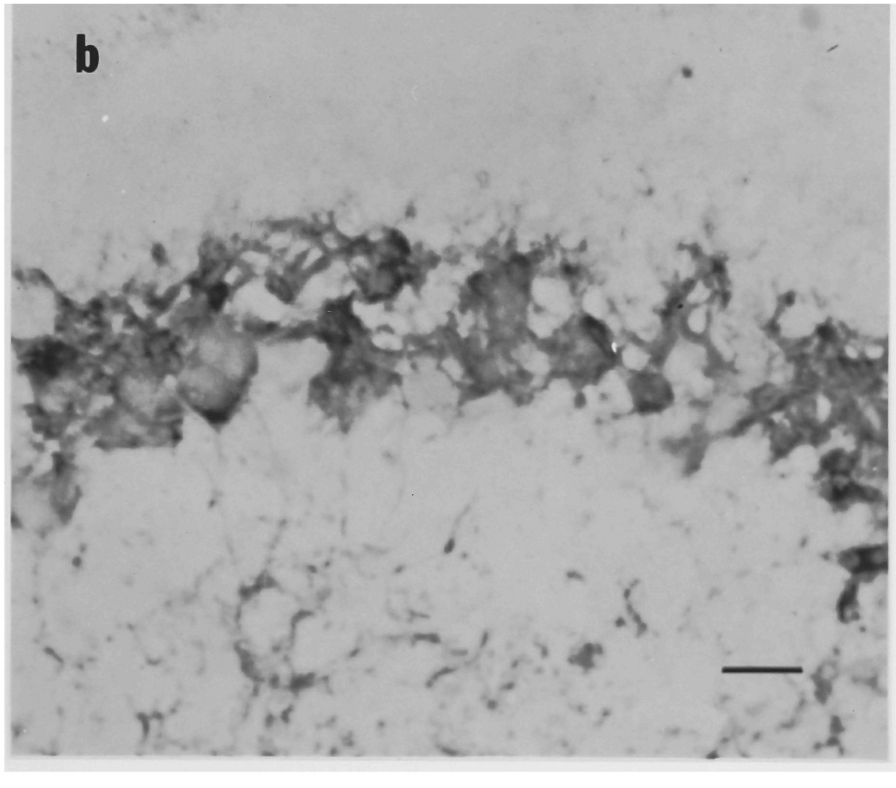
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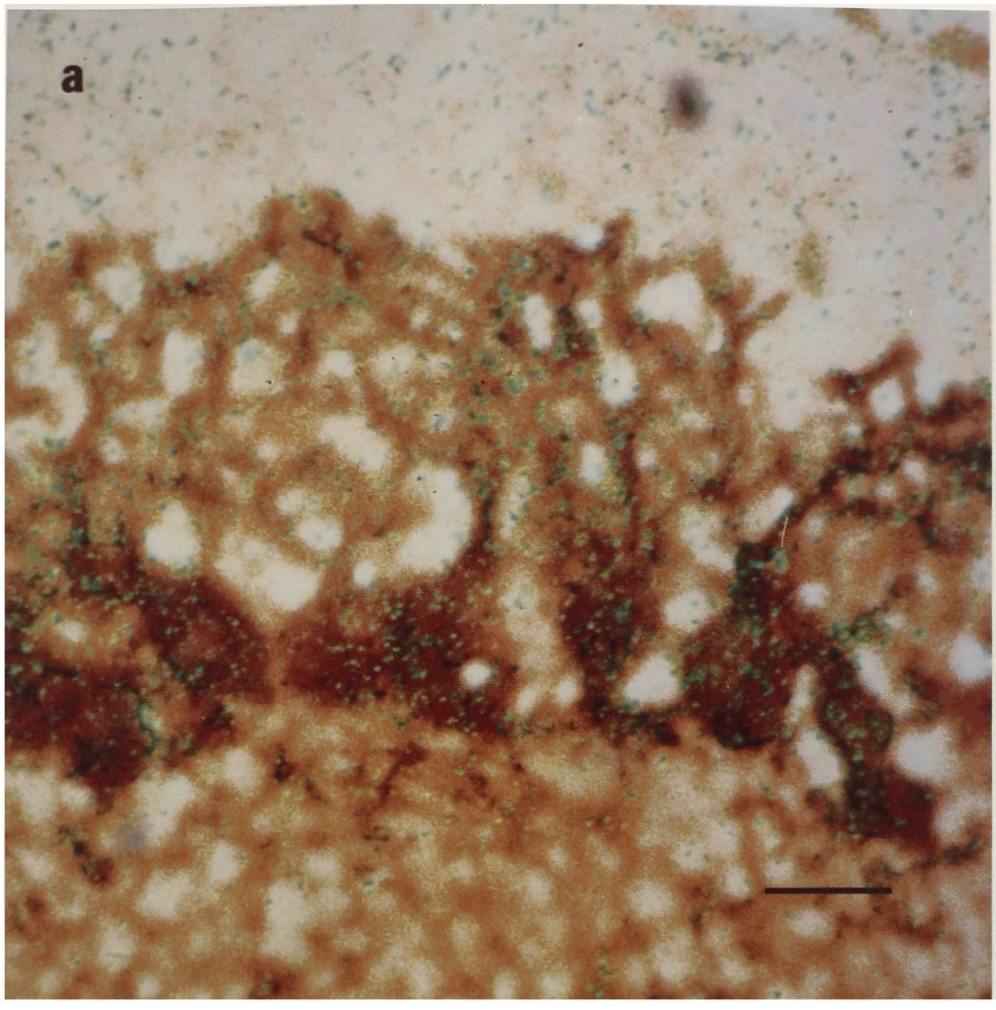
Figure 5.- High-affinity binding sites on Purkinje cells in the postnatal day 10 rat cerebellum. High-affinity binding sites were detected by exposure to a chase of unlabeled NGF after incubation with the radiolabeled ligand (see methods). (a) ^{125}I-NGF binding is visualized over cell bodies and dendrites of Purkinje cells. Silver grains appear as green particles overlying brown, CaBP-immunopositive cells, and are distributed over the cell body and dendritic processes of Purkinje cells. Silver grains were also detected in the external granular layer (EGL), a proliferative and premigratory zone that lies above the developing Purkinje cell dendritic trees. Note that the density of silver grains in the high-affinity group is lower than in the low-affinity group (Fig. 3a). (b) Control slices, only background binding is detected. Scale bar, 20μm.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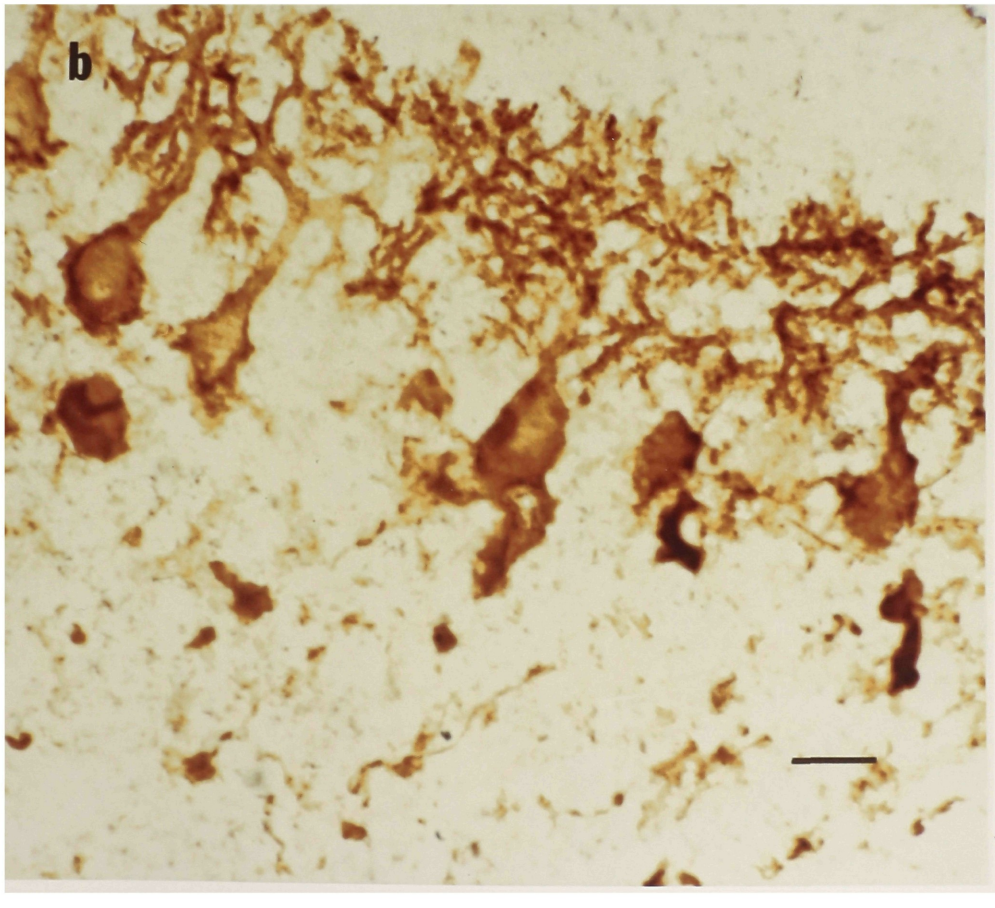
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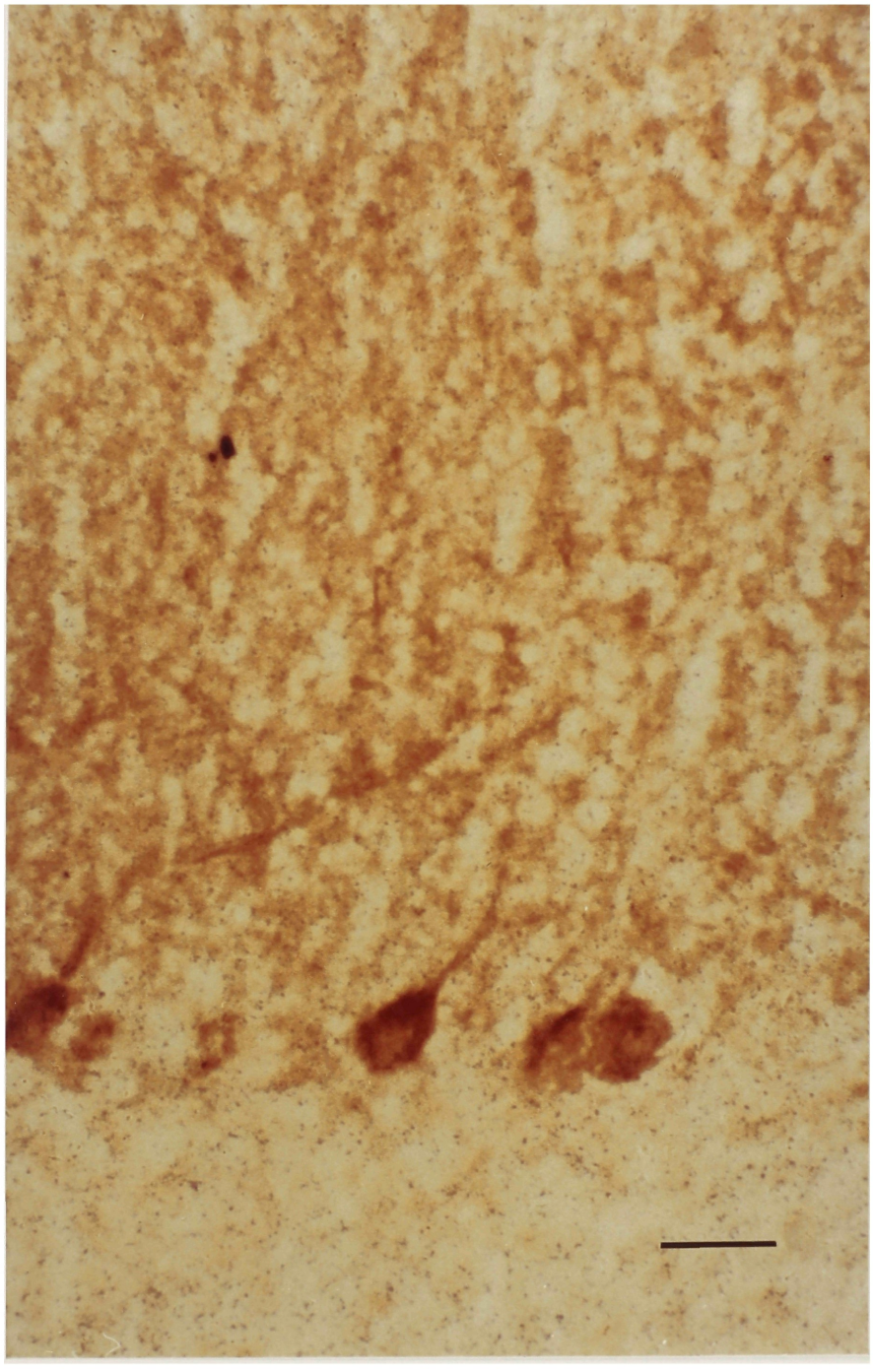
Figure 6.- Photomicrograph of Purkinje cells in the adult rat cerebellum. Adult rat cerebellar slices were incubated with ^{125}I-NGF to label both low- and high-affinity binding sites. No specific binding was observed in this case. Note that only background grains are observed. Scale bar, 40μm.

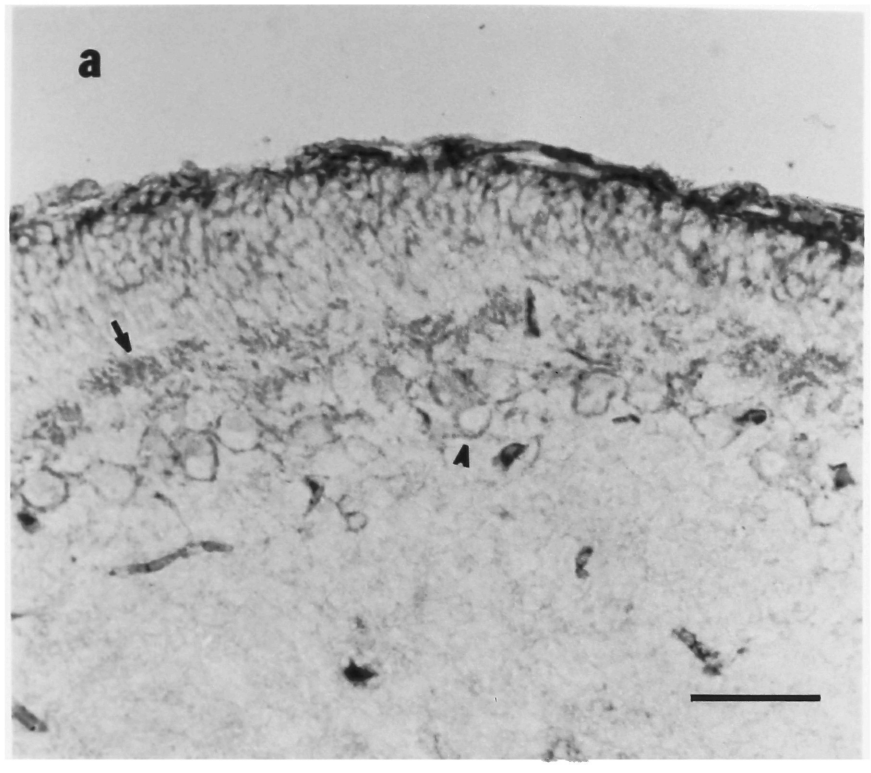


parallel to visualization of binding sites. Table 1 shows the results obtained by analyzing ^{125}I-NGF binding to P10 and adult rat cerebellum. This experiments confirmed specificity of binding in the postnatal rat cerebellum, that in the adult becomes undetectable.

Immunocytochemical localization of NGF receptors

Expression of NGF receptors by developing Purkinje cells was also analyzed in this study by immunocytochemical staining with the monoclonal antibody 192-IgG, directed to the rat NGF receptor. Ten micron cryostat sagittal sections of the E18 and P10 rat cerebellum were immunostained using the avidin-biotin peroxidase technique. At P10, immunostaining of Purkinje cell bodies and scattered staining of dendrites was seen (Fig 7a). Undefined staining in the external granular layer was also observed. This finding confirm previous observations that Purkinje cells and cells in the external granular layer are immunoreactive to NGF-R antibodies during the early phase of postnatal development (Eckenstein, 1988; Schatteman et al., 1988; Yan and Johnson, 1988). In the embryonic day 18 rat cerebellum, very light staining could be observed in some areas of the developing cerebellar primordium (7b). The staining was almost undistinguishable from background staining, thus suggesting that expression of NGF-R at this time in development is low, if present at all.

Figure 7.- Photomicrograph of NGF-R immunoreactive cells in the developing rat cerebellum. Ten micron cryostat sagittal sections from E18 (b) and P10 (a) rat cerebellum were immunostained with the NGF-R antibody 192-IgG and visualized with the avidin/biotin technique. (a) In the P10 rat cerebellum, NGF-R immunoreactive Purkinje cells are observed. Immunoreactivity is associated to the cell body (arrowhead) and dendrites (arrow) of Purkinje cells. In addition, pericellular immunoreactivity associated to the external granular layer can be observed. (b) In the E18 rat cerebellar primordium, weak NGF-R immunoreactivity can be observed in areas rich in migrating neurons well as in the forming cortex (area delineated by double arrowheads; cf. Fig 1A). Scale bar, 50μm.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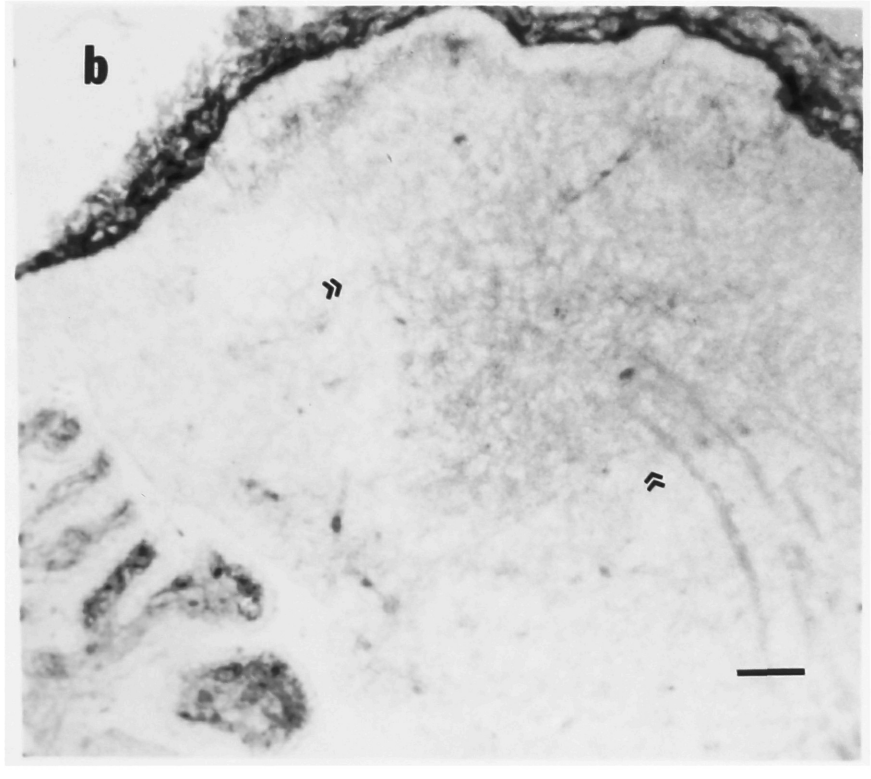
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Table 1. Specific ^{125}I-NGF binding to rat cerebellar slices

	Postnatal day 10	Adult
	cpm/mg protein	*cpm/mg protein*
Experimental group		
Control (L+H)	610.50±11.39	632.0±49.0
Low+High Affinity (L+H)	1437.25±275.91**	570.5±18.5
Control (H)	491.75±94.63	615.2±213.0
High-Affinity (H)	1118.25±279.15*	731.4±196.5

^{125}I-NGF binding experiments were performed as described in the methods section. After incubation with radiolabeled NGF (L+H), and after exposure to excess unlabeled NGF following incubation with the radioiodinated ligand (H), the tissue slices were washed 6 times in PBS. Five slices from each experimental group were pooled, and radioactivity was determined in a gamma-counter. Each value represents the result from four independent experiments normalized to cpm/mg wet weight, and is expressed as mean ± standard error of the mean. ** Significantly different from controls by Scheffe-F test. * Significantly different from controls by Fischer test. $P<0.05$

DISCUSSION

Results presented in this chapter can be briefly summarized as follows: 1) Developing Purkinje cells can be readily identified by the specific expression of vitamin D-dependent calcium binding protein at early stages during differentiation. 2) Purkinje cells transiently express NGF receptors during development, as defined by NGF binding. 3) The biologically active form of the NGF receptor, the high-affinity subtype, is associated with developing Purkinje cells at a time of active differentiation and synaptogenesis.

