Order Number 9218246

Epidermal growth factor and transforming growth factor-alpha gene expression in the normal and neurodegenerate murine central nervous system

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City University of New York, 1992

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EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-ALPHA GENE EXPRESSION IN THE NORMAL AND NEURODEGENERATE MURINE CENTRAL NERVOUS SYSTEM

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1992

This manuscript has been read and accepted by the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Acknowledgements

I would like to thank the many people who have enriched my life, both personally and scientifically, during my graduate training years: members of the Supervisory Committee whose guidance and seemingly endless enthusiasm contributed greatly to the evolution and realization of the dissertation; members of the Fishberg Neurobiology "family" who created a wonderfully unique environment in which to learn and enjoy science and my husband Eric, sister Patty and parents for their love, encouragement and extreme patience.

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Thesis Summary

During the past decade, it has been recognized that the primary pathologic abnormality observed in neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease is a progressive degeneration of distinct neuronal pathways. It has been further demonstrated that specific factors exist which are responsible for the development, maintenance, survival and recovery from injury of particular neuronal populations in the mammalian CNS. It has therefore been suggested that the induction, progression or amelioration of the neurodegenerative disease process is a result of changes in the availability of pathway-dependent trophic substances.

Epidermal growth factor (EGF) is one such agent that may play a neurotrophic role during the natural course of neurodegenerative events. A mitogenic polypeptide originally isolated from the male mouse submaxillary gland, EGF has been shown to enhance the survival and process outgrowth of neurons grown in culture and to promote glial proliferation and differentiation *in vitro*. Investigations into the localization of EGF within mammalian brain, however, have produced conflicting results and have led some to suggest that transforming growth factor-α (TGF-α), an equal competitor for EGF receptor binding and a neuropeptide demonstrated to be synthesized in rodent brain, is the physiological ligand for EGF receptor activation in the CNS. The assignment of EGF-specific trophic interactions to discrete neuronal or glial populations in brain, then, has remained difficult without direct biochemical evidence for the synthesis of epidermal growth factor itself in the developing or mature nervous system.

Our investigations into the brain-specific expression of epidermal growth factor have therefore focused on demonstrating EGF mRNA in mouse brain, providing direct evidence of synthesis, and correlating changes in levels of EGF gene expression with normal and abnormal developmental events. With the seemingly redundant potential for co-expression of transforming growth factor-α and epidermal growth factor in brain, our studies have further compared differences in the levels and patterns of their respective mRNAs in an effort to differentiate roles for these ligands sharing a common membrane receptor. In particular, cerebellar mouse mutants demonstrating distinct and well-characterized neurological deficits were employed in our exploration of the effects of selective neuronal loss and reactive glial events on patterns of growth factor gene expression.

Using a highly sensitive and specific solution-hybridization ribonuclease-protection assay, we have detected EGF mRNA in anatomically-defined regions of adult mouse brain including brainstem, cerebellum, cerebral cortex, hippocampus, basal hypothalamus, olfactory bulb, olfactory tubercle, striatum and thalamus. While TGF-α mRNA was detectable in all brain areas as well, EGF mRNA levels were determined to be 16 - 170 times lower. An examination of growth factor mRNA expression during embryonic and early postnatal mouse brain development similarly revealed co-expression of EGF and TGF-α mRNA in the CNS and again, TGF-α mRNA levels were above those observed for EGF. Taken together, these findings provide direct biochemical evidence for the synthesis of EGF in brain and suggest, on the basis of observed differences in absolute levels of growth factor mRNAs, that these putative EGF receptor ligands play distinct roles throughout mammalian CNS development.

As EGF receptor immunoreactivity has previously been reported to be present on cerebellar Purkinje cells and in astroglial cell cultures derived from