Visualization of Purkinje cells

The study presented in this thesis is concerned with the development of cerebellar Purkinje cells and the effects of several epigenetic signals during differentiation. Therefore, the need to reliably identify and characterize this neuronal population through different stages in development. Vitamin D-dependent Calcium Binding Protein (CaBP or Calbindin D_{28k}), proved to be an excellent marker to identify Purkinje cells. Expression of CaBP commences early in ontogeny (Legrand et al., 1983), and within the cerebellum is restricted to the Purkinje cell population (Christakos et al., 1987). Immunostaining with antibodies to CaBP allowed visualization of cell bodies, axons and dendrites of developing Purkinje cells. As reported in Chapter 3 of this thesis, this marker also proved essential for the identification and study of NGF effects on developing Purkinje cells in culture.

Expression of NGF receptors by developing Purkinje cells

Previous evidence on the role of NGF during cerebellar development indicates that NGF-R protein, NGF-R mRNA and NGF protein are transiently expressed in this brain region. The aim of the experiments presented in this chapter was to define the NGF-R

subtypes expressed by developing Purkinje cells as a measure to determine whether this neuronal population is potentially responsive to NGF. The evidence presented in this chapter demonstrates, for the first time, that Purkinje cells transiently express the two NGF receptor subtypes, high- and low-affinity. The transient expression of high-affinity NGF receptors by Purkinje cells suggests that NGF receptors may be functional during development.

Evidence obtained mainly from studies in the peripheral nervous system (PNS) demonstrates that biological actions of NGF are correlated with high-affinity binding (Greene and Shooter, 1980; Thoenen and Barde, 1980). NGF binding and internalization through high-affinity receptors mediates the majority of NGF's effects (Bernd and Greene, 1984, Green et al., 1986). Therefore, the expression of high-affinity receptors by Purkinje cells suggests that NGF may regulate the ontogeny of this neuronal population.

Elevated expression of NGF receptors by Purkinje cells occurred during early postnatal development, and markedly decreased to undetectable levels in the adult rat cerebellum by binding criteria. These results confirmed previous evidence indicating that NGF receptor message levels become almost undetectable in the adult rat cerebellum (Buck et al., 1988). However, in a recent report, Pioro and Cuello (1988) have shown that Purkinje cells in the adult rat cerebellum are immunopositive for NGF-R in animals treated with colchicine. Detection of NGF-R on treated animals may be due to constitutive, low expression of the NGF-R protein, that after treatment with colchicine accumulates and becomes immunocytochemically detectable. Another possibility is that colchicine-induced toxicity may mediate *de novo* expression of NGF-R. In any event, whether or not Purkinje cells retain the ability to express NGF receptors through adulthood, expression is markedly lower than in the developing animal, suggesting that Purkinje cells are maximally responsive to NGF during the early stages of differentiation.

The transient expression of NGF messenger RNA within the cerebellum (Lu et al.,

1989), and of NGF receptors by Purkinje cells (Cohen Cory et al., 1989) coincides with the period of active differentiation and synaptogenesis. The localization of NGF receptor binding sites to cell bodies and dendrites of Purkinje cells in the postnatal rat cerebellum, suggests, that NGF may play an important role during morphogenesis and/or synaptogenesis. It is now of interest to define the cellular sources of NGF and characterize NGF actions to further elucidate the role of NGF in the context of cerebellar development. These questions are addressed in the following chapters.

Summary

The results presented in this chapter confirm previous immunohistochemical data suggesting that NGF may be a regulatory agent in developing Purkinje cells and demonstrate that Purkinje cells express both low- and high-affinity NGF receptor sites that may be functional during the active phases of cerebellar differentiation and synaptogenesis.

CHAPTER 3

NGF AND EXCITATORY NEUROTRANSMITTERS REGULATE SURVIVAL AND MORPHOGENESIS OF CULTURED CEREBELLAR PURKINJE CELLS

Localization of the biologically active, high-affinity NGF binding sites to developing Purkinje cells suggested that the trophic factor regulates the ontogeny of this specific neuronal population. A system readily accessible to manipulation was required to evaluate the potential roles of trophic and excitatory signals in the development of Purkinje cells. To define mechanisms by which these signals potentially regulate Purkinje cell ontogeny, I studied the effects of NGF and excitatory neurotransmitters on the survival and differentiation of Purkinje cells in dissociated cell culture. In this study, CaBP immunoreactivity was used as the criteria to identify Purkinje cells in culture, and throughout this chapter the term Purkinje cell will be used as an operational definition.

A culture system to study Purkinje cell growth

To evaluate the potential role of NGF during Purkinje cell ontogeny, a culture system allowing analysis of responses to trophic factor stimulation was established. Dissociated embryonic rat cerebellar cell cultures maintained in serum-containing medium fostered survival and differentiation of Purkinje cells. The use of mixed cultures containing neurons and glia (Figs 8, 9), promoted differentiation of Purkinje cells in a relatively rich environment, where interactions between neuronal and glial cell types proved to be essential for normal development of neuritic processes (see below). In this study, Neuron Specific Enolase (NSE) immunostaining (Fig 9) was used to recognize and analyze several

distinct neuronal populations in culture, including Purkinje cells. Neuronal identity of NSE positive cells was confirmed by phase microscopy (Fig 8; phase bright cells), morphological features of cells in culture (Fig 9; process-bearing cells), and absence of glial associated markers (not shown). To visualize Purkinje cells, cultures were immunostained with Vitamin D-dependent calcium binding protein (CaBP) antibodies. Phase microscopy confirmed immunopositivity of the entire cell body and processes of CaBP-positive cells in culture. Immunopositive cells appeared as phase bright neuron-like cells, exhibiting morphology characteristic of developing Purkinje cells (Fig 10).

A detailed characterization of neuronal survival was established in order to define the optimal time in culture for the analysis of NGF actions. Table 2 compares the survival rate of neurons, identified by neuron specific enolase (NSE) immunoreactivity, and Purkinje cells (CaBP-immunoreactive), in a representative set of cultures.

TABLE 2.- Time course of neuronal and Purkinje cell survival in culture

	NSE	**CaBP**	**CaBP/NSE**
Days in culture	cells/dish	cells/ dish	CaBP/NSEx100
1	120,600	27,350	22.60
3	90,140	15,700	17.41
5	71,860	15,220	21.10
7	40,180	2,191	5.45

Figure 8.- Phase photomicrograph of dissociated cerebellar cells in culture. Dissociated E18 rat cerebellar cells were grown in mixed culture for 7 days in the absence (A), or presence of NGF (B), potassium (C), potassium with NGF (D), aspartate (E), or aspartate with NGF (F). Note the presence of phase bright neuron-like cells overlying the phase-dark support cells. Marked differences in the maturation of phase bright neuron-like cells in the potassium with NGF(D), and aspartate with NGF (F) groups are observed. Scale bar, 100μm.

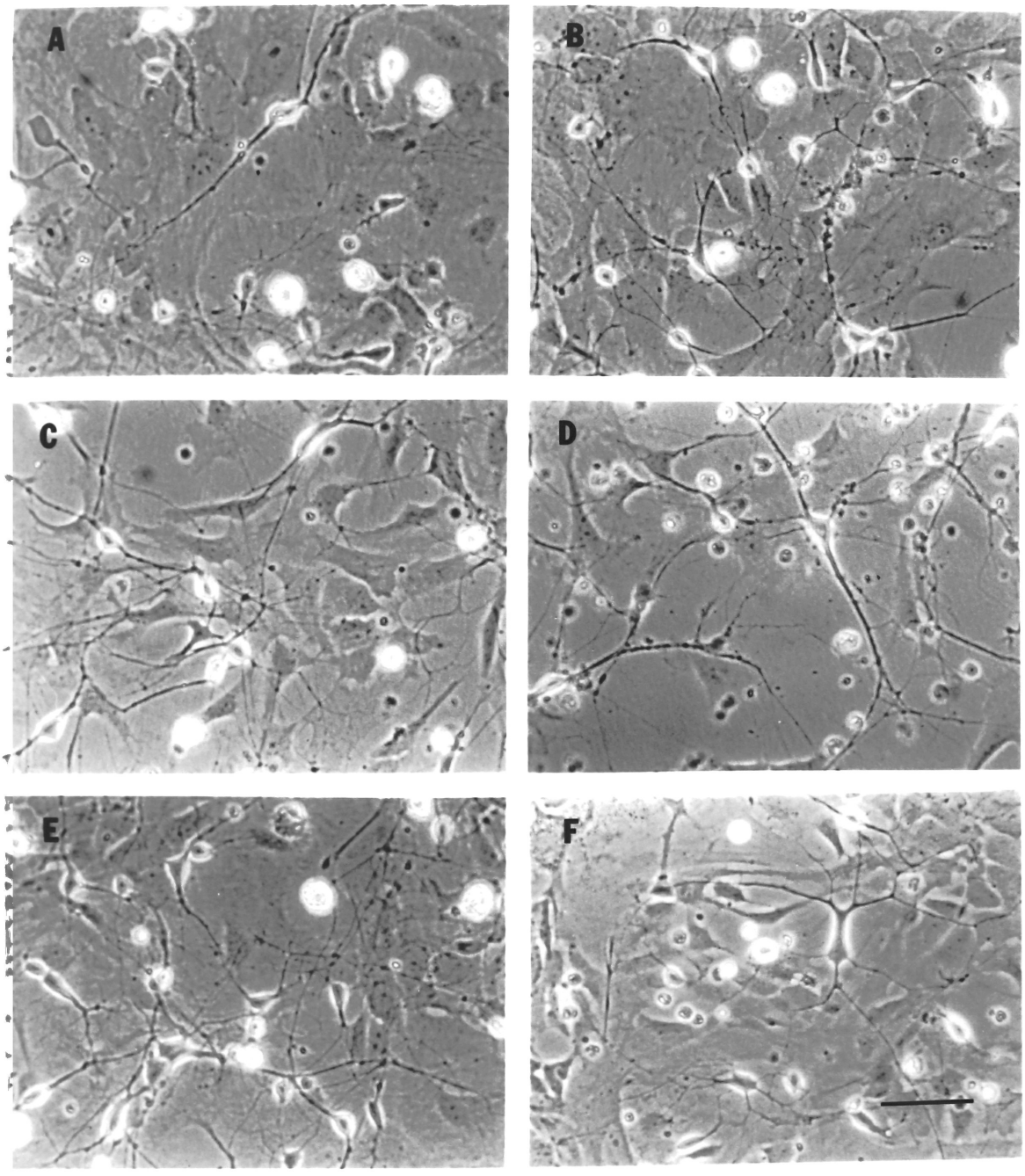
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Figure 9.- Visualization of neurons in culture by immunocytochemical staining with NSE antibody. Cerebellar cells were grown for 7 days in culture under standard conditions (A) or in the presence of NGF (B), potassium (C), potassium with NGF (D),aspartate (E), or aspartate with NGF (F). Note the increase in neuronal cell number and neurite length by treatment with potassium or potassium with NGF. Scale bar, 100μm.

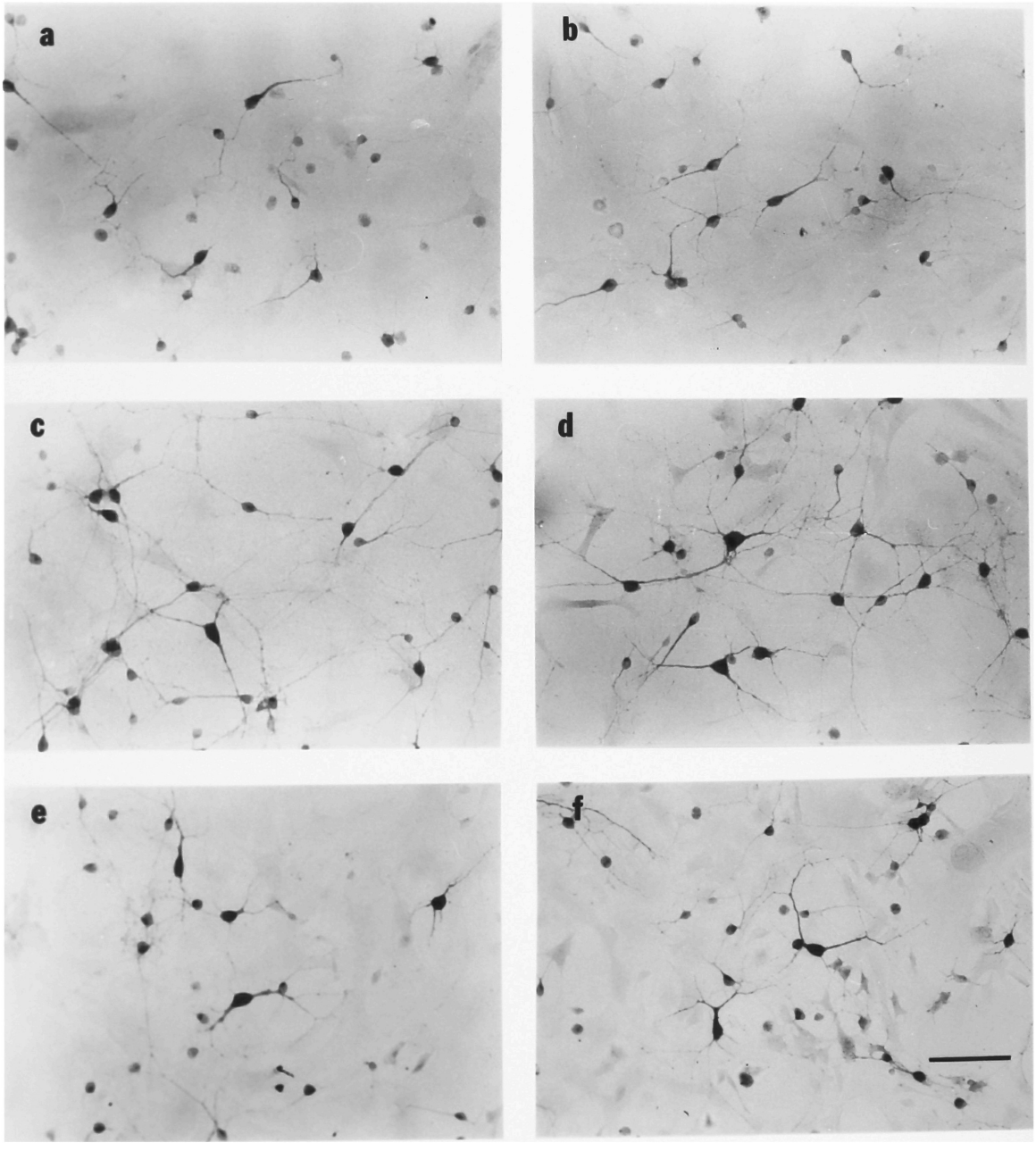
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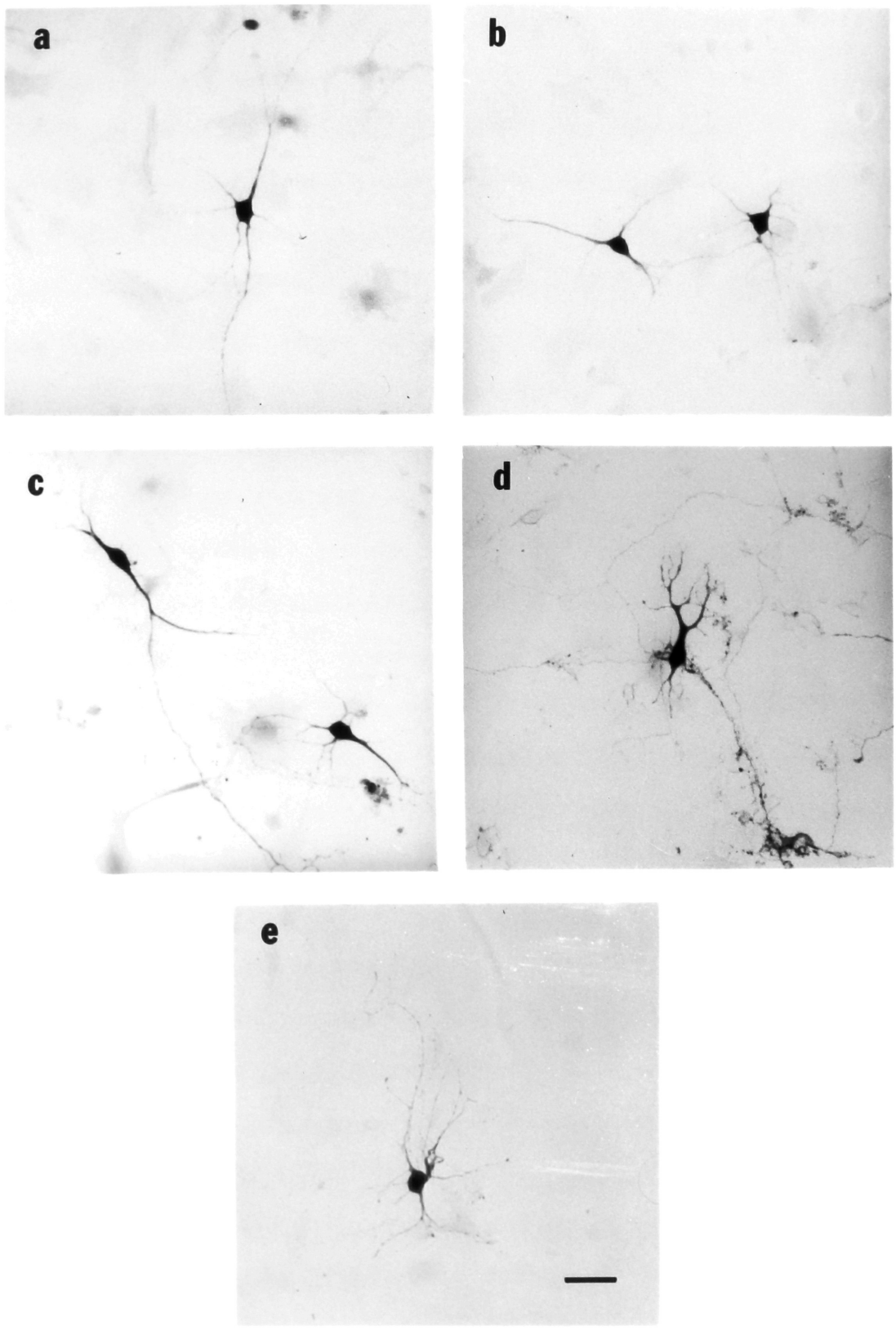
Figure 10.- Immunocytochemical visualization of Purkinje cells in culture. Cerebellar cell cultures were grown for 7 days in the absence (A), or presence of either NGF (B), potassium (C) potassium with NGF (D), or aspartate with NGF (E), and immunostained with Vitamin D-dependent Calcium Binding Protein (CaBP) antibodies. Note the marked enhancement in neurite elaboration by treatment with potassium + NGF (D) or aspartate + NGF (E). Scale bar 40μm.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Neuronal survival declined with time in culture. After one week, only a fraction (10-20%) of the cells plated survived. The decrease in neuron survival was more apparent between days 5 and 7, a time when non-neuronal cells reached confluency. Although survival of all neuronal populations seemed to be affected by unrestricted non-neuronal cell proliferation, a more marked effect was elicited on the Purkinje cell population. The most dramatic effect was seen between days 6 and 7, defining this, as a critical time for Purkinje cell survival. Neuronal survival continued to decline gradually after one week in culture, and Purkinje cells survived up to two weeks. In spite of a marked decrease in survival after 6 days in culture, Purkinje cells continued to differentiate.

The conditions for Purkinje cell growth and differentiation in mixed cultures were compared to those of pure neuronal cultures. Purkinje cell survival was prolonged in cultures grown in serum-free medium, a condition that prevents glial proliferation. However, under these conditions, Purkinje cell differentiation was impaired, since neurons grown up to 22 days failed to develop dendrites (not shown). Consequently, I focused on the study of NGF effects in the development of Purkinje cells under conditions that would allow both the survival and differentiation of this neuronal population. The mixed cultures provided an optimal system to analyze the effects of NGF and excitatory neurotransmitters on survival and differentiation of individual neurons.

Purkinje cells in culture express NGF receptors

While initial studies indicated that Purkinje cells express NGF receptors *in vivo*, expression in culture had yet to be delineated. To determine whether Purkinje cells retain the ability to express NGF receptors in culture, expression of NGF receptors was examined by immunocytochemical criteria. Neurons in culture expressing NGF receptor immunoreactivity were identified as Purkinje cells both morphologically and by CaBP immunoreactivity. Using the optimal culture conditions, the anti-rat NGF-R monoclonal

antibody, 192-IgG, revealed the presence of positive cells that were morphologically identified as developing Purkinje cells (Fig 11). In all cases examined, NGF-R antigenicity co-localized with CaBP immunoreactivity (Fig 12C,D). However, when grown under control conditions, not all CaBP-positive cells were NGF-R positive (see page 56, Chapter 4). Expression of NGF-R by CaBP-positive neurons in culture was confirmed by ^{125}I-NGF binding studies. Incubation with radioactive NGF specifically labeled binding sites on CaBP-positive cells (Fig. 12A). Silver grains were restricted to CaBP-immunopositive cells, suggesting that expression of NGF-R is limited to the Purkinje population. Specific ^{125}I-NGF binding was completely abolished in control cultures incubated with 1000-fold excess unlabeled NGF (Fig 12B). The expression of NGF receptors by Purkinje cells in culture suggests that these cells retain the potential ability to respond to NGF in vitro.