rodent cerebellum, our observation of EGF mRNA in normal mouse cerebellum suggested regional co-localization of EGF synthesis and receptor expression and thus, predicted an intra-cerebellar pathway for the potentially trophic effect of EGF in the CNS. We therefore analyzed the expression of EGF and TGF-α mRNA in mutant mice exhibiting selective losses of specific neuronal populations and demonstrating well-described glial events in an effort to correlate specific neurodegenerative processes with alterations in growth factor mRNA. Two cerebellar mutant strains were examined, the Purkinje cell degeneration (Pcd) and the weaver mouse. For Pcd mutant mice, cerebellar Purkinje cells are typically lost, beginning around postnatal day 18, so that by the fifth postnatal week, very few Purkinje neurons remain. In weaver cerebellar mutant mice, there is a rapid degenerative loss of granule neurons in the vermis (midline region) during the first postnatal month. An examination of EGF gene expression in Pcd mutant (genotype *pcd/pcd*) and control (*+/+* and *+/pcd*) cerebellum revealed significantly higher concentrations of EGF mRNA in affected mutants from postnatal day 39 onward. A corresponding increase in total EGF mRNA content was observed between P39 and P62. For TGF-α gene expression, mRNA concentrations were higher in mutant cerebellum beginning on postnatal day 65. However, while TGF-α mRNA concentrations continued to rise, TGF-α mRNA content was only transiently elevated (P70 to P99). In the weaver mutant (*wv/wv*) cerebellum, both EGF and TGF-α mRNA concentrations did not differ from non-ataxic homozygous (*+/+*) or heterozygous (*+/wv*) mice. Mutant cerebellar levels of total EGF or TGF-α mRNA, however, were consistently below the levels present in *+/+* and *+/wv* cerebellum. In view of the differences in EGF and TGF-α gene expression resulting from abnormalities in genetically-determined neurodevelopmental programs, these putative EGF receptor ligands are likely to have distinct physiologic roles both in normal

development and in response to injury in the mammalian CNS. In addition, since alterations in growth factor gene expression occur relatively late with respect to neuronal degeneration, it appears that a biological response of newly synthesized growth factor would be related to removal of neuronal debris, formation of glial scarring or induction of compensatory sprouting for functional recovery of the affected neuronal circuits and not necessarily to the prevention of neuronal cell loss.

Introduction

Origin of Epidermal Growth Factor in the Mammalian Central Nervous System

Recent investigations into the localization of epidermal growth factor (EGF) within the mammalian central nervous system (CNS) have identified regions of the mouse and rat brain containing relatively high levels of EGF-immunoreactive material (Fallon et al., 1984; Lakshmanan et al., 1986; Schaudies et al., 1989). Fallon et al (1984), using an indirect immunofluorescence histochemical technique, have detected EGF cross-reactive material in pallidal areas of the adult albino rat brain with apparent restriction of immunoreactivity to neuronal fibers and terminals of the globus pallidus, ventral pallidum, entopeduncular nucleus, substantia nigra (pars reticulata), and the islands of Calleja. Lakshmanan et al (1986), employing a highly specific and sensitive EGF radioimmunoassay, have demonstrated the presence of epidermal growth factor within synaptosomal preparations of mouse cerebral cortex. In a recent study by Schaudies et al (1989), multiple species of EGF-like immunoreactive material were identified using a homologous radioimmunoassay in extracts of rat brainstem, cerebellum, hippocampus, diencephalon and telencephalon.

In contrast to these findings, Probstmeier and Schachner (1986) have reported their inability to detect epidermal growth factor in the developing and adult rodent CNS using a highly sensitive and specific double-site enzyme immunoassay. Previous attempts by various investigators to demonstrate EGF in rodent and human brain tissue using less sensitive and specific

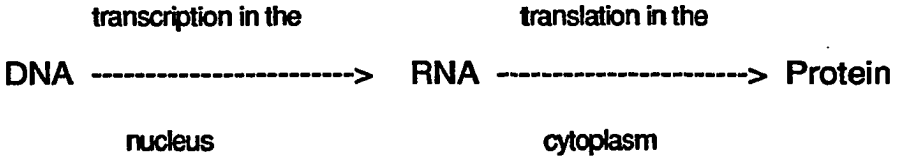
immunological techniques have produced disparate results as well (Byyny et al., 1972; Hirata and Orth, 1979; Hirata et al., 1982; Roberts et al., 1981).

While such conflicting reports might be based solely on the sensitivity and specificity of the various techniques employed, the inability of some investigators to detect epidermal growth factor in brain tissue and CSF has led to the suggestion that epidermal growth factor is not the physiological ligand of the putative EGF receptor in brain (Probstmeier and Schachner, 1986; Ojeda et al., 1990). Alternative candidates for endogenous EGF receptor activation in the mammalian central nervous system, then, might include the EGF-related peptides derived from the preproEGF precursor protein (Scott et al., 1983; Gray et al., 1983) or, more likely, transforming growth factor-α (TGF-α), an EGF-like transforming agent previously shown to bind the EGF receptor in transformed and normal cell lines (Matrisian et al., 1982; Massagué, 1983; Carpenter et al., 1983; Marquardt et al., 1983, 1984). With respect to the latter, TGF-α mRNA has been detected in both developing and adult rodent brain and therefore, there is a biochemical basis for the expression of TGF-α in the CNS (Lee et al., 1985a; Wilcox and Derynck, 1988; Kudlow et al., 1989; Ojeda et al., 1990; Seroogy et al., 1991).

Still, the reported immunocytochemical demonstrations of EGF-like material in rodent brain implicate epidermal growth factor itself in some undefined role in the nervous system. Some have argued that the detection of EGF-like immunoreactivity within the rodent CNS reflects a sequestering of epidermal growth factor from the peripheral circulation (Plata-Salamán, 1991) and that EGF may gain slow access to the interstitial fluid of the brain via the circumventricular organs lacking a blood-brain-barrier (for example, the area postrema, median eminence, organum vasculosum laminae terminalis) (Blatteis et al., 1989). Alternatively, EGF may enter the cerebrospinal fluid (CSF) by way

of the choroid plexuses, which serve as a barrier between the blood and the CSF (Wright and Saito, 1986; Zlokovic et al., 1988). The inability of EGF to cross the blood-brain or blood-CSF barrier, however, has been demonstrated by injecting radio-labelled EGF into the peripheral circulation of adult rats (Nave et al., 1985; Jørgensen et al., 1988). Since no specific uptake system has been recognized that accounts for the concentrations of EGF detected in brain and CSF, these observations suggest that EGF or EGF-like activities in the mammalian CNS are primarily neural in origin.

Perhaps one of the most direct and definitive approaches to the demonstration that a particular protein is synthesized in a particular tissue is to identify within that tissue the mRNA specifically encoding the protein. The underlying assumption here is the basis of genetic Central Dogma (Crick, 1958):



Based on the flow of genetic information, messenger RNA molecules serve as templates for the production of a specific protein. Such protein gene products may subsequently be used for basic intracellular processes or may leave the cell to be used in a local or distant region of the organism. In contrast to proteins, however, mRNA transcripts remain sequestered within the cytoplasm of the cells from which they derive.

It follows then that the detection of EGF mRNA transcripts in specific cells of the brain provides direct biochemical evidence for the potential synthesis of EGF therein. A single incidental observation of low levels of preproEGF mRNA in mouse brain has been reported (Rall et al., 1985). This study, however,

employed the technique of dot-blot hybridization and could not exclude the possibility of cross-hybridization of their preproEGF cDNA probe to other similar, non-EGF mRNA sequences. We have therefore sought stronger evidence for the expression of EGF mRNA in the normal adult mouse brain and new evidence that EGF mRNA is expressed in prenatally developing brain using a more sensitive and specific solution hybridization-ribonuclease protection assay.

Expression of Transforming Growth Factor-α, an EGF-like Neuropeptide, in Brain

Transforming growth factor-α (TGF-α) is a 50 amino acid polypeptide that is structurally and functionally related to epidermal growth factor (Marquardt et al., 1983, 1984). This EGF homolog, previously shown to bind the EGF receptor (Massagué, 1983), has been localized by immunocytochemistry within the developing and adult rodent CNS (Fallon, 1987; Kudlow et al., 1989; Fallon et al., 1990; Brown et al., 1990). With respect to its expression in neurons, TGF-α immunoreactive material has been detected in the brainstem, cerebellum and hypothalamus (Kudlow et al., 1989) as well as in the striatum (caudate-putamen), nucleus accumbens, olfactory tubercle, amygdala, and the bed nucleus of the stria terminalis (Fallon, 1987). In addition, TGF-α immunoreactivity has been observed to co-localize in neurons expressing enkephalin in both the basal ganglia and septal regions (Fallon, 1987; Code et al., 1987). Recently, regions previously shown to contain TGF-α in neurons (Fallon, 1987) have been shown to contain TGF-α-precursor immunoreactivity in astrocytes as well (Fallon et al., 1990). Perhaps most intriguing are reports that TGF-α and EGF immunoreactive materials in brain regionally overlap,

including the globus pallidus, ventral pallidum, entopeduncular nucleus and substantia nigra (Fallon et al., 1984; Fallon, 1987).