NGF and excitatory signals regulate Purkinje cell survival

Effects of NGF and depolarizing stimuli on Purkinje cell survival

To begin defining the trophic mechanisms that may regulate Purkinje cell development, the possibility that NGF may alter survival was investigated initially. As noted in the introduction, impulse activity apparently regulates Purkinje cell development *in vivo*. Although dissociated cell culture allows the study of neuronal survival in a rigorously controlled series of environments, normal afferents and cell-cell interactions are disrupted. Consequently, I investigated the possibility that NGF and excitatory signals may interact in the regulation of development. Cerebellar cells were grown under depolarizing conditions, and NGF effects were analyzed. Neuronal cell number was markedly increased in cultures grown for 7 days in high-potassium medium (25 mM KCl) (Figs. 9 and 13). Treatment with high-potassium alone increased CaBP cell number by 40% (Fig 13). Moreover, NGF in combination with potassium significantly increased cell number by 75% over control with no potassium (Fig 13). However, NGF had no effect in the absence of high-

Figure 11.- Immunocytochemical visualization of NGF receptor positive cells in culture. Cerebellar cultures were grown for 7 days in the presence of potassium and NGF, and immunostained with the anti-rat 192-IgG antibody. NGF-R immunopositive cells were recognized as Purkinje cells by morphology and similarity to CaBP-positive cells in sister cultures. Scale bar 20μm.

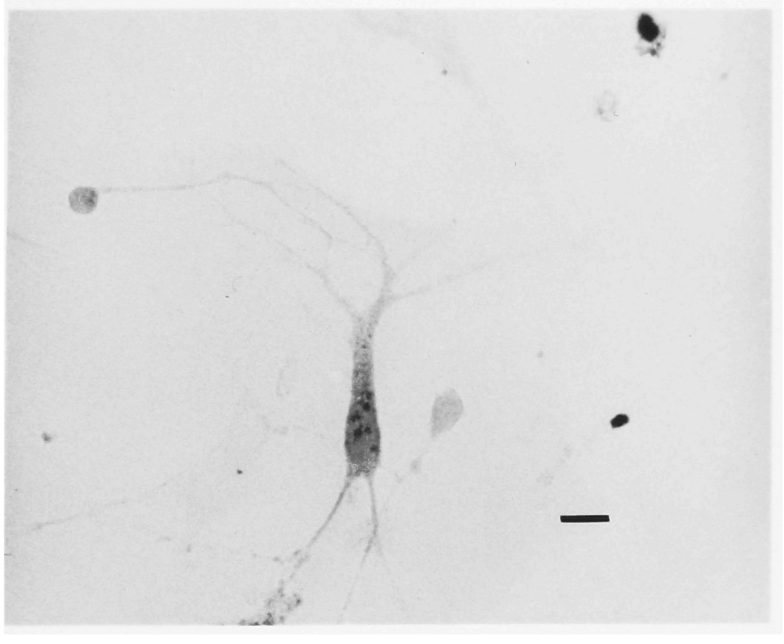
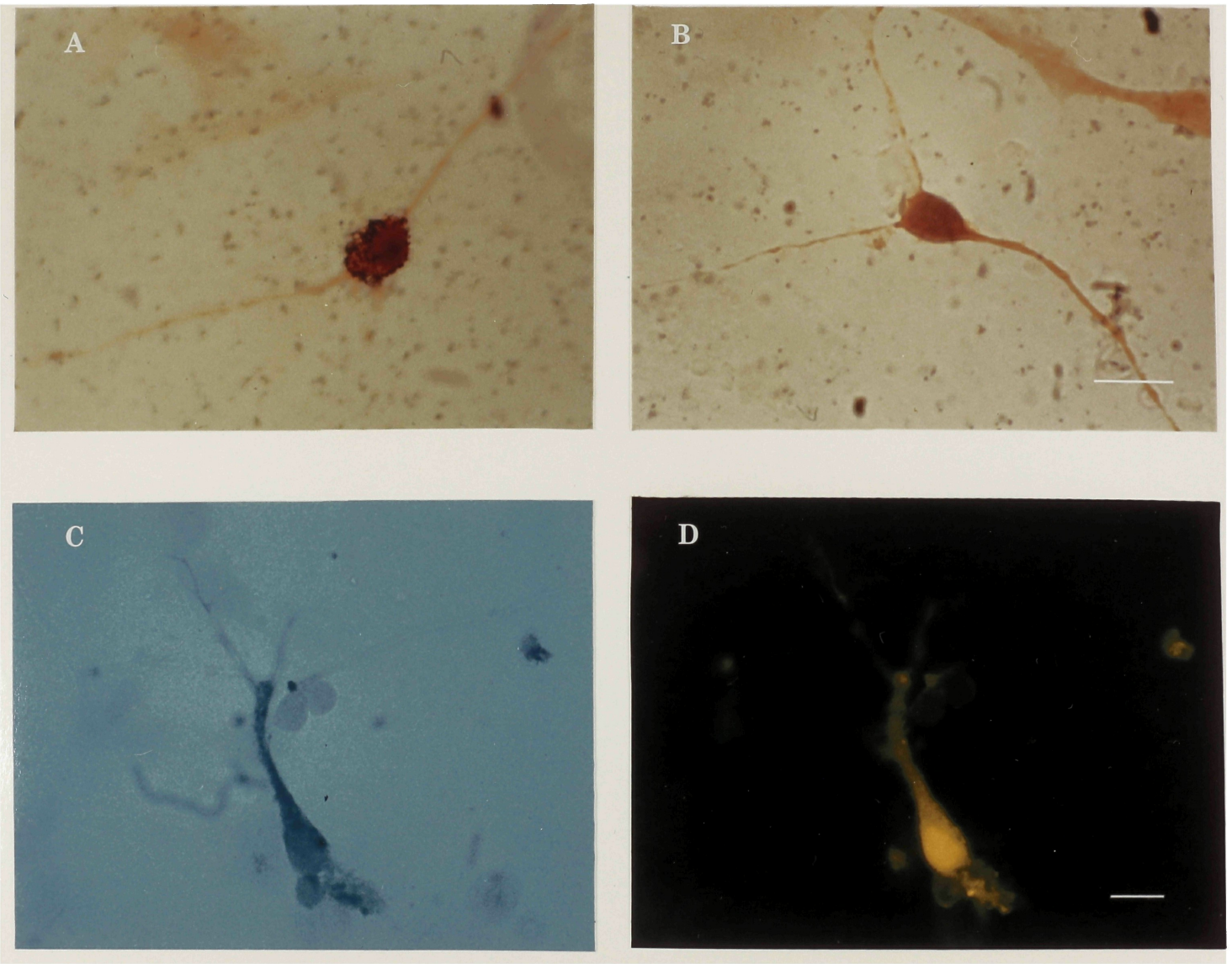


Figure 12.- Localization of NGF receptors to Purkinje cells in culture.

(A, B) ^{125}I-NGF binding to Purkinje cells in culture Cerebellar cultures were grown for 7 days under standard conditions and incubated with ^{125}I-NGF as described in Methods. ^{125}I-NGF binding was identified as silver grains over CaBP immunopositive cells in culture. (A) Bright field illumination reveals the presence of silver grains over an immunoreactive Purkinje cell. (B) In control cultures, only background grains are observed. For binding studies, the ^{125}I-NGF binding protocol was used, cultures were then fixed and immunostained for CaBP and treated for autoradiography. Exposure time 17 days. Scale bar, 20μm.

(C, D) Immunocytochemical visualization of NGF receptor-positive cells in culture. Cerebellar cultures were grown for 7 days in the presence of potassium and NGF, and double stained with the anti-rat NGF receptor and CaBP antibodies. 192-IgG immunopositive cells were visualized by the ABC-diaminobenzidine reaction product, and CaBP-positive cells were visualized with a fluorescein-coupled anti-rabbit IgG. Scale bar, 20μm.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D

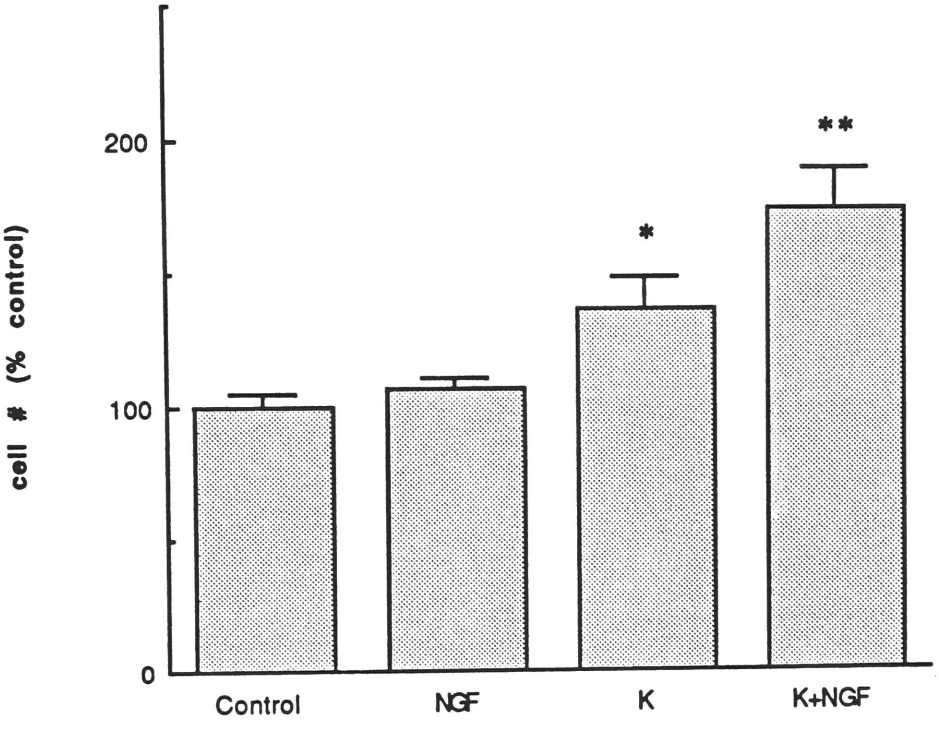
potassium. The effect of NGF and potassium in combination was specific and significantly different from that elicited by either agent alone (Fig 13).

To determine whether depolarization *per se* was responsible for the potassium-elicited effect, cultures were grown in the presence of veratridine. The alkaloid depolarizes neurons by opening sodium channels, a mechanism distinct from that of potassium. To analyze NGF effects in combination with the depolarizing agent, a dose of 0.5 μM veratridine was chosen from dose response curves (Fig 14). At this dose, veratridine elicited a half maximal effect on survival of neurons in culture without affecting their morphological development. Veratridine depolarization increased survival of all neurons, mimicking potassium. Veratridine treatment alone increased CaBP cell survival by 130% (Fig 15). Moreover, a further and significant 70% increase in survival was elicited by NGF in the presence of veratridine (Fig 15). Consequently, exposure to either high-potassium or veratridine was required for NGF actions on cell survival. This observations raised the possibility that afferent excitatory transmitters and trophic agents act in concert to regulate survival.

Effects of NGF and excitatory neurotransmitters on Purkinje cell survival

As noted before, in the developing rat cerebellum, synaptogenesis largely occurs during the first postnatal weeks, coincident with high NGF-R expression. However at birth, prior to the establishment of cerebellar excitatory afferent synapses, developing Purkinje cells are already sensitive to L-aspartate and L-glutamate (Woodward et al., 1971; Dupont et al., 1987). To determine whether normal innervation is necessary for trophic regulation of Purkinje cell development, I analyzed the role of both aspartate and glutamate on survival. The combination of NGF and either aspartate or glutamate markedly enhanced survival of CaBP cells in culture (Figs. 16 and 17). A specific, 100% increase in survival was observed after exposure to aspartate and NGF (Fig 16). A similar effect was obtained

FIGURE 13.- Effects of Potassium and NGF on Purkinje cell survival. E18 rat cerebellar cells were grown in culture in the presence or absence of elevated potassium, NGF or potassium with NGF and immunostained for CaBP. Numbers of positive cells were obtained by analyzing 10% of the dish area. Triplicates of 4 independent experiments were analyzed per condition. Data is expressed as percent CaBP cell number compared to controls grown alone. Each experimental value represents mean cell number ± s.e.m. Statistical analysis was by one-way ANOVA and Scheffe F-test. Doses of 25 mM KCl (K) and 200 U/ml NGF were used. Values depicted are as follows: Control 100±4.89; NGF 108.25±4.17; K 137.71±12.49; K+NGF 175.43±15.44. Actual number of cells in representative control cultures 1923.2±35.21. *Differs from control and NGF; ** Differs from control, NGF, and K by $P<0.05$.


cell # (% control)
200
100
0
*
**
Control
NGF
K
K+NGF

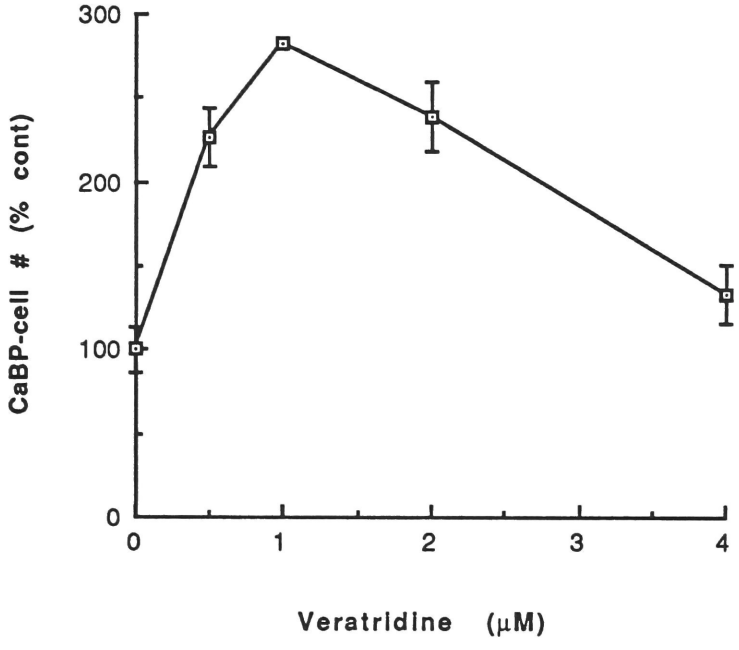


FIGURE 14.- Survival of CaBP-positive cells at different doses of veratridine. A dose-response curve to vertatridine was established by growing E18 rat cerebellar cells in culture in the absence (0) or in the presence of 0.5, 1.0, 2.0 and 4.0 μM veratridine. After 7 days, cultures were immunostained for CaBP, and immunopositive cell numbers were obtained as described in figure 13. Triplicates of two independent experiments were analyzed per condition. Values are expressed as percent CaBP cell number compared to controls grown alone. Each experimental value represents mean cell number ± s.e.m.

FIGURE 15.- Effects of Veratridine and NGF on Purkinje cell survival. E18 rat cerebellar cells were grown in culture and immunostained for CaBP. Numbers of positive cells were obtained as described in figure 13. In this case, duplicates of 4 independent experiments were analyzed per condition. Doses of 0.5 μM Veratridine (VRT) and 200 U/ml NGF were used. Values depicted are as follows: Control 100±5.42; NGF 122.71±6.29; VRT 234.85±8.24; VRT+NGF 286.85±8.02. Actual number of cells in representative control cultures: 1527±64.17. *Differs from control and NGF; **Differs from control, NGF, and veratridine by $P<0.05$.

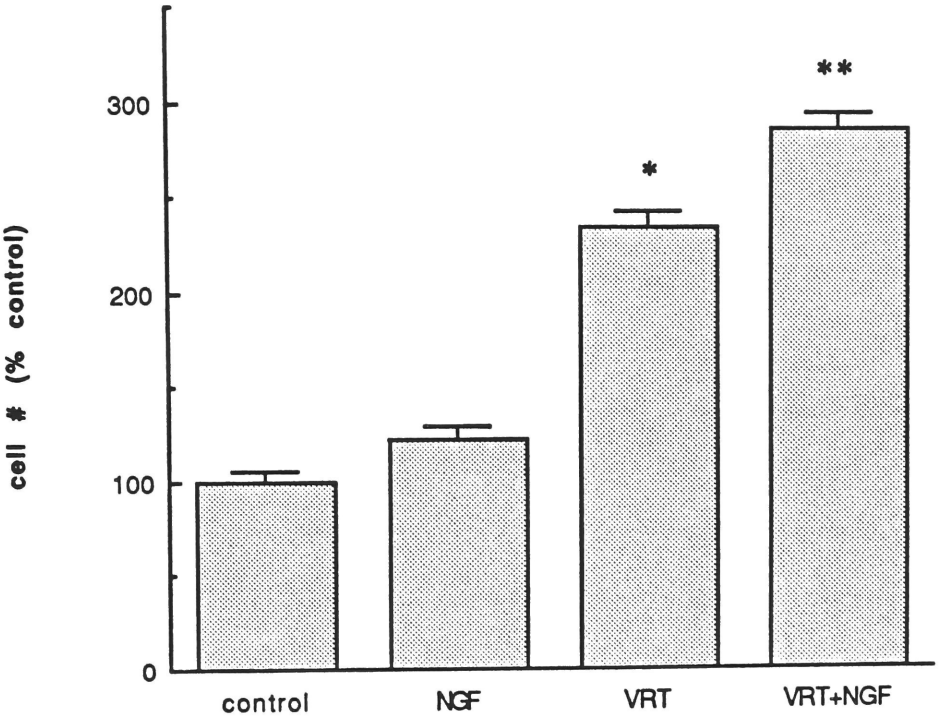

cell # (% control)
300
200
100
0
*
**
control
NGF
VRT
VRT+NGF

FIGURE 16.- Effects of Aspartate and NGF on Purkinje cell survival. E18 rat cerebellar cells were grown in culture, immunostained for CaBP, and analyzed as described for figure 13. In this case, duplicates of 2 independent experiments were analyzed per condition. Doses of 10μM aspartate (Asp) and 200 U/ml NGF were used for the time in culture. Values depicted are as follows: Control 100±4.49; NGF 115.0±6.72; Asp 99.75±8.42; Asp+NGF 194.0±15.70. Actual number of cells in representative control cultures 1688±99.42. *Differs from control, NGF, and aspartate by $P<0.05$.