The distribution of transforming growth factor-α gene transcripts in the murine CNS has more recently been defined. With the aid of *in situ* and Northern hybridization techniques, TGF-α mRNA has been localized to discrete, yet scattered regions of the adult rodent brain including the caudate nucleus, dentate gyrus, anterior olfactory nuclei, and mitral cells of the olfactory bulb (Wilcox and Derynck, 1988) and most recently, in the hypothalamus, cerebral cortex, brainstem and cerebellum (Ojeda et al., 1990; Seroogy et al., 1991). The physiological function of this endogenous CNS factor, however, remains unknown.

Due to the potential for EGF and TGF-α to share a common receptor *in vivo*, we have examined the quantitative relationship between EGF and TGF-α mRNAs in defined regions of the adult mouse CNS and in whole developing brain in an effort to gain insight into their physiological potentials in brain.

Application of Cerebellar Neurodegenerative Mutant Mice to the Study of Epidermal Growth Factor and Transforming Growth Factor-α Gene Expression in the CNS

It is often possible to gain insight into the normal functions of a biological system by examining abnormally functioning models as well. The aberrant biochemical pathways or morphologies observed in a diseased tissue, for example, are often not unique to the particular disease process, but merely reflect an alteration, loss or accentuation of a normally existing pathway or structure (Hill RB, 1980). From this observation, it follows that the study of a pathophysiological process involving deviations from normal structure or cell

biology has the potential to clarify our understanding of a biological norm (Robbins et al., 1984).

Cerebellar mouse mutants demonstrating distinct and well-characterized neurodegenerative deficits have been particularly valuable in the exploration of factors involved in both normal and abnormal neurodevelopmental processes. The Purkinje cell degeneration (Pcd) mouse (Mullen et al., 1976) is one such cerebellar mutant resulting from a spontaneous gene mutation occurring on mouse chromosome 13 (Green, 1981). As the original characterization of this mouse line was reported more than a decade ago, the genetic, behavioral and histopathological traits characterizing the Pcd mutant are relatively well described (Mullen et., 1976; Landis and Mullen, 1978). In brief, the autosomal-recessive Pcd mutant mouse (*pcd/pcd*) is identified by a characteristic ataxia of gait as early as 22 days past birth. The histopathological changes, evident by light microscopy, typically begin around 15 to 18 days postnatally with a two week period of rapid Purkinje neuronal degeneration. By the end of the first postnatal month, histologic examination reveals a nearly complete loss of the cerebellar Purkinje population, with a superimposed glial proliferative reaction occurring in the overlying molecular layer. An additional histopathological change occurring in Pcd mutants is the slower neurodegenerative loss of olfactory bulb mitral cells apparent at ~10 months of age. In contrast to the degenerating cerebellar cortical efferent neurons described for Pcd mutants, weaver mice (genotypically defined as an autosomal recessive mutation, *wv/wv*) display a severe loss of granule neurons in the vermal (midline) region (Rakic and Sidman, 1973; Herrup and Trenkner, 1987). The time course of this loss is relatively rapid during the first 2 postnatal weeks so that by the end of the first postnatal month, only remnants of these cortical afferent neurons remain.

The characteristic ataxia and fine tremor may be observed approximately 14 days past birth.

The application of such models to our studies stems from recent demonstrations of EGF receptors in the rodent cerebellum. Specifically, EGF-receptor immunoreactivity in rat brain has been observed on Purkinje cell bodies and dendritic processes (Gómez-Pinilla et al., 1988). Earlier investigations into the effects of EGF on primary cultures of early postnatal mouse cerebellum revealed astrocyte-specific EGF binding with stimulatory actions on astroglial DNA synthesis (Leutz and Schachner, 1981, 1982). In addition, EGF has been shown to be trophic for neonatal rat cerebellar neurons, most likely Purkinje cells, maintained in culture (Morrison et al., 1988).

Therefore, in our final studies, we have examined the expression of EGF and TGF-α mRNAs in both the normal and neurodegenerate cerebellum (Pcd and weaver) and olfactory bulbs (Pcd), in an effort to correlate potential changes in growth factor mRNA levels with well-characterized neurodevelopmental events.