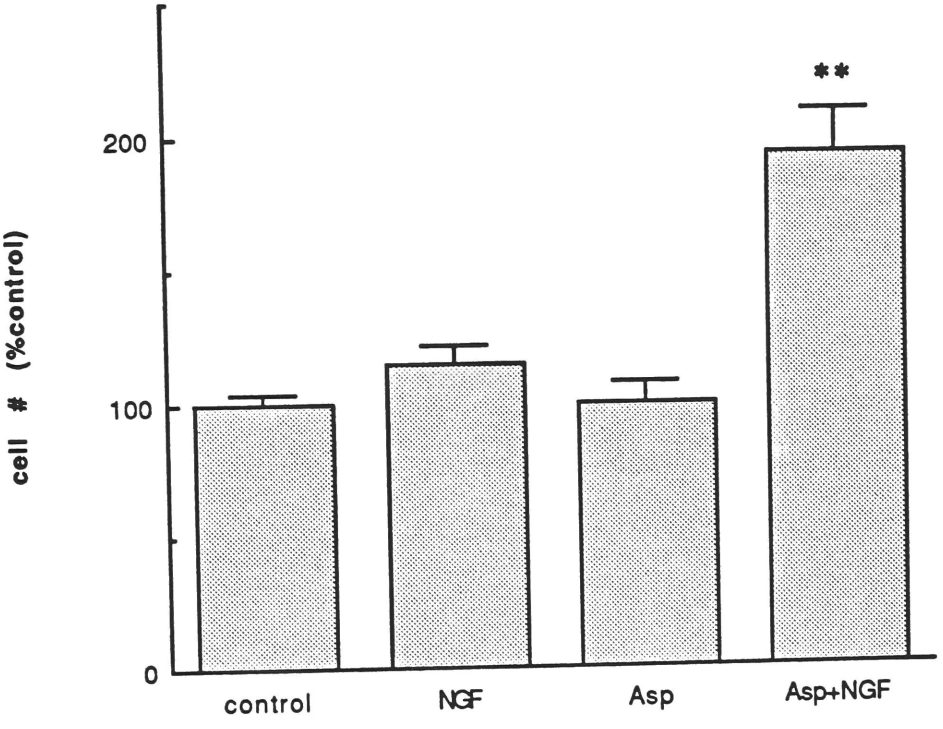
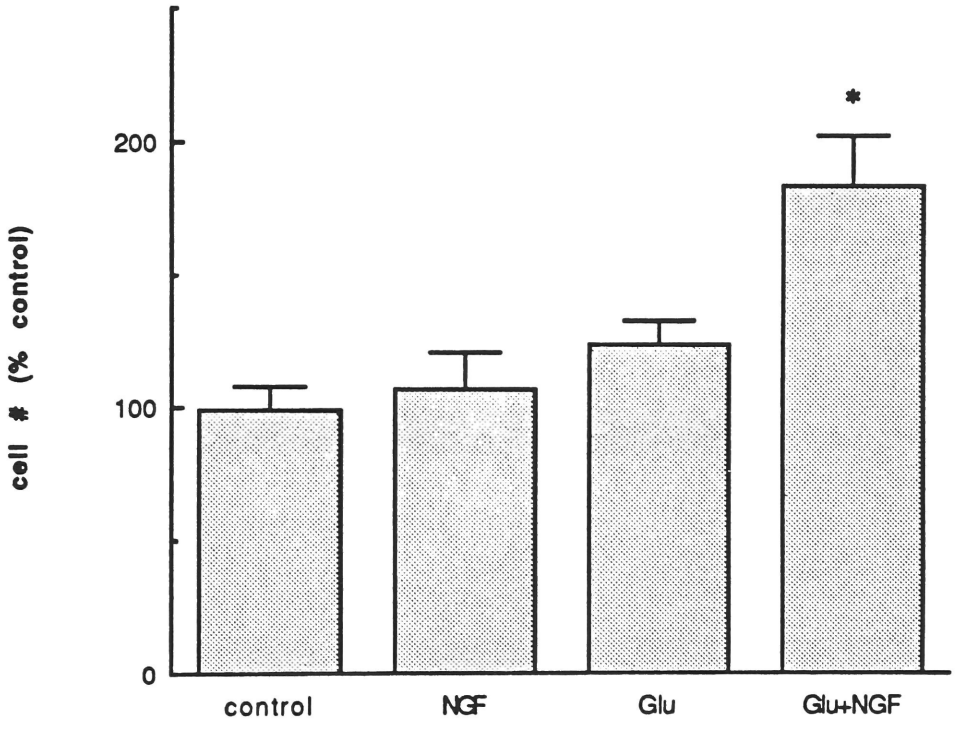

cell # (%control)
200
100
0
**
control
NGF
Asp
Asp+NGF

Figure 17.- Effects of Glutamate and NGF on Purkinje cell survival. E18 rat cerebellar cell cultures were immunostained for CaBP, and analyzed as described for figure 13. Duplicates of 2 independent experiments were analyzed per condition. Doses of 10μM glutamate (Glu) and 200 U/ml NGF were used. Values depicted are as follows: Control 100±8.96; NGF 107.33±14.19; Glu 124.41±9.52; Glu+NGF 184.23±19.02. Actual number of cells in representative control cultures 1691.4±150.95. *Differs from control by $P<0.05$.


cell # (% control)
200
100
0
*
control
NGF
Glu
Glu+NGF

with glutamate and NGF (Fig 17). Unlike effects noted with the pharmacologic agents, the specific transmitters L-aspartate and L-glutamate alone had no effect on the survival of CaBP-positive cells (Figs. 16 and 17). Moreover, upon exposure to higher doses of aspartate (0.10- 1 mM) or glutamate (0.05-1 mM), a toxic effect was observed on all cells. Consequently, *in vivo* excitatory neurotransmitters, in concert with the trophic protein, may regulate Purkinje cell survival.

NGF and depolarizing agents regulate cell size and shape

To further define NGF actions on Purkinje cell development, I examined the effects of NGF and depolarizing agents on the morphological maturation of cells in culture. Several parameters were evaluated by direct comparison of cells in culture (photomicrographs and camera lucida tracings), and by morphometric measurements (Tables 3 and 4).

Neuritic morphology

Potassium and NGF: Treatment with potassium alone significantly increased neurite outgrowth in all CaBP-positive cells (Table 3, Fig 10). Moreover, treatment with both potassium and NGF elicited specific changes, increasing neurite number per cell (Table 3, cf: control) and neurite branching (Table 3, Fig 10). In contrast, NGF alone had no significant effect on neurite number or neurite branching. Exposure to NGF and high-potassium increased the proportion of cells with spiny neurites (Table 3).

Veratridine and NGF: Veratridine alone promoted neurite outgrowth in similarity to high-potassium (not shown). However, in this case, dendrites were less developed than in control, or potassium-treated cultures. Nevertheless, exposure to veratridine and NGF in combination resulted in increased proportion of cells with spiny neurites (Fig 18).

Aspartate/Glutamate and NGF: In agreement with the survival results, treatment with aspartate or glutamate had only a moderate but not statistically significant effect on neurite morphology. However, treatment with excitatory neurotransmitters and NGF markedly enhanced neuritic length and complexity (Figs 10 and 19). NGF, in combination with aspartate, significantly increased neurite branching (Table 4). The treatment similarly increased the proportion of CaBP-positive cells with spiny neurites (Table 4).

Cell size

NGF, in combination with high-potassium (Table 3), aspartate (Table 4, Fig 19) or glutamate (Fig 19) significantly increased the size of the soma of CaBP cells. These effects were not observed by exposure to aspartate, glutamate or potassium alone.

Table 3.- Effects of Potassium and NGF on Purkinje cell size and neurite elaboration.

	CONTROL	NGF	K^+	K^++NGF
Cell diameter (μm)	18.5±0.5	20.4±0.6	20.9±0.5	22.4±0.6*
Neurite Number	4.5±0.2	5.6±0.2	5.6±0.3	6.3±0.2*
Neurite length (μm)	218±24	339±36	521±49*a	592±40*a
Branch Point Number	3.8±0.4	5.1±0.4	5.2±0.4	7.4±0.5*b
Neurons with spiny processes	8%	30%	38%	78%

E18 rat cerebellar cells were grown in culture and immunostained for CaBP. Fifty cells, in 3 separate dishes prepared on the same day, were analyzed per experimental condition. Doses of 25 mM KCl and 200 U/ml NGF were used. Statistical analysis was by one-way ANOVA and Scheffe F-test. Each value represents mean ± s.e.m. *Significantly different from control; *aSignificantly different from control and NGF; *bSignificantly different from control, NGF and potassium. $P<0.05$.

Table 4.- Effects of Aspartate and NGF on Purkinje cell size and neurite branching.

	CONTROL	NGF	ASP	ASP+NGF
Cell diameter (mm)	17.6±0.3	18.1±0.3	19.1±0.4	21.0±0.3*a
Neurite Number	4.3±0.1	4.6±0.1	4.5±0.2	4.8±0.2
Neurite length (mm)	263±38	302±36	352±36	404±36
Branch Point Number	3.8±0.4	4.4±0.3	5.0±0.4	5.8±0.3*
Neurons with spiny processes	1%	2%	12%	26%

E18 rat cerebellar cells were grown in culture and immunostained for CaBP. Fifty cells in 3 separate dishes prepared on the same day, were analyzed per experimental condition. Doses of 10 μM aspartic acid and 200 U/ml NGF were used. Statistical analysis by one-way ANOVA and Scheffe F-test. Each value represents mean ± s.e.m. *Significantly different from control; *aSignificantly different from control, NGF, and aspartate. $P < 0.05$.

Figure 18.- Immunocytochemical visualization of Purkinje cells in veratridine treated cultures. Cerebellar cell cultures were grown for 7 days in the presence of veratridine (a) or veratridine with NGF (b), and immunostained with CaBP antibodies. Note the marked enhancement in formation of spiny-like processes after treatment with veratridine + NGF. Scale bar, 40μm.

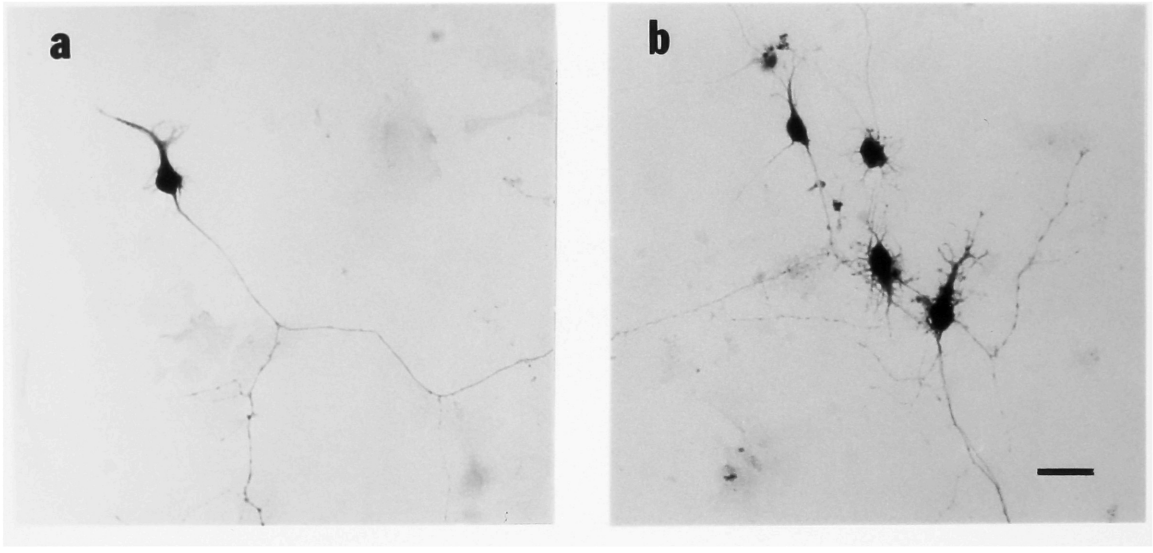
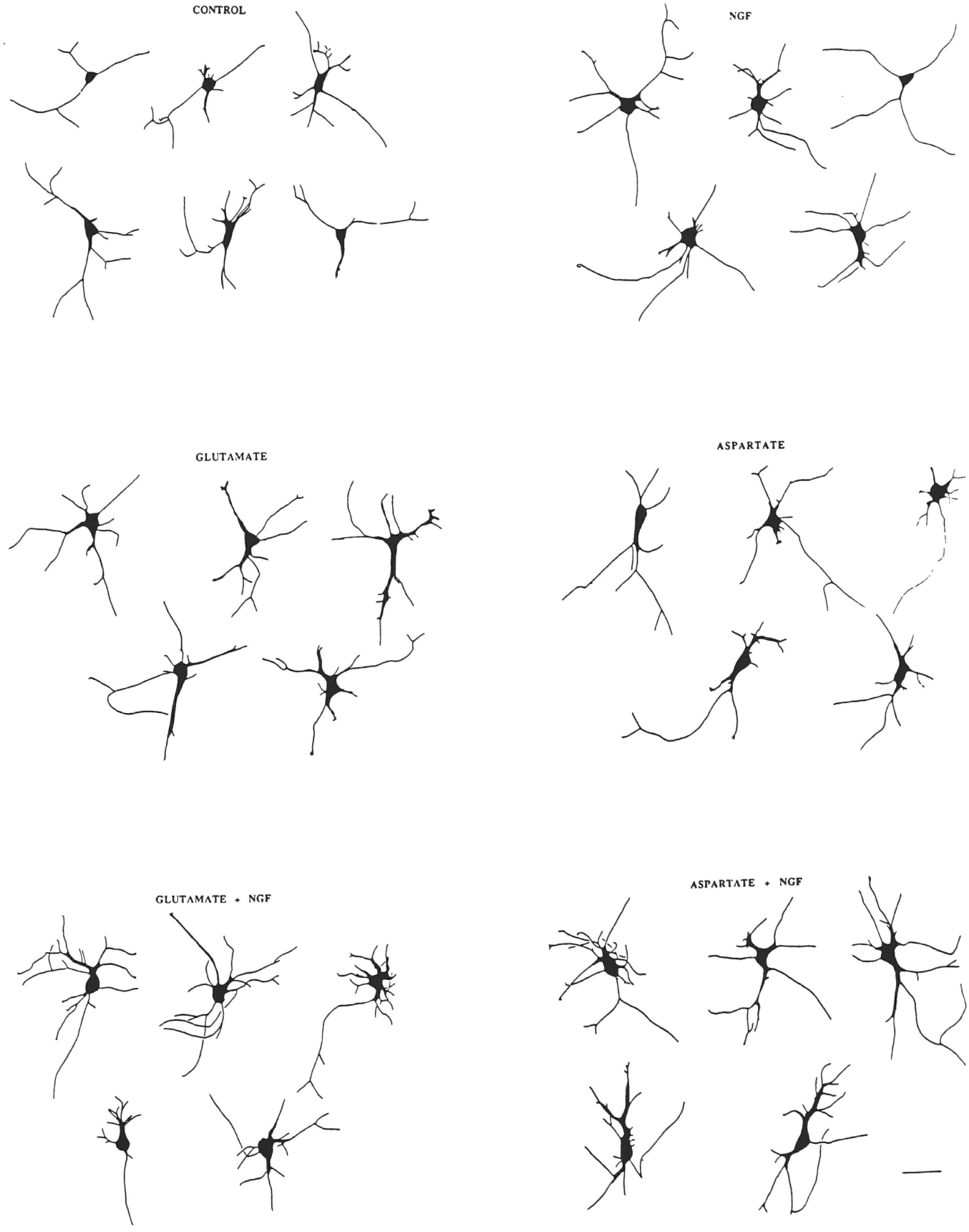

a
b

FIGURE 19.- Morphological maturation of Purkinje cells elicited by excitatory neurotransmitters and/or NGF. Camera lucida tracings of representative CaBP-positive cells were obtained from each experimental condition. Note the marked enhancement in neurite elaboration and in cell size after treatment with glutamate + NGF, or aspartate + NGF. L-aspartate and L-glutamate were used at 10 μM final concentration and NGF was used at 200 U/ml. Scale bar, 40μm.


CONTROL
NGF
GLUTAMATE
ASPARTATE
GLUTAMATE + NGF
ASPARTATE + NGF

DISCUSSION

Localization of the biologically active, high-affinity NGF binding sites to developing Purkinje cells suggested that the factor regulates ontogeny of this specific neuronal population. The evidence presented in this chapter indicates that NGF and depolarizing signals influence survival and morphological differentiation of Purkinje cells in culture.

A culture system to study Purkinje cell growth

To begin defining the trophic mechanisms that may regulate Purkinje cell development, a cell culture system that allowed the study of NGF and excitatory signals in the regulation of Purkinje cell development was established. Dissociated embryonic cell cultures provided a particularly advantageous model, since survival and differentiation of Purkinje cells paralleled that observed *in vivo*. In culture, Purkinje cells developed and expressed several traits normally present *in vivo*. This allowed the immunocytochemical visualization of Purkinje cells with Vitamin D-dependent Calcium Binding Protein (CaBP) antibodies (Christakos et al., 1987) that in turn, permitted me to reliably follow their development in culture.

As an initial approach to define NGF effects during development, the possibility that NGF regulates Purkinje cell survival was examined. In control experiments, Purkinje cell survival declined after one week in culture, coincident with unrestricted non-neuronal proliferation. At this time, only a fraction of the neurons plated survived. Indeed, decreasing survival with time is characteristic of cerebellar macroneurons grown in mixed cultures (Hockberger et al., 1989), and has been associated with a neuronotoxic activity released to the culture medium (Grau-Wagemans et al., 1984). Efforts to eliminate glia by the growth of pure neuronal cultures, or to stop cell division by addition of anti-mitotic

drugs, was harmful for the Purkinje cell population. Interactions between Purkinje cells and glial cells proved to be required for their differentiation in culture.

Dissociated cell cultures proved to be a particularly advantageous model to study Purkinje cell differentiation and survival. However, some of the epigenetic signals known to be important for Purkinje cell development are absent in culture. For example, afferent innervation is known to play a critical role during development, and in cell culture normal circuitry is disrupted. Consequently, I examined the role of afferent neurotransmitters as well as NGF in the regulation of Purkinje cell ontogeny.

During normal cerebellar development, differentiation of Purkinje cells is influenced by two distinct afferent systems, the parallel and climbing fibers. In classical studies, Ramón y Cajal provided the first evidence that climbing fibers guide Purkinje dendritic growth (Ramón y Cajal, 1911); subsequent evidence indicated that climbing fibers play an important role in neurite branching (Bradley and Berry 1976a, 1976b; Sotelo and Arsenio-Nunes, 1976). More recently, Mason and Blazeski (1989) have shown that initial afferent interactions between climbing fibers and undifferentiated Purkinje cells occurs during late embryonic stages, prior to dendritic differentiation. During this early stage of cerebellar development, the local expression of NGF message (Lu et al., 1989), and the expression of NGF receptors by Purkinje cells (Eckenstein 1988, Schatteman et al., 1988, Yan and Johnson 1988; Cohen-Cory et al., 1989), coincide with the establishment of initial climbing fiber contacts. Consequently, these epigenetic signals may interact during development.

Study of cerebellar mutants (Rakic and Sidman, 1973; Sotelo, 1975; Sadler and Berry, 1989), and of granule cell ablation (Berry and Bradley, 1976b; Crepel et al., 1980) have also indicated that excitatory stimulation by parallel fibers is required for normal maturation of Purkinje cells. Indeed, parallel fiber stimulation coincides with the peak of NGF-R message expression (Buck et al., 1988). Therefore, the interaction of NGF and

excitatory influences in the regulation of Purkinje cell development was examined. Initially, receptor expression *in vitro* was examined.

Purkinje cells express NGF receptors in culture

During early development *in vivo*, Purkinje cells express low- and high-affinity NGF receptors (Chapter 2), and in culture, they retain the ability to express NGF receptors. A highly sensitive ^{125}I-NGF binding assay revealed NGF receptors localized to Purkinje cells in control cultures. Moreover, receptor number was enhanced after treatment with depolarizing agents (see below and chapter 4), allowing the detection of NGF receptors by monoclonal antibody binding. In all cases examined, expression of NGF receptors co-localized with CaBP immunoreactivity. The selective localization of NGF receptors to Purkinje cells suggested that receptor expression is specifically limited to this population. In turn, selective expression suggested that Purkinje cells might be responsive to NGF in culture.

NGF and pharmacologic depolarizing agents enhance Purkinje cell survival

The selective expression of NGF receptors by Purkinje cells in culture led me to analyze the putative effects of NGF on the development of this population. Since presynaptic innervation regulates neuronal differentiation in several systems (Black, 1978), and potentially guides Purkinje cell development, the roles of NGF and depolarizing signals were examined. Treatment with either high-potassium or veratridine alone increased numbers of all neuronal types, as indicated by an increase in NSE-positive cells (Fig. 9) and CaBP-positive cells (Fig 13). This result confirmed the well-known effects of depolarizing agents in the promotion of survival of many different populations in culture (Lasher and Zagon, 1972; Nishi and Berg, 1981; Hockberger et al., 1987; Gallo et al.,

1987; Moran and Patel, 1989; Hockberger et al., 1989).

In addition, however, simultaneous exposure to depolarizing agents and NGF specifically increased Purkinje cell numbers in these cultures. NGF, in combination with either high-potassium or veratridine, increased survival of CaBP-positive neurons, an effect significantly different from that of potassium or veratridine alone. Yet, NGF alone did not increase survival of Purkinje cells. The observation that depolarizing signals are required for NGF-induced survival suggests that neuronal depolarization may influence receptivity to the trophic agent. This raised the possibility that afferent excitatory signals in combination with the trophic factor may govern survival.

Although it is apparent from the data that NGF, in combination with depolarizing agents, regulates Purkinje cell survival, two other possibilities warrant consideration. First, NGF could act to induce cell proliferation, potentially resulting in increased Purkinje cell number. This is a less-likely possibility, however, since Purkinje neurons are postmitotic at the time of dissociation. A second possibility is that NGF could act to induce neuronal differentiation, and therefore expression of CaBP. While the latter possibility has not definitively been excluded, published results indicate that CaBP expression is restricted to Purkinje cells in the cerebellum (Christakos et al., 1987), and that expression commences early in differentiation (Legrand et al., 1983). Future studies are needed to discern between these different mechanisms.

NGF and excitatory neurotransmitters enhance Purkinje cell survival

Climbing and parallel fibers constitute the two major excitatory afferents of Purkinje cells; L-aspartate and L-glutamate are generally regarded as the respective excitatory transmitters (Ito, 1984). Therefore, I examined the effects of aspartate and glutamate on cell survival. Treatment with aspartate or glutamate, in combination with NGF, markedly enhanced survival of Purkinje cells. This observation suggests that the combination of

excitatory transmitter and NGF is required to enhance survival. Although Purkinje cells are sensitive to excitatory amino acid iontophoresis at the time of dissociation (Woodward et al., 1971; Dupont et al., 1987), the transmitters alone do not alter Purkinje cell number (Hockberger et al., 1989; S. Cohen-Cory, personal observations). This evidence suggests a novel mechanism, in which trophic factors and afferent stimulation interact to promote survival of developing Purkinje cells.

The effects of excitatory neurotransmitters on the survival of Purkinje cells, apparently differ from those of the general pharmacologic depolarizing agents. While potassium and veratridine alone increased cell survival, aspartate and glutamate elicited effects only in combination with NGF. The fact that naturally occurring transmitters had more restricted effects, suggests that these agents act through specific receptors, expressed only by restricted populations in culture. Preliminary data suggests that aspartate effects are mediated, at least in part, through non-NMDA receptors (see future perspectives in chapter 5). Alternatively, aspartate and glutamate may act through a mechanism distinct from depolarization. A putative role for excitatory neurotransmitters as trophic agents during development remains a possibility.

Differences of effects of pharmacologic depolarizing agents and excitatory neurotransmitters may also be attributable to different mechanisms of action. Elevated potassium is known to act through a more general mechanism to alter survival (Lasher and Zagon, 1972; Kingsbury et al., 1985; Moran and Patel, 1989; Hockberger et al., 1989). Potassium and veratridine, consequently, may stimulate multiple neuronal and non-neuronal populations, leading to manifold indirect effects. In contrast, since NGF receptors seem to be exclusively expressed by Purkinje cells in culture (see above) the trophic factor probably acts directly on this specific population.

NGF and excitatory neurotransmitters regulate cell size and shape

While afferents are known to regulate the morphological differentiation of Purkinje cells (Rakic and Sidman, 1973; Bradley and Berry, 1976a, 1976b; Berry and Bradley 1976a; Sotelo and Arsenio-Nunes, 1976; for review see Ito, 1984), underlying mechanisms remain unclear. To define mechanisms by which the two epigenetic signals, NGF and innervation, may regulate differentiation, the effects of trophic and excitatory stimulation on morphological maturation were examined.

In fact, NGF increased cell size and promoted neurite elaboration of developing Purkinje cells. The effects required simultaneous exposure to NGF and either aspartate, glutamate or pharmacologic depolarizing agents. Although moderate effects were seen after exposure to excitatory neurotransmitters or NGF alone, statistically significant effects were observed when these agents were used in combination. These results suggest that presynaptic transmitters and NGF act in concert to induce differentiation. The morphological features exhibited by these neurons in culture are similar to the features of Purkinje cells developing *in vivo* (Ramón y Cajal 1911, Altman, 1972; Berry and Bradley, 1976b; Morris et al., 1985).

Regulation of neurite elaboration by NGF in combination with aspartate, the putative climbing fiber transmitter, or with glutamate, the putative parallel fiber transmitter, suggests that early afferent stimulation and NGF interact to regulate dendritic development *in vivo*. In that event, the temporal appearance of the two afferent signal categories may fine tune the regulation of dendritic arborization.

A potential mechanism for the interaction of depolarizing signals and trophic influences.

In this chapter, I have presented evidence indicating that NGF, in combination with the putative excitatory neurotransmitters, aspartate and glutamate, promotes the survival

and morphological maturation of Purkinje cells in culture. This evidence suggested a novel mechanism for regulation of development, in which trophic factors and afferent stimulation interact. It was therefore of interest to define the potential mechanisms by which these epigenetic signals regulate brain neuron development.

The apparent interaction of neurotransmitters and trophic signals in the regulation of development may potentially be attributable to multiple mechanisms. For example, the two epigenetic signals may share a common intracellular regulatory locus, the level of which may dictate the developmental effects.

A second possibility is that one factor may modify the response to the second factor, thus potentiating the effects of the latter. For example, impulse activity may directly increase responsiveness to the trophic agent. This theoretically can be achieved in at least two ways: Excitatory neurotransmitter stimulation may induce modifications in the affinity of the receptor and/or in receptor coupling to intracellular signal transducers, leading to a cascade of events that result in physiological responses to trophic factor stimulation. Another possibility is that excitatory neurotransmitters may regulate the specific expression of receptors, therefore increasing the number of receptor sites on responsive cells.

During the final phase of my doctoral research, the possibility that excitatory influences may modulate the expression of NGF receptors by Purkinje cells in culture was investigated. In addition, to begin identifying the potential cerebellar source for NGF synthesis, and to examine the possibility that NGF expression may also be subject to regulation by depolarizing signals, I studied the possibility that NGF message is expressed by cerebellar cells in culture. The results of these studies are presented in the following chapter.

EXCITATORY STIMULI REGULATE THE EXPRESSION OF THE GENES FOR NGF AND ITS RECEPTOR

To define mechanisms by which trophic and excitatory signals interact to promote survival and morphogenesis of Purkinje cells, the possibility that depolarizing influences increase NGF receptor expression in the cerebellum in culture was examined. As a parallel approach to define the potential mechanism by which NGF may regulate Purkinje cell development, the nature of the putative source of NGF during cerebellar development was also investigated.

Purkinje cells express NGF receptors in culture

The studies presented in chapter 2 of this thesis indicate that Purkinje cells express NGF receptors *in vivo*. In addition, initial studies in culture indicated that Purkinje cells retained the ability to express NGF receptors after dissociation (chapter 3). In these studies, expression of NGF receptors by Purkinje cells in culture was confirmed immunocytochemically using an NGF receptor antibody. In these cultures, 192-IgG immunoreactivity co-localized with the expression of CaBP, a specific marker for Purkinje cells within the cerebellum (see Fig 12 C,D in chapter 3).

During early cerebellar development *in vivo*, Purkinje cells exhibit biochemical and morphological heterogeneity (Wassef and Sotelo, 1984; Wassef et al., 1985), perhaps reflecting differences in the time of origin among distinct subpopulations of Purkinje cells (Altman, 1978). Consequently, not all Purkinje cells may be equally responsive to NGF at the same time in development. To begin investigating this possibility, NGF receptor

expression by Purkinje cells was examined further. In cultures grown under control conditions, the number of neurons immunopositive to NGF-R was markedly smaller than the number of CaBP-positive cells present in sister cultures (Fig 20). However, exposure to high-potassium markedly increased the number of NGF-R positive cells (Fig 20) while the number of CaBP-positive cells did not change significantly (see Table 5). This result suggests that depolarizing agents may regulate receptor expression in culture.

Pharmacologic depolarizing agents increase detectability of NGF receptor protein

To begin defining whether depolarizing agents regulate NGF receptor expression, the level of detectable NGF receptor protein was determined initially. The method of affinity cross-linking of ^{125}I-NGF to receptor sites allows quantitation of NGF/receptor complexes. To evaluate NGF receptor protein levels in cultures exposed to depolarizing signals, embryonic day 18 rat cerebellar cells were grown for 6 days in dissociated cell culture and assayed for NGF binding. Cells in suspension were incubated with ^{125}I-NGF at a concentration that would bind specifically to both low- and high-affinity sites. Using the soluble cross-linker EDAC, a specific cross-linked protein complex of 100 kDa was purified electrophoretically from cultured cerebellar cells (Fig. 21A). The cross-linked complex migrated identically to that obtained from cultured rat PC12 cells (Green and Greene, 1986; Grob et al., 1983; see also Fig. 21A), and correlated with the size of the NGF/receptor complex purified from adult rat brain (Taniuchi et al., 1986). This result indicates that in the cerebellum, detectable NGF receptors correspond to those of other systems.

The specificity of the NGF/ NGF-R complex was further confirmed by displacement of the radiolabeled NGF after incubation with an excess unlabeled NGF. Exposure of cultures to depolarizing concentrations of potassium (25 mM KCl) increased

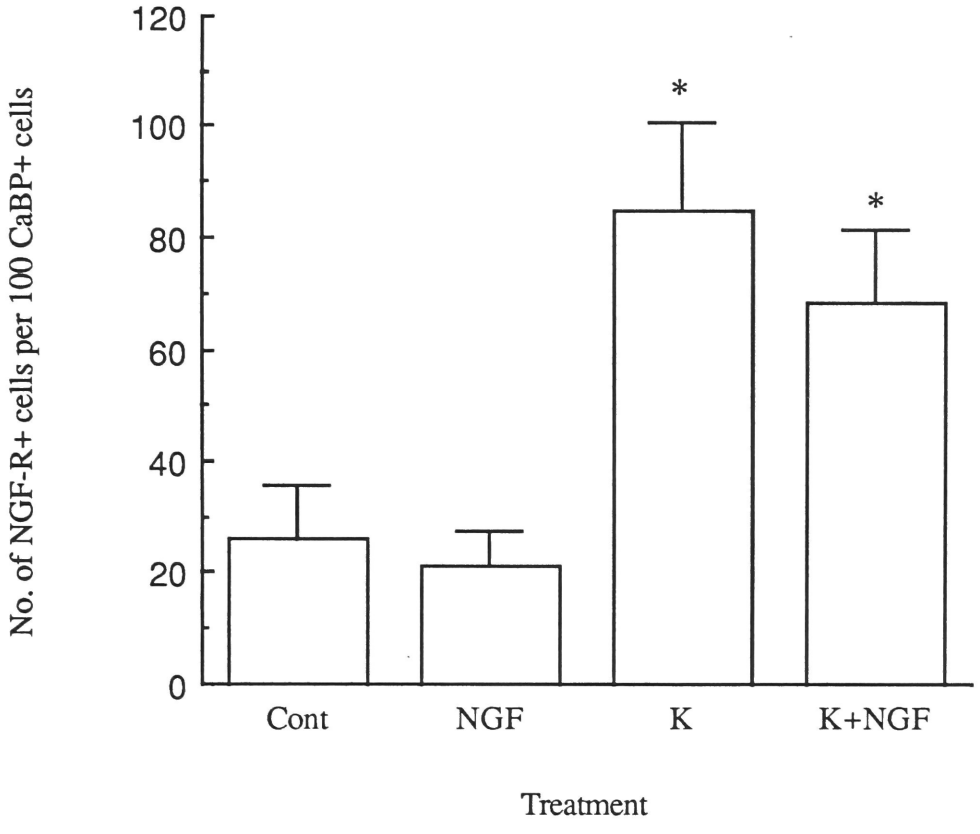
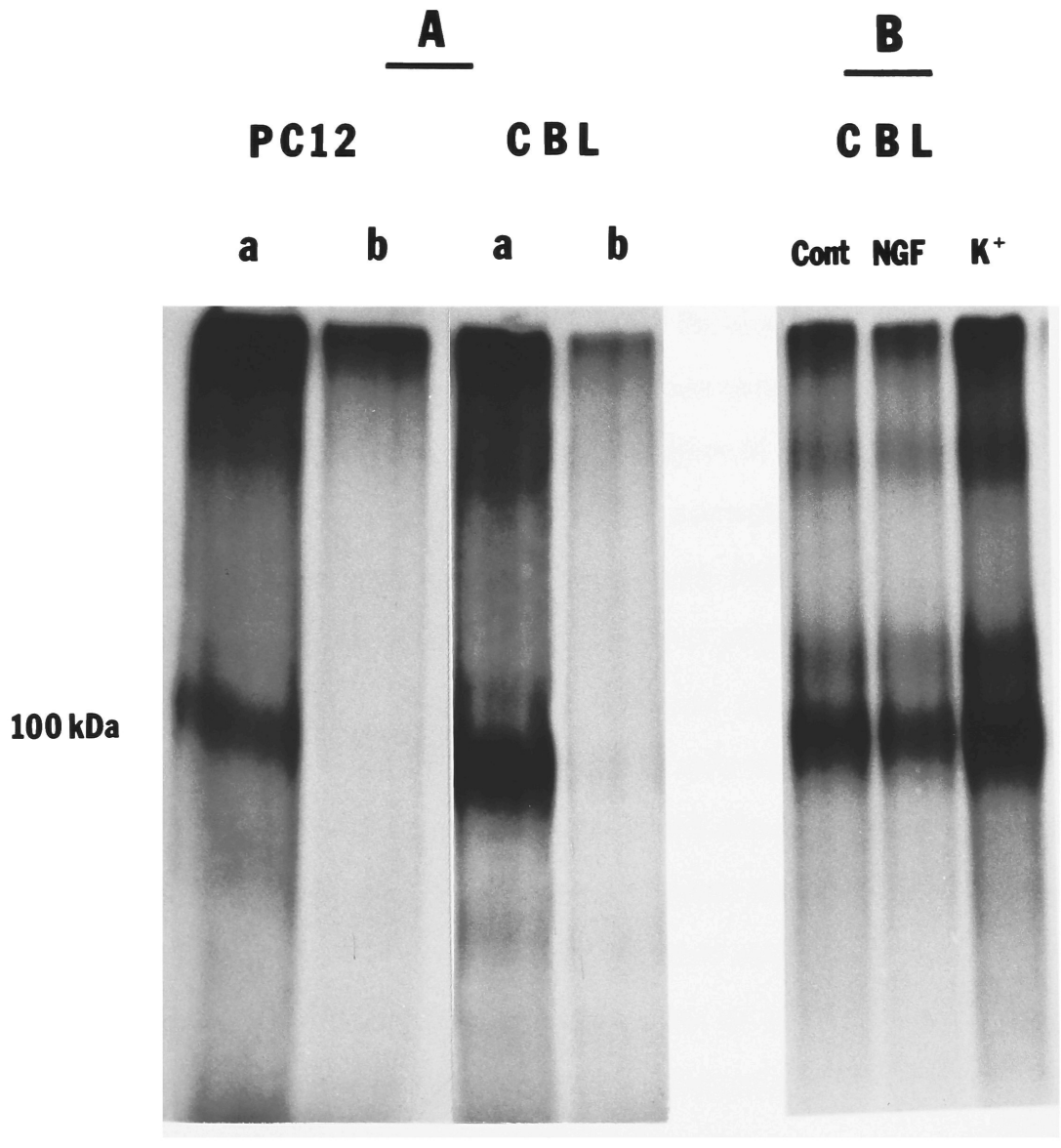


Figure 20.- Comparison between CaBP-positive and NGF-R-positive cell numbers in sister cultures. Embryonic cerebellar cell cultures were grown for 7 days under standard conditions or in the presence of NGF, potassium (K) or potassium with NGF (K+NGF). Sister cultures were immunostained with either CaBP or 192-IgG antibodies and immunopositive cell numbers were determined. For each experimental condition, values were adjusted to number of NGF-R positive cells per 100 CaBP-positive cells. Values represent results obtained from 4 independent experiments and are depicted as follows: Control 26±9.67; NGF 21±6.45; K 85± 15.9; K+N 68.33±13.32. Statistical analysis was by one-way ANOVA and Scheffe F-test. * Differs significantly by $P<0.05$.

Figure 21.- Affinity cross-linking of NGF receptors from cerebellar cells in culture. A) PC12 cells (PC12) and embryonic cerebellar cells in culture (CBL) were incubated with 1 nM ^{125}I-NGF, in the absence (a) or presence (b) of 1μM unlabeled NGF, and cross-linked with the specific agent EDAC. The samples were washed, suspended in sample buffer, and subjected to electrophoresis on a 7% polyacrylamide gel. The gel was exposed for autoradiography. Note the presence of a specific 100kDa protein complex in both PC12 and CBL cells, that is specifically displaced by incubation with excess unlabeled NGF (b). B) Cerebellar cells were grown under standard conditions (Cont) or in the presence of NGF, or high-potassium (K). After 7 days in culture, cells were detached, incubated with ^{125}I-NGF and cross-linked in the presence of EDAC. The equivalent to 0.3 mg of total cellular protein was loaded per sample, and electrophoresed as described above. Compare the intensity of the band in potassium versus control conditions.


A
B
PC12
CBL
CBL
a
b
a
b
Cont
NGF
K+
100 kDa

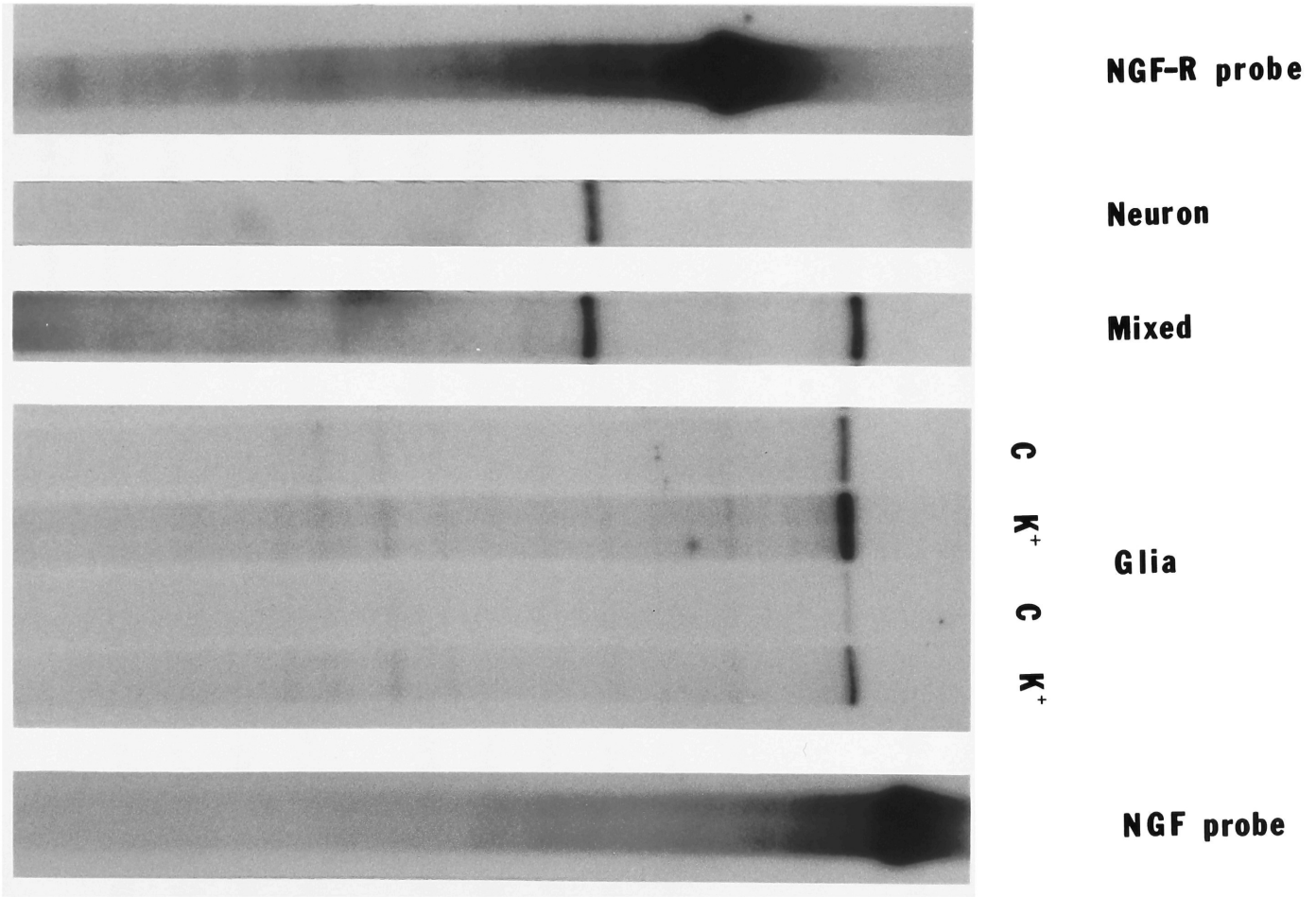
detectable receptor levels two-fold (Fig. 21B), thus indicating that depolarizing agents may modulate NGF receptor levels on Purkinje cells.

Purkinje cells express the NGF receptor gene, while glia in culture express the NGF gene.

To begin defining mechanisms responsible for elevated NGF receptor protein, gene expression was examined. A sensitive ribonuclease protection assay (Lu et al., 1989; Lu et al., in press) was employed to study expression of the NGF-R gene in mixed cerebellar cell cultures (containing neurons and non-neuronal cells). Total RNA obtained from embryonic cultures was hybridized with both NGF and NGF-R riboprobes. Following hybridization, any remaining unhybridized single stranded sequences were digested with RNAse T2. The resulting protected fragments were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography (see Chapter 6 for a description and a diagram of the technique). This method allowed the simultaneous analysis of both NGF and NGF-R message levels from individual sets of cultures. Expression of both NGF and NGF-R mRNA's was detected in mixed cerebellar cultures grown for 6 days under control conditions (Fig. 22). Expression of NGF message indicated the presence of a cellular source for NGF in the system, suggesting that within the cerebellum there is a capacity for trophic regulation of neuronal function .

To define specific cell types expressing NGF-R, gene expression was studied in isolated neuronal and glial populations. Virtually pure neuronal cultures were established by taking advantage of the fact that non-neuronal cells do not proliferate in the absence of serum. Embryonic rat cerebella were dissociated and grown in serum free medium (SFM) for 6 days. At this time, less than 4% of the population in culture were non-neuronal cells. NGF-R message was detected in both neuron-enriched and mixed cultures (Fig. 22).

Figure 22.- Expression of NGF and NGF-R mRNA's in cerebellar cells in culture. Mixed, neuronal, or glial cerebellar cells were grown in culture for 6 days. Glial cultures were grown in the absence (C) or in the presence of 25 mM KCl (K), at medium (first two bands) or high density (last two bands). RNA extraction and ribonuclease protection assays were performed as described in the method section. Twenty micrograms of total RNA were assayed simultaneously with NGF and NGF-R probes. Double stranded protected fragments were separated and analyzed in polyacrylamide sequencing gels, and exposed for autoradiography. Undigested NGF and NGF-R riboprobes were run in parallel as molecular weight controls. The NGF protected fragment (411 base fragment) is detected in mixed and glial cultures, while NGF-R protected fragment (270 base fragment) can only be detected in mixed and neuronal cultures. Note increased NGF message levels after treatment of glial cultures with high-potassium.


NGF-R probe
Neuron
Mixed
C
K+
C
K+
Glia
NGF probe

However, no NGF message was detected in neuron-enriched cultures. Consequently, while expression of the NGF-R gene was associated with the neuronal population, the NGF gene was expressed by a different population in culture.

To further characterize the cellular source of NGF, I examined gene expression in glia-enriched cultures. Specific expression of the NGF gene was observed in glial cultures (Fig. 22). Consequently, while expression of NGF-R is restricted to the neuronal population, glial cells expressed the NGF gene. Consequently, these results suggest that during cerebellar ontogeny, glia may provide a local source of NGF, at a time when Purkinje cells are responsive to trophic stimulation.

Depolarizing signals regulate expression of the NGF receptor gene

To investigate potential mechanisms by which depolarizing signals modulate trophic responsiveness, the possibility that excitatory signals regulate NGF-R gene expression was examined. NGF-R message levels were analyzed in mixed cerebellar cultures grown in the presence of depolarizing signals. Exposure of mixed cultures to high-potassium resulted in a 2- to 3-fold increase in NGF-R mRNA levels (Figs. 23, 24), suggesting that depolarizing signals regulate receptor gene expression.

To determine whether depolarization *per se* elicited the effect, mixed cultures were grown in the presence of veratridine and analyzed for NGF-R message expression. Exposure to veratridine resulted in a two-fold increase in NGF-R message levels (Fig. 25). Consequently, depolarizing signals may regulate responsiveness to the trophic agent by increasing NGF-R gene expression by Purkinje cells.

I have previously reported that depolarizing signals and NGF interact to regulate survival of Purkinje cells in culture (Chapter 3). Consequently, the apparent increase in NGF-R message levels may simply be attributable to increased cell survival. To examine this possibility, NGF receptor gene expression was characterized in cultures grown under

conditions that minimize survival effects (high density, 6 days in culture). Although CaBP-positive cell numbers increased by 40% after exposure to veratridine alone or potassium in combination with NGF (Table 5), exposure to depolarizing agents increased NGF-R message levels by 200 - 300% (Fig. 24). Consequently, these results suggest that increased NGF-R message levels result, at least in part, from increased gene expression.

Excitatory signals directly affect neurons

Interactions between developing neurons and supportive glia may be required for the modulation of NGF-R expression by depolarizing signals. Alternatively, depolarizing agents may act directly on the neuronal population. To examine this possibility, NGF-R expression was analyzed in virtually pure neuronal cultures. As observed for mixed cultures, treatment of neurons with high-potassium resulted in a 4 fold increase in NGF-R message levels (Fig. 23). This result indicates that depolarizing signals may act directly on the neuronal population and that interactions between glial and neuronal cells are not required for these effects.

Excitatory neurotransmitters regulate expression of the NGF receptor gene.

During early development in vivo, expression of NGF receptors by developing Purkinje cells coincides with the establishment of excitatory afferent contacts. Purkinje cell differentiation begins with the early formation of afferent climbing fiber contacts. As described in chapter 3, aspartate, the putative climbing fiber transmitter, interacts with NGF to promote Purkinje cell development in culture. Thus, excitatory neurotransmitter stimulation may regulate Purkinje cell development by influencing receptivity to the trophic agent. As described above, depolarizing agents may influence receptivity to NGF by increasing expression of the NGF-R gene by potentially responsive neurons. To examine the possibility that afferent excitatory neurotransmitters may elicit responsiveness to NGF

by a mechanism similar to that of the pharmacologic depolarizing agents (i.e. by increasing NGF-R gene expression), the role of aspartate on NGF-R gene expression was analyzed. Treatment with 10μM aspartate resulted in a two-fold increase in message levels (Fig 26). This effect was observed in both neuron-enriched or mixed cultures, suggesting that aspartate acts directly on the neuronal population.

Purkinje cells receive inhibitory and well as excitatory innervation during the postnatal period of development (see Chapter 1). To investigate the possibility that inhibitory signals also modulate receptor expression, cerebellar cells in culture were exposed to GABA, the major inhibitory transmitter candidate in the cerebellum. Exposure to GABA had no effect on the expression of NGF receptor gene in mixed culture (Fig 25).

During cerebellar development, the establishment of excitatory synaptic contacts on Purkinje cells is followed by the formation of inhibitory synapses. Prior exposure to excitatory signals may be required for inhibitory agents to modulate gene expression. To test this possibility GABA was added to cultures in combination with high-potassium and NGF-R message levels were determined. No difference in message levels was seen after exposure of cultures to GABA in combination with potassium (Fig 25). Consequently, it appears that at least in the presence of general depolarizing agents, GABA does not modulate the effects of the excitatory agent.

Does NGF regulate expression of its receptor ?

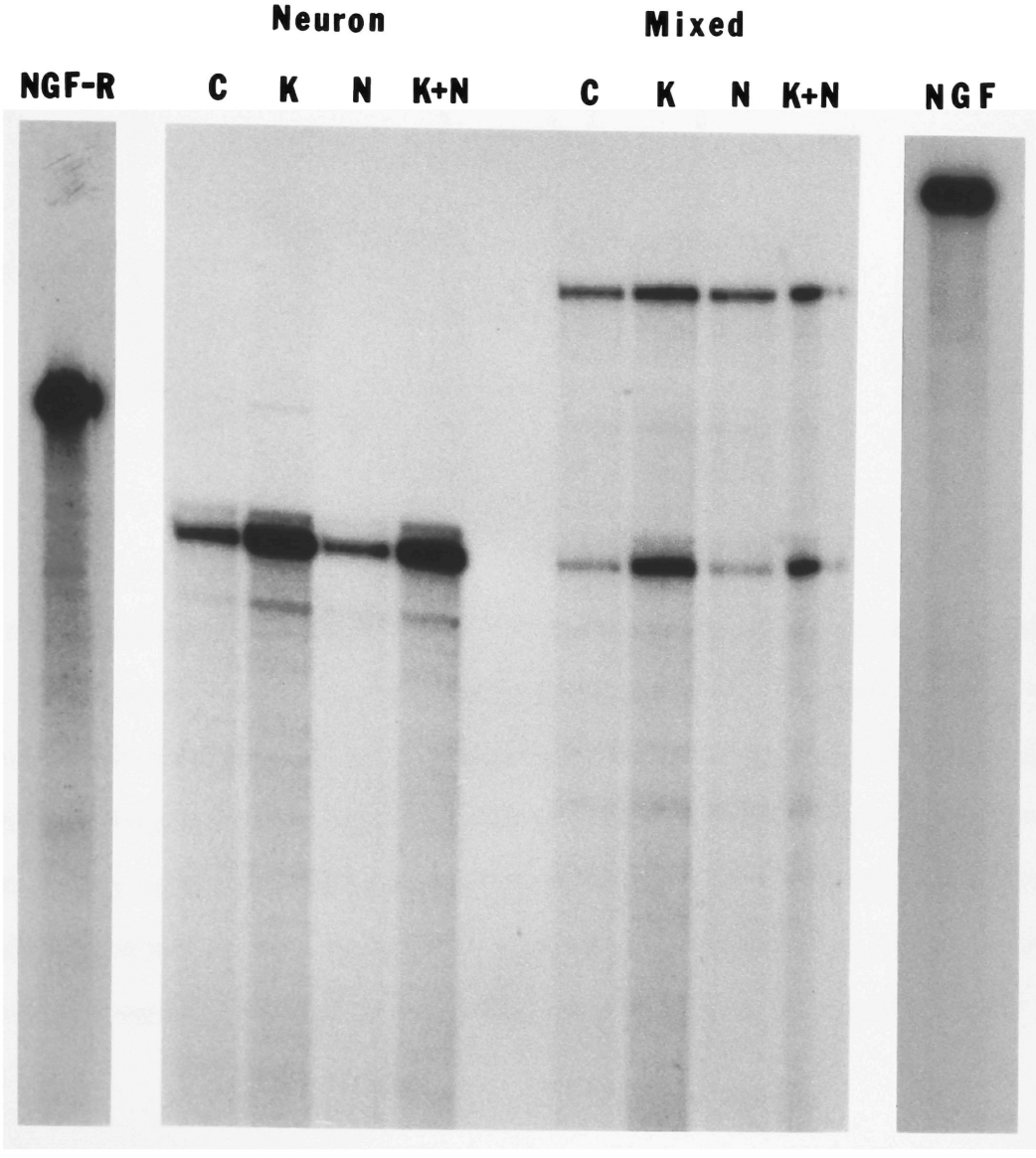
Recent evidence indicates that NGF may up-regulate NGF receptor expression in some neuronal systems (Elliott et al., 1990; Lindsay et al., 1990). These observations raised the possibility that NGF may also modulate NGF receptor gene expression by Purkinje cells. In contrast to observations in other systems, we have found that NGF did not alter NGF receptor message levels in the cerebellum in culture, either alone, or in combination with depolarizing agents (Figs. 23, 24). Consequently, in the cerebellum, a

distinct mechanism of trophic factor regulation may apply.

Expression of the NGF gene is regulated by depolarizing agents

The regulation of trophic function by excitatory signals may occur at multiple levels. For example, in addition to modulation of NGF-R gene expression, impulse activity may regulate the expression and/or release of the trophic agent itself, thus influencing its availability to neurons that are dependent of trophic support. Indeed, preliminary evidence obtained in this study indicates that expression of the NGF gene by cerebellar glia is subject to regulation by depolarizing agents. Densitometric analysis revealed a two-fold increase in NGF mRNA levels was observed after exposure of glia in culture to high-potassium (Fig 22). Consequently, impulse activity may influence trophic support by increasing expression of both trophic and receptor proteins.

Figure 23.- Effects of high-potassium on NGF and NGF-R message levels. Expression of NGF and NGF-R mRNA's was analyzed in pure neuronal and mixed cultures grown under control conditions (C) or in the presence of potassium (K), NGF (N), or potassium with NGF (K+N). Twenty micrograms of total RNA were assayed simultaneously with NGF and NGF-R riboprobes, and analyzed as described for figure 23. Undigested NGF and NGF-R riboprobes were used as molecular weight markers. Note the marked increase in the intensities of the bands after treatment with K or K+N.


Neuron
Mixed
NGF-R
C
K
N
K+N
C
K
N
K+N
NGF

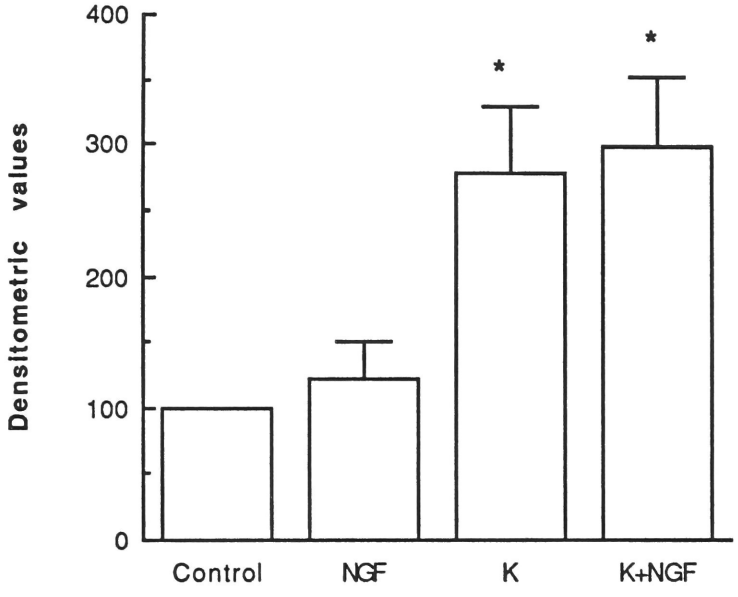


Figure 24.- Densitometric analysis of NGF-R mRNA levels in mixed cerebellar cell cultures. Cerebellar cell cultures were grown for 6 days under control conditions or in the presence of NGF, potassium (K), or potassium with NGF (K+NGF), and RNA samples were obtained. Autoradiographs of NGF-R ribonuclease protection assays were analyzed by densitometry, and arbitrary units of 100 were assigned to control lanes. Four independent experiments were analyzed. Values represent the mean ± s.e.m. Statistical analysis was by one-way ANOVA and Scheffe F-test. *Differs from control and NGF by $P<0.05$.

Figure 25.- Comparison of veratridine, high-potassium, and GABA effects on NGF and NGF-R mRNA levels. Mixed cerebellar cell cultures were grown for 6 days in the absence (Cont) or in the presence of 0.5μM veratridine (Vrt), 25 mM KCl (K), 50 μM GABA (GABA), or potassium + GABA (K+GABA). RNA was purified and twenty micrograms from each sample were analyzed by the ribonuclease protection assay. The arrows point to the digested NGF and NGF-R protected fragments, respectively.

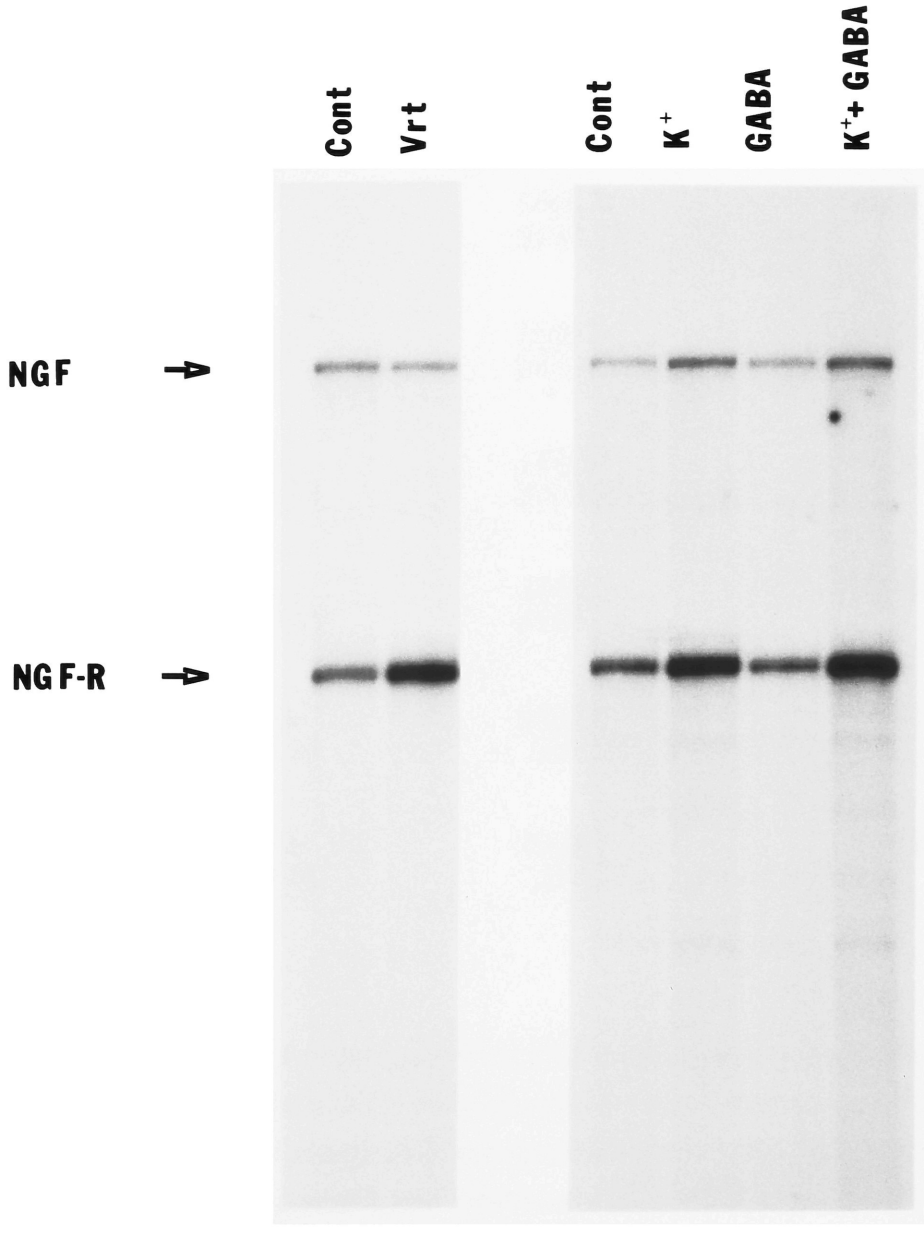
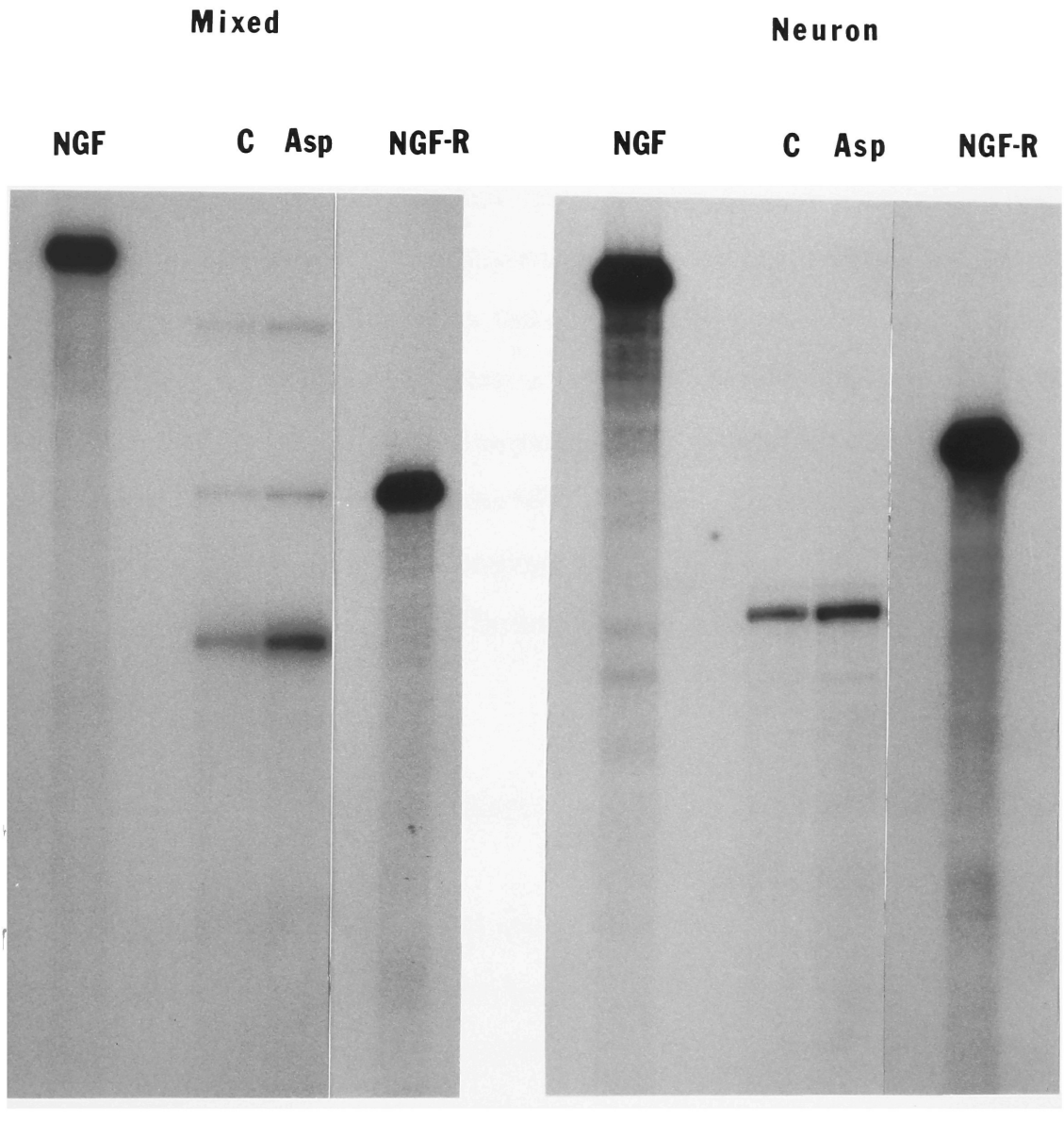

Cont
Vrt
Cont
K+
GABA
K++ GABA
NGF
NGF-R

Table 5. Purkinje cell numbers in representative cerebellar cultures after 6 days in vitro.

Condition	CaBP+	NSE+	CaBP/NSE
Control			
mean	100	1490.3	6.7%
SE		±136.72	
NGF			
mean	110.0	1566.3	7.02%
SE	±15.30	±217.67	
K			
mean	102.6	1430.0	7.13%
SE	±9.82	±118.87	
K+NGF			
mean	147.0	1441.3	10.2%
SE	±3.15	±167.74	
VRT			
mean	146.0	1722.3	8.47%
SE	±17.05	±87.21	
VRT+NGF			
mean	151.6	1322.3	11.42%
SE	±9.35	±75.29	

Purkinje and neuronal cell numbers surviving after 6 days in representative mixed cultures. Sister cultures to those assayed for NGF-R mRNA expression were immunostained for CaBP or NSE to identify Purkinje cells and neurons respectively. Immunopositive cells in 10% of the dish area were counted, and values were adjusted relative to 100 CaBP positive cells in control cultures. Three independent experiments were analyzed. Values represent mean cell number ± standard error of the mean. Actual number of cells in a 100 mm^2 area in control cultures was; CaBP = 558±53.7, NSE = 8691±680.02.

Figure 26.- Effects of aspartate on NGF-R mRNA levels. Mixed and pure neuronal cerebellar cultures were grown under control conditions (C) or in the presence of 10 μM L-aspartate (Asp) for 6 days. Twenty micrograms of total RNA were assayed simultaneously with NGF and NGF-R riboprobes. Undigested NGF and NGF-R probes are used as molecular weight markers. Note the increase in intensity of the NGF-R protected fragment bands after aspartate treatment. (On the Mixed group, an excess of undigested NGF-R riboprobe is seen).


Mixed
Neuron
NGF
C
Asp
NGF-R
NGF
C
Asp
NGF-R

DISCUSSION

As noted in the Introduction, the acquisition of NGF responsiveness by Purkinje cells is developmentally coincident with differentiation and the establishment of initial afferent contacts. Indeed, the evidence presented in Chapter 3 suggests that afferent innervation and NGF interact in the regulation of Purkinje cell development. Afferent regulation of trophic function may constitute one mechanism through which trophic and transmitter signals interact to modulate neuronal development and function. The results presented in this chapter provide evidence that supports such a mechanism, and suggest that excitatory signals increase responsiveness to NGF by inducing Purkinje cell NGF receptor gene expression. Moreover, the data presented also indicate that cerebellar glia in culture have the capability of expressing the NGF gene, and that expression may also be regulated by depolarizing signals. Consequently, impulse activity may regulate both receptor expression and synthesis of NGF by local glia, a potential cerebellar source of the trophic agent.

Purkinje cells express NGF receptors, while cerebellar glia express the NGF gene

During cerebellar development in vivo, expression of NGF receptors has been detected on cells in the external granular layer, Purkinje cells, and cells in the deep cerebellar nuclei (Eckenstein, 1988; Schatteman et al., 1988; Yan and Johnson, 1988). As shown in chapter 3, developing Purkinje cells retain the ability to express NGF receptors in culture and are responsive to trophic factor stimulation. Studies on the selective localization of NGF receptors to Purkinje cells were extended in this last part of the work. The data presented in this chapter suggest that expression of the NGF-R gene is restricted to the neuronal population: specific message could be detected in either neuron-enriched or mixed

cultures, but not in glial cultures (Fig 22). Moreover, NGF receptor immunoreactivity co-localized with CaBP in all cases (Fig 12). Consequently, NGF-R expression is associated with the Purkinje cell population in culture.

In the developing cerebellum, NGF is expressed coincidently with the peak in NGF receptor expression, suggesting that NGF may act as a local trophic agent (Lu et al, 1989). Although expression of NGF mRNA in the developing cerebellum had been demonstrated, the nature of its cellular source remained to be determined. To begin identifying the cell populations that express the NGF gene, NGF mRNA expression in isolated neuronal and glial populations was studied. Selective expression of NGF was restricted to the glial population in culture.

Expression of the NGF gene has been associated with several glial populations both *in vivo* (Finn et al., 1987; Lu et al., in press) and in culture (Lindsay, 1979; Norrgren et al., 1980; Furukawa et al., 1987; Assouline et al., 1987; Lu et al., in press), in addition to some adult neuronal populations (Ayer-LeLievre et al., 1988; Whittemore et al., 1988). Although I have observed that NGF expression is restricted to cerebellar glia in culture, I can not exclude the possibility that NGF is also expressed by neurons, since expression may be associated with a neuronal population absent in the culture system, or may be expressed at very low and undetectable levels. The definitive localization of NGF gene expression in the developing cerebellum *in vivo* remains to be determined. Future *in situ* hybridization studies may help characterize cell types expressing NGF in the developing cerebellum. Nevertheless, the results presented in this chapter suggest that, at least in part, proximate glia may foster Purkinje cell development by providing a source of the trophic agent.

Depolarizing signals regulate NGF receptor gene expression

During development, the influences encountered by a growing neuron and its immediate environment may ultimately determine cell fate. In addition, interactions between distinct epigenetic signals may influence their normal developmental program. Presynaptic innervation plays a major role in neuronal differentiation (Black, 1978), and may also modulate trophic support during ontogeny (Purves, 1988). Consequently, the possibility that impulse activity influences NGF receptor expression by developing Purkinje cells was examined. Treatment with high-potassium increased the number of NGF-R-immunopositive cells in culture relative to the number of CaBP-positive cells, suggesting that the proportion of Purkinje cells that express NGF receptors may increase by the treatment. In addition, a significant increase in detectable cross-linked receptor protein complex was observed after exposure to the depolarizing agent, indicating that depolarizing signals may increase the level of NGF-R protein. These observations suggested that NGF-R expression was indeed regulated by depolarizing signals; however, the level at which regulation takes place remained unclear.

Increased NGF-R protein levels reflected an increased NGF-R gene expression. Exposure of cultures to pharmacologic depolarizing agents resulted in a significant increase in NGF-R message levels. Although high-potassium and veratridine increased neuronal survival, these effects were smaller than effects on NGF-R message levels, suggesting that depolarizing agents increase NGF-R message levels per cell. The increase in NGF-R gene expression suggests that afferent innervation may influence receptivity to the trophic agent.

Afferent transmitters regulate NGF receptor gene expression

Afferent innervation is known to influence the morphological differentiation of Purkinje cells in vivo (Ramón y Cajal, 1911; Rakic and Sidman, 1973; Berry and Bradley, 1976; Bradley and Berry, 1976 a,b; Sotelo and Arsenio-Nunes, 1976). I have found that in

culture, aspartate and glutamate, interact with NGF to induce differentiation of Purkinje cells. One possible mechanism by which afferent neurotransmitters regulate trophic function is by directly regulating receptivity to the trophic agent. In this study, the effects of aspartate on the expression of the NGF-R gene were examined. Treatment with aspartate increased NGF-R gene expression two fold. This effect was observed whether neurons were grown in isolation or in the presence of cerebellar glia, suggesting that the excitatory neurotransmitter acts directly on the neuronal population. Not surprisingly, direct effects on the neuronal population were also observed after treatment with pharmacologic depolarizing agents.

In addition to excitatory stimulation, innervation by inhibitory interneurons may potentially modulate trophic function. Consequently, the effects of GABA, the major inhibitory neurotransmitter in the cerebellum, on the regulation of NGF-R gene expression were examined. Message levels were not affected by treatment with GABA, suggesting that inhibitory signals do not affect receptor expression.

Expression of the NGF gene is regulated by depolarizing signals.

It has been proposed that impulse activity may modulate post-synaptic synthesis and/or release of trophic factors, which in turn, act on presynaptic terminals to stabilize connections (Henderson, 1987; Purves, 1988). Present studies indicate that in the cerebellum, glial expression of the NGF gene is subject to regulation by high-potassium. High-potassium, acting as an impulse activity-generated intercellular signal may promote this effect. A potential mechanism by which depolarizing signals may regulate NGF gene expression by cerebellar glia deserves further and detailed exploration.

A potential mechanism for the regulation of trophic function

The evidence presented in Chapter 3 indicate that normal afferent neurotransmitters interact with NGF to regulate Purkinje cell development. These agents may exert their effects by acting in combination, or sequentially. One example of a sequential event is that afferent neurotransmitters may increase NGF receptor gene expression, thus increasing receptor protein synthesis and in turn, increasing responsivity to NGF. Indeed, I have found that excitatory signals increase detectability of the NGF receptor protein. Another mechanism of receptor regulation that still deserves further investigation is whether receptor affinity is also subject to regulation by epigenetic signals, and if this event is independent of receptor expression.

In addition to its effects on receptor regulation, neural activity may modulate the availability of the trophic agent itself, by specifically increasing its synthesis and/or release from a target or local source. Indeed, my preliminary results indicate that cerebellar glia in culture have the capability of synthesizing NGF, and that expression of NGF may also be subject to up-regulation by depolarizing signals. Based on the evidence presented, a potential mechanism of epigenetic interaction may be proposed: Impulse activity from afferent fibers may increase Purkinje cell responsiveness to trophic agent stimulation by specifically regulating receptor expression. Simultaneously, neural activity modulates the synthesis and release of the trophic agent by surrounding glia, increasing its availability in the local environment. The coincident increase in trophic factor availability, and in the number of functional receptor sites, results in trophic support of Purkinje cells. This in turn, may lead to neuronal differentiation and strengthening of synaptic connections.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

It has long been suggested that nerve growth factor plays a major role in the development of the central nervous system. Indeed, increasing evidence indicates that NGF regulates the ontogeny of central cholinergic neurons (Thoenen et al., 1987; Whittemore and Seiger, 1987; Dreyfus, 1989). A potential role for NGF in the development of other brain areas has been proposed (Large et al., 1986; Escandón and Chao, 1989; Lu et al., 1989; Allendoerfer et al., 1990). However, its role during cerebellar ontogeny remained to be defined.

The work presented in this thesis offers direct evidence that NGF regulates the development of cerebellar Purkinje cells in culture and suggests possible mechanisms. Initial evidence indicated that Purkinje cells have the capability of expressing high-affinity NGF receptors during development. Biological actions of NGF are correlated with high-affinity binding (Greene and Shooter, 1980; Thoenen and Barde, 1980). Thus, the transient expression of high affinity receptors by Purkinje cells suggested that receptor sites might be functional at the time of active differentiation and synaptogenesis. It was then of interest to define cellular sources of NGF in the cerebellum and characterize NGF actions to further elucidate the role of NGF in the context of cerebellar development.

During development, individual neurons are exposed to many environmental signals that eventually determine cell fate. Interactions among signals, and appropriate temporospatial expression, may regulate normal, orderly neuronal development and function. The concept that neural activity modulates trophic interactions during development has gained considerable support over the last few years (Purves, 1988). Consequently, it became important to define the role that afferent neurotransmitters as well as NGF play in the regulation of Purkinje cell ontogeny. By studying Purkinje cell

development in culture, I obtained direct evidence that NGF regulates survival and differentiation of Purkinje cells, and that for this to occur, the presence of excitatory neurotransmitters or pharmacologic depolarizing signals is required. This evidence suggested that afferent excitatory neurotransmitters and NGF interact to regulate the survival and morphological differentiation of developing Purkinje cells.

The apparent interaction of neurotransmitter and trophic agents in the regulation of neuron development may potentially be attributable to multiple mechanisms. The studies presented in the last part of this thesis, in fact, suggest a novel mode of interaction. Afferent excitatory influences may increase receptor expression by Purkinje cells, making these neurons more receptive to trophic factor stimulation. Moreover, cerebellar glia, acting as a potential source of NGF may respond to neural activity by increasing trophic factor synthesis and providing trophic support for developing neurons.

The results presented in the last part of this work indicate that depolarizing agents may increase expression of NGF by glia in culture. It is possible then that in mixed culture, depolarizing signals in the absence of exogenous NGF, may be able to promote some of the "trophic" effects of NGF. This indirect effect, mediated by increased NGF release by glia, may account , at least in part, for the observed effects on neuronal survival, or morphological differentiation of Purkinje cells. The molecular mechanism by which excitatory signals may regulate Purkinje cell responsiveness to NGF, deserves further exploration.

Together, the evidence presented in this doctoral dissertation suggest that afferent innervation in combination with trophic support may govern Purkinje cell survival and differentiation *in vivo*. Moreover, these findings open new avenues to further investigate how these interactions take place in the developing animal, and how their perturbation may alter the normal developmental program.

Future perspectives

Several new avenues of research derive from the evidence presented in this thesis. The mechanism by which NGF and excitatory neurotransmitters regulate Purkinje cell survival can be further explored to define the level of specificity at which both trophic and excitatory signals exert their effects. Here, I present two sets of preliminary data that may provide new insights. One supports the concept that NGF is critical for survival of Purkinje cells, and the second further investigates the mechanism by which excitatory amino acids act.

Anti-NGF antibodies decrease the survival of Purkinje cells

One possible way to define the level of dependency of trophic factor stimulation by developing neurons is to block trophic activity by the use of specific antibodies (Levi-Montalcini and Booker, 1960). In preliminary experiments, cerebellar cell cultures were treated with polyclonal antibodies directed against NGF, and Purkinje cell survival was evaluated. Anti-NGF diminished cell survival by 40% in cultures grown under control conditions (Fig 27). These results not only suggested a dependency for trophic stimulation, but also indicated that a local source of NGF might be present in mixed cultures. The latter possibility was further investigated, and the results are presented in chapter 4.

Future antibody perturbation studies will prove helpful in the further elucidation of the mechanisms by which NGF regulates Purkinje cell development *in vivo*.

Specific bockade of the aspartate-mediated survival effects by excitatory amino acid antagonists

A deeper understanding of the mechanism by which excitatory neurotransmitters regulate development can be obtained by elucidating whether these effects are mediated through specific excitatory amino acid receptor activation. Traditionally, excitatory amino

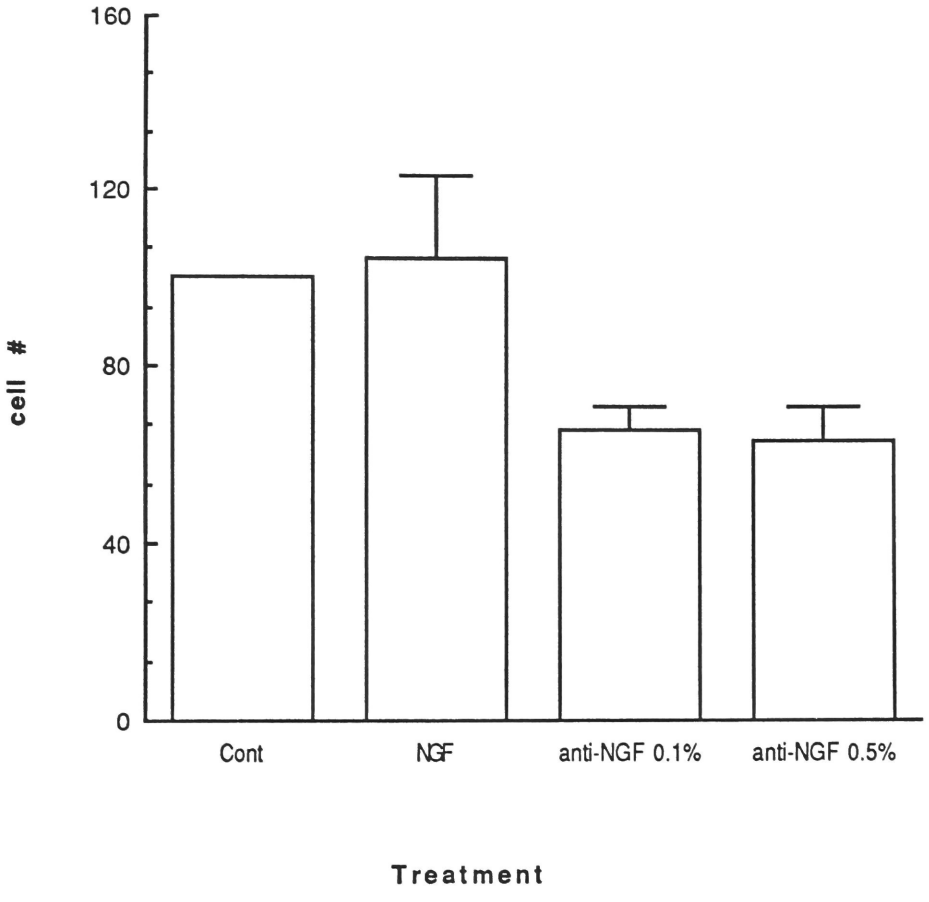


Figure 27.- Effects of anti-NGF antibodies on Purkinje cell survival. E18 rat cerebellar cell cultures were grown under standard conditions or in the presence of NGF, or anti-NGF antisera at 0.1 or 0.5%. Cultures were immunostained for CaBP and numbers of positive cells were obtained by analyzing 10% of the dish area. Three independent experiments were analyzed. Data is expressed as percent CaBP cell number compared to controls grown alone. Each experimental value represents mean cell number ± s.e.m. Statistical analysis was by one-way ANOVA and Scheffe F-test. * Differs from control and NGF by $P<0.05$.

acid receptors are divided into three major subtypes based on selective responses to the agonists N-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QA). These receptors can be selectively blocked by specific antagonists that act either on NMDA (as is the case of 2-amino 5-phosphovalerate or APV) or on non-NMDA (as 6,7-Dinitroquinoxaline-2,3-dione or DNQX) receptor subtypes. Preliminary experiments devoted to identify the nature of the receptor subtype by which aspartate exerts effects, showed a marked tendency for DNQX to block the survival effect elicited by aspartate in combination with NGF (Fig 28). On the other hand, addition of APV to aspartate + NGF treated cultures did not decrease survival significantly. These results suggest that, at least in part, aspartate effects are mediated through receptors of the non-NMDA subclass.

It has been suggested that glutamate channels of the NMDA type are involved in the synaptic organization of cerebellar circuitry (Garthwaite and Garthwaite, 1986; Garthwaite et al., 1987; McDonald and Johnston, 1990). However more recent reports indicate that during early development, Purkinje cell responses to excitatory stimulation may be mediated by non-NMDA channel activation (Audinat, 1990). My preliminary observations agree with early activation of non-NMDA receptors, and suggests that the developmental effects of aspartate may indeed be mediated through these receptors. More studies are needed to identify the mechanism by which excitatory amino acids regulate development.

The findings presented in this dissertation open several new questions concerned with the role of trophic factors in the development and function of central neurons. Some of these questions and their rationale are briefly addressed in the following paragraphs.

Possible role of calcium in trophic factor mediated survival

The effects of excitatory amino acids and trophic agents on neuronal survival may differ mechanistically from those on morphological differentiation. Increased neuronal

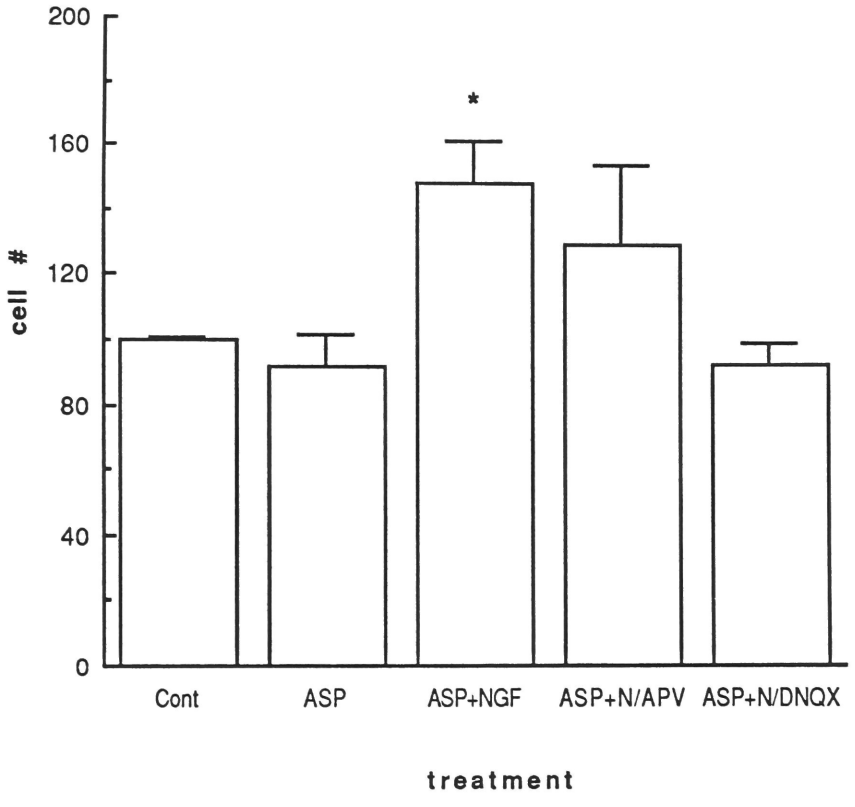


Figure 28.- Blockade of the aspartate survival effect by excitatory amino acid receptor antagonists. Cerebellar cells were grown in culture under standard conditions, or in the presence of aspartate (ASP), or aspartate +NGF (ASP+NGF). To examine the effects of the specific excitatory amino acid receptor blockers, APV or DNQX were added to aspartate + NGF-treated cultures at the time of plating. Cultures were grown for 7 days and immunostained for CaBP. Numbers of positive cells were obtained by analyzing 10 % of the dish area. Three independent experiments were analyzed. Data is expressed as percent CaBP cell number compared to controls grown alone. Statistical analysis was by one-way ANOVA and Scheffe F-test. * Differs from control, aspartate and ASP+NGF/DNQX by $P<0.05$.

survival may be due to a rescue from neurotoxicity. This can be explained by the capacity of excitatory and trophic signals to modulate intracellular calcium buffering systems, thus making cells more resistant to toxic influences. It is particularly interesting in this regard that, recently, it has been found that the effects of NGF and depolarizing signals on the survival of sympathetic neurons may be explained by their capacity to modulate intracellular Ca^{2+} levels (Koike et al., 1989). Moreover, NGF has been found to increase mRNA species encoding calcium binding proteins in PC12 pheochromocytoma cells (Masiakowski and Shooter, 1988), and CaBP is first detected in Purkinje cells at very early stages during development (Legrand et al., 1983). It will now be of interest to define whether these epigenetic signals regulate the expression of calcium binding proteins during the development of Purkinje cells. Preliminary experiments examining effects of NGF and depolarizing signals on the expression of CaBP, suggest that this might be a case.

A putative role for NGF in the regulation of neuronal excitability

It has been suggested that NGF may influence neuronal excitability (Dichter et al., 1977; Chalazonitis et al., 1986). Studies done mainly in PC12 cells have shown that NGF increases electrical excitability by specifically inducing synthesis of sodium channels (Rudy et al., 1982; Mandel et al., 1988; Pollock et al., 1990). This, in turn, allows the generation of action potentials. Since induction of electrical excitability may be a key feature of NGF-induced neuronal differentiation, it is then possible that NGF may also influence excitability of central neurons. In such a case, just as depolarizing signals regulate NGF receptor expression, so may NGF increase responsiveness to afferent innervation by altering the excitability state of neurons. This is a new possibility that is now open for experimentation.

A putative role for trophic factors in adult life

In this thesis I have presented evidence that the development of Purkinje cells is subject to regulation by trophic signals, in particular NGF. At the time when I started this project, no evidence on a putative role for NGF in the adult cerebellum was available. Virtually no NGF-R expression could be detected either by molecular, immunocytochemical or NGF binding techniques. However, with the advent of more sensitive markers, a number of groups have recently demonstrated that Purkinje cells retain the ability to express NGF receptors in the adult, although at markedly lower levels (Figuereido et al., 1990; Mufson et al., 1990). To date, high-affinity receptors have not been associated with Purkinje cells. Therefore, functional receptors and the putative role of NGF in adult cerebellum remain to be defined.

It is interesting to note that it is not yet even clear which trophic factor may interact with putative receptor sites. For example, there is no evidence that NGF is present in the adult cerebellum. However, other members of the newly described neurotrophin family, such as BDNF, which have been proposed to interact with NGF receptor sites (Rodriguez-Tébar et al., 1990), are highly expressed in adult life (Hofer et al., 1990; Wetmore et al., 1990). It remains to be determined whether these, or other factors, may be critical for normal Purkinje cell function. This new avenue in research remains for future investigations.

CHAPTER 6

MATERIALS AND METHODS

Animals

Sprague Dawley rats were used throughout this study. Pregnant rats were from Hill Top Laboratories; embryonic day 1 (E1) was the first day a vaginal plug was seen, birth was usually on E22. The day of birth was considered as Postnatal day 0 (P0). Postnatal animals used for NGF binding studies were sacrificed by decapitation. Adult normal and pregnant rats were sacrificed by carbon dioxide exposure.

Preparation of NGF

The β subunit of nerve growth factor (NGF) was prepared from adult mouse salivary glands as modified by Dr. K. Wu following published procedures (Mobley et al., 1976). In brief, total protein was obtained from 30-50 grams of adult mouse salivary glands, and NGF protein was purified by serial steps of dialysis and ion-exchange chromatography. Yields and concentration of the NGF preparation were determined by the method of Lowry (1951), and purity was confirmed by SDS-polyacrylamide electrophoresis. Bioactivity was determined following published procedures (Fenton, 1970).

NGF Binding Assay

The ^{125}I-NGF used in this studies was kindly provided by Dr. P. Bernd, and the NGF binding protocol was modified after that previously described (Bernd et al., 1988).

1) Tissue slices: Sprague-Dawley rats were sacrificed, the cerebellum was dissected as a unit, and 400μm sagittal slices were obtained with the use of a grid-based

tissue slicer (Katz, 1987). Slices were immediately transfered to well plates (22 mm diameter) and pre-incubated in minimal essential medium (MEM, Earle's salts; Gibco labs.), 0.6% glucose and 5 mg/ml bovine serum albumin, for 15 min at 37°C in a 5% CO_2 /100% humidity atmosphere. For ^{125}I-NGF binding, slices were incubated with 0.2nM ^{125}I-NGF for 1hr under the same conditions. In control slices, 1000 fold excess unlabeled NGF was added at the time of incubation with the radioactive ligand. Following incubation, sections were rapidly washed six times in ice cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde. Ten micron cryostat sections were obtained from each cerebellar slice, the sections were immunostained with Vitamin D-dependent Calcium Binding Protein antibodies (CaBP), dipped in L-4 emulsion (Ilford labs.) and exposed for autoradiography.

To define high-affinity receptor sites, cerebellar slices were exposed to a chase of 0.2μM unlabeled NGF for 30 min at 4°C following incubation with the iodinated ligand, and were processed as above.

2) Cerebellar cells in culture: Embryonic day 18 rat cerebellar cell cultures were grown for 7 days (see below), nutrient media was removed, and cultures were washed with 3 changes of MEM, 0.6% glucose for 2 hrs at 37°C to remove any endogenous NGF. For ^{125}I-NGF binding, cultures were incubated with 0.2nM ^{125}I-NGF for 1 hr at 37°C in MEM, 0.6% glucose, 5 mg/ml bovine serum albumin. Following incubation, cultures were rinsed six times in ice cold PBS and fixed in 4% paraformaldehyde. Cultures were immunostained and processed for autoradiography.

Tissue dissociation and cell culture

Cerebella from embryonic day 18 (E18) Sprague Dawley rats were used to establish dissociated cell cultures. Timed pregnant rats were sacrificed by exposure to CO_2 vapor. Fetuses were removed by cesarean section and transfered to a sterile petri dish with

phosphate buffered saline (PBS). Fetal cerebella were dissected from surrounding brain tissue. In brief, the skull was removed and the midbrain was dissected to expose the cerebellum and underlying brainstem. Special care was taken to avoid contamination with surrounding tissues. Only cerebellar tissue medial to the cerebellar peduncles was dissected, and the meninges were completely removed. Pooled tissue from 2-4 litters was minced into 0.5-1.0 mm pieces and mechanically dissociated in 2 ml of nutrient medium. Culture conditions for neuronal and non-neuronal cells were modified after those described (Hatten, 1985). Mixed cultures containing neurons and non-neuronal cells were maintained in nutrient medium (NM) containing Minimal Essential Medium with Earle's salts supplemented with 2 mM glutamine (MEM; Gibco, Grand Island, NY), glucose (6 mg/ml), heat inactivated horse serum (10% vol/vol;' Hazelton Inc.), and low concentrations of penicillin (0.5 U/ml), and streptomycin (0.5 μg/ml). Cells were counted and plated on poly-D-lysine-coated petri dishes (35 mm) at a final density of $1x10^6$ cells/dish. For RNA purification purposes, cells were plated on 100 mm diameter petri dishes at a final density of 10 x 10^6 cells per dish. Cultures were maintained in nutrient medium at 37°C in a 95% air / 5% CO_2 humidified incubator. The concentration of the drugs used in this study was determined from dose response experiments. Doses yielding optimal effects without toxicity were chosen. As indicated for each experiment, NGF (200U/ml), potassium chloride (25 mM), veratridine (0.5μM), aspartic acid (10μM), and glutamic acid (10μM) were added to the medium at the time of plating. In addition, where indicated, antibodies to NGF (Sigma or Collaborative Research) at 0.1-0.5% final volume, 2-amino-5-phosphovalerate (APV; Sigma) at 50μM, or 6,7-Dinitroquinoxaline-2,3,-dione (DNQX; Tocris Neuramin) at 20μM final concentration, were added to the culture medium. The medium was replaced once after 4-5 days in culture, with the addition of fresh drugs.

Neuron-enriched cultures were obtained from E18 rat cerebellum following published procedures (Fischer, 1982). Neurons were maintained in serum free medium

(SFM), containing Basal Medium Eagle with Earle's salts supplemented with 2mM glutamine (BME; Gibco), 1 mg/ml bovine serum albumin, 10 μg/ml insulin, 0.1 nM thyroxine, 0.1 mg/ml human transferrin, 30 nM selenium, 0.25% glucose, 0.5 U/ml penicillin and 0.5 μg/ml streptomycin. Conditions for growth were as described above.

For glia-enriched cultures, E18 rat cerebella were dissociated and plated in 100 mm diameter petri dishes and maintained in nutrient medium (NM) for 15 days. At this time, cultures became fully confluent and neurons were detached by gentle agitation. Following removal of neurons, the nutrient medium was eliminated and glia were detached from the substrate by pipeting up and down with a solution of 2.5 mg/ml trypsin, 0.2 mg/ml EDTA in MEM. Cells were washed in NM, counted and replated at low, medium, and high densities ($1x10^6$, $5x10^6$, and $10x10^6$ cells per dish, respectively). Glial cells were harvested for RNA preparation once the high-density cultures reached confluency (from 3 to 4 days).

Immunocytochemistry

Preparation of tissue sections

Postnatal and adult animals were perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer pH7.4, the brains were removed, and immersed in 4% paraformaldehyde overnight. Embryonic tissues and cerebellar slices were fixed by immersion in 4% paraformaldehyde. Following fixation, tissues were immersed in PBS, 30% sucrose overnight, and embeded on OCT compound (Tissue-Tek). Ten micron cryostat sections were obtained, air dried and immediately stained, or stored at -20°C in air tight boxes until use.

Preparation of cell cultures

After seven days in culture, dissociates were rinsed in PBS and fixed for 3 hrs at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer, pH7.4. Cultures were rinsed twice with ice cold PBS and immediately stained, or stored in PBS at 4°C in humidified chambers until use.

Antibodies and staining procedure

Cryostat sections and cell cultures were washed several times in PBS, and pre-incubated for 1 hr with PBS containing 5% Normal Goat Serum (NGS). Incubation in primary antibody was carried out for 1 hr at room temperature, with the following antibodies diluted to the appropriate concentration (in PBS, 5%NGS 0.3% triton X-100). To specifically detect the Purkinje cell population, a polyclonal antibody to Vitamin D-dependent calcium binding protein (CaBP), generously provided by Dr. S. Christakos, was used at a 1:2000 dilution (Christakos et al.; 1987). The 192-IgG monoclonal antibody directed against the rat NGF-R (Yan and Johnson, 1988) was used at a 1:10 dilution (a gift from Dr. M. Chao). A polyclonal antibody against neuron specific enolase (NSE) (Polysciences, Inc. laboratories, 1:1000 dilution) was used for immunostaining of the neuronal populations in culture. After incubation with the primary antibody, the samples were rinsed 3 times with PBS, 5% NGS and were incubated for 1 hr with biotinylated secondary antibodies (Vector labs). The avidin-biotin complex (ABC) technique (Vectastain ABC kit, Vector labs.) was used to visualize staining. For double labeling experiments, fluorescein conjugated anti-rabbit IgG was used to discern CaBP staining.

Morphometric analysis and assessment of cell survival

Cultures were examined using a Nikon Labophote 100 microscope. Immunopositive cell counts were obtained by analyzing an area equivalent to 10% of the dish surface. The number of CaBP-positive cells was expressed as percent of cells in experimental versus control cultures. Morphometric measurements were performed using representative, isolated CaBP-immunopositive cells in sparse cultures. Cell diameter and neurite length were measured with an eyepiece micrometer. Numbers of neurites and branch points per cell, were determined by counting. Neurites were defined as those processes 20 μm or longer protruding from the cell body. Neuritic length refers to length of longest neurite. "Neurons with spiny processes" were defined as those CaBP-positive cells with processes 0.5-1.0 μm in length protruding from central neurites. A drawing tube coupled to the Nikon microscope was used for tracing CaBP-positive cells. An analysis of variance program (ANOVA) was used for the statistical analysis of data.

Affinity cross-linking

Cerebellar cells were grown in culture for 6 days. At this time, cells were washed 3 times with MEM, 0.6% glucose to remove any endogenous NGF, and cells were briefly incubated in 25 mM EDTA, 0.6% glucose in PBS and harvested by aspiration with a pasteur pipet. Cells were washed in PBS, pelleted and resuspended in 0.5 ml of 20 mM KH_2PO_4, 160 mM NaCl, pH 6.8. Affinity cross-linking was carried out according to Escandón and Chao (1989). In brief, cells in supension were incubated with 1 nM ^{125}I-NGF for 1hr at 37°C to allow binding of NGF to receptor sites. Following incubation, the NGF/receptor complex was cross-linked with the specific agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDAC), and samples were washed of excess unbound iodinated ligand. Affinity cross-linked products were subjected to electrophoresis on an SDS-polyacrylamide gel and exposed for autoradiography.

RNAse Protection Assay

RNA purification

Total RNA was extracted by the method of Chirgwin (Chirgwin, 1979) and purified through CsCl gradient. For dissociated cell cultures, media was removed and lysis buffer (4 M guanidinium thiocynate, 25 mM sodium citrate, pH7.0, 0.1M ß-mercaptoethanol) was added. Cultures were scraped in the lysis buffer with an RNAse-free rubber-policeman. The lysates were immediately loaded onto a CsCl cushion (50 mM NaAc, pH7.5, 50 mM EDTA, pH8.0, 5.7 M CsCl) and spun for 16 hrs at 35,000 rpm with an SW50.1 rotor. The pellets were resuspended in diethylpirocarbonate (DEP) treated H_2O and ethanol precipitated. The yield and purity of RNA was determined spectrophotometrically by measuring OD_{260nm} and OD_{280nm}.

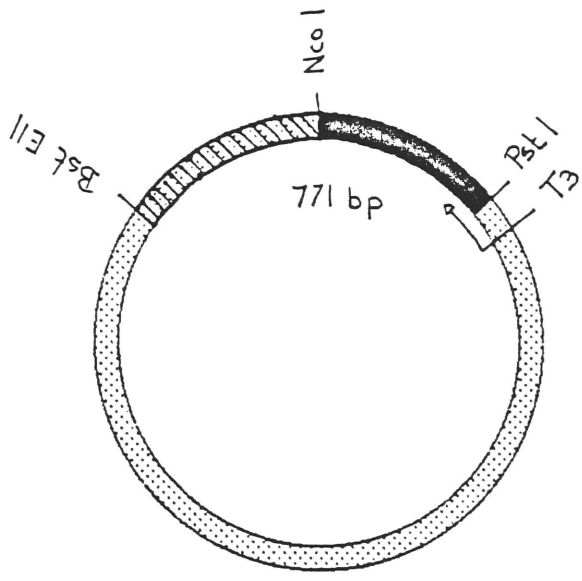
NGF and NGF-R probes

The NGF cDNA construct pBRsNGF contains a 771bp BstEII-PstI insert coding for the entire rat prepro-NGF (Whittemore et al., 1988). The rat NGF-R cDNA, a gift of Dr. M. Chao, consists of 270bp BamHI-EcoRI fragment coding for the extracellular cystein-rich domain of the receptor (Buck et al., 1988; see figure 29 for schematic diagram of the plasmids).

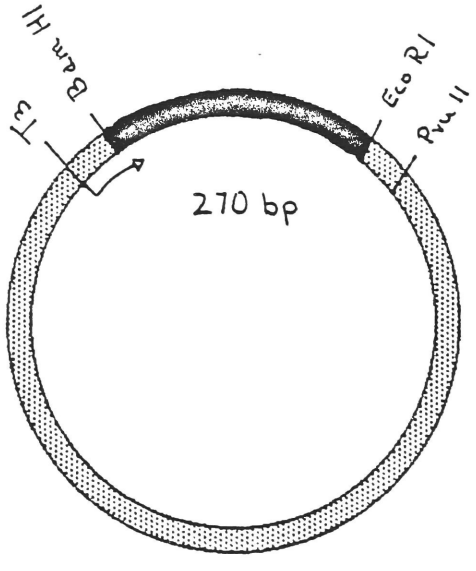
Ribonuclease protection assay

A ribonuclease protection assay was used to determine NGF and NGF-R mRNA levels in cerebellar cell cultures. Ribonuclease protection experiments were a modification of those described (Lu et al., 1989). Briefly, ^{32}P labeled antisense riboprobes was generated by T3 in vitro transcription from the rat cDNA construct, pBRsNGF, and the rat NGF-R cDNA construct. Total RNA samples were simultaneously hybridized with both

NGF and NGF-R probes, followed by digestion of any remaining unhybridized single stranded sequences by RNAse T2 (see figure 30 for diagram of the procedure). The protected fragments were resolved by electrophoresis in polyacrylamide-sequencing gels, exposed for autoradiography and analyzed with densitometry. RNA hybridization with the NGF probe generated a 411 base NGF-specific protected fragment while the NGF-R antisense riboprobe generated a 270 base NGF-R-specific protected fragment. Consistent results from experiments repeated at least 3 times are presented in either gel photographs or densitometric graphs.



pBRs NGF



035A NGF-R

Fig.29.- Schematic diagram of plasmids.

A) Rat NGF cDNA construct, pBRs NGF. B) NGF-R cDNA construct, 035A NGF-R.

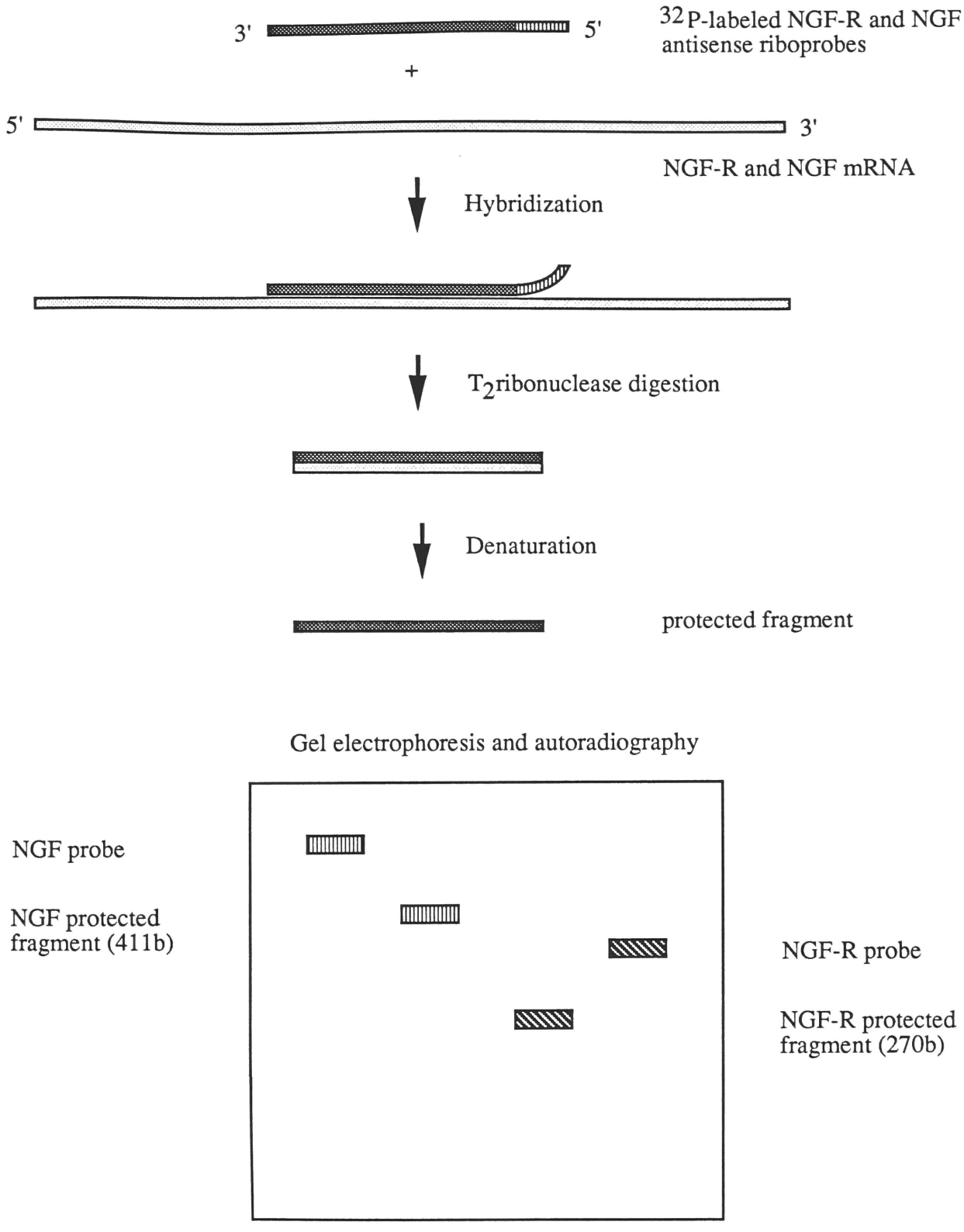


Fig. 30.- Ribonuclease protection assay - Hybridization and digestion

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