CHAPTER 1

Regional Distribution and Developmental Expression of Epidermal Growth Factor and Transforming Growth Factor-α mRNA in Mouse Brain by a Quantitative Nuclease Protection Assay

(in press)

Abstract

A solution-hybridization ribonuclease-protection assay was used to identify epidermal growth factor (EGF) mRNA in mouse brain and to compare the regional and developmental levels of EGF gene expression in the CNS with those of its structural homolog, transforming growth factor-α (TGF-α). Adult brain regions examined included brainstem, cerebellum, cerebral cortex, hippocampus, basal hypothalamus, olfactory bulb, olfactory tubercle, striatum and thalamus. While both EGF and TGF-α mRNAs were detected in all regions, TGF-α mRNA levels were 15-170 times higher, ranging from 0.39 (cerebellum and cerebral cortex) to 2.93 (striatum) pg TGF-α mRNA per μg total cytoplasmic RNA. In contrast, EGF mRNA levels ranged from 11 to 36 fg EGF mRNA per μg, with the highest regional concentrations observed in olfactory bulb, basal hypothalamus and cerebellum. In our comparison between sexes, no significant male-female differences in EGF or TGF-α mRNA levels were observed for any region of adult brain. However, in the pituitary gland, consisting of both endocrine and neural elements, EGF and TGF-α mRNA levels were significantly higher in males (234 and 215 fg/μg, respectively) than in females (172 and 118 fg/μg, respectively). An examination of growth factor gene expression in the developing CNS revealed EGF and TGF-α mRNAs detectable as early as embryonic day 14 (earliest time point studied). While gene expression for both peptides continued into the postnatal period, EGF and TGF-α mRNA levels were nearly equal to adult concentrations by postnatal day 10. Taken together, our findings provide evidence for the synthesis of EGF in brain and suggest a role for both EGF and TGF-α in the development and support of the mammalian central nervous system.

Introduction

Epidermal growth factor (EGF) is a potent mitogenic peptide, first isolated from the male mouse submaxillary gland (Cohen, 1962), influencing the growth and development of a variety of tissues (reviewed in Carpenter and Cohen, 1979; Carpenter and Wahl, 1990). From observations in culture, EGF has been shown to promote the proliferation and differentiation of glia (Leutz and Schachner, 1981; Simpson et al., 1982; Honegger and Guentert-Lauber, 1983) and to enhance neuron survival and process outgrowth (Morrison et al., 1987, 1988; Casper et al., 1991). The normal physiologic function of EGF in brain, however, has not been determined despite the presence of epidermal growth factor receptors (EGF-R) in both the developing and mature CNS (Adamson and Meek, 1984; Hiramatsu et al., 1988; Gómez-Pinilla et al., 1988; Werner et al., 1988; Chabot et al., 1988).

Perhaps one of the greatest obstacles to elucidating roles for EGF in the nervous system has been the inability to firmly establish its presence in the CNS. Indeed, published reports concerning the immunological detection of EGF in brain have remained inconsistent and controversial. The intense EGF immunostaining observed in pallidal and cortical regions of adult rodent brain by Fallon et al (1984), for example, has not been observed by other investigators employing comparably sensitive and specific immunohistochemical techniques (Poulsen et al., 1986; Beerstecher et al., 1988). Attempts to immunologically identify EGF in extracts of both rodent and human brain have produced conflicting results as well (Byyny et al., 1972; Lakshmanan et al., 1986; Probstmeier and Schachner, 1986; Schaudies et al., 1989). With only one incidental observation of low EGF messenger RNA (mRNA) levels in whole adult mouse brain (Rall et al., 1985), no evidence for the expression of EGF mRNA in fetal brain (Carpenter and Wahl, 1990) and the

inability of EGF to cross the blood-brain barrier (Nave et al., 1985; Jørgensen et al., 1988), the source of the intense EGF immunostaining observed in the CNS remains unclear.

The apparent discrepancy among these findings has led to speculations that epidermal growth factor is not endogenously synthesized in brain, but that other peptides sharing EGF-like structural characteristics serve as the physiological ligands for EGF-R activation in the CNS (Probstmeier and Schachner, 1986; Ojeda et al., 1990). Transforming growth factor-α (TGF-α), a structural homolog of EGF which is able to compete equally well in EGF-R binding studies (Massagué, 1983; Carpenter et al., 1983; Marquardt et al., 1983, 1984), has been considered the likeliest of such candidates based on relatively strong immunological and molecular evidence supporting its synthesis in both the developing and mature CNS (Lee et al., 1985a; Fallon, 1987; Wilcox and Derynck, 1988; Kudlow et al., 1989; Ojeda et al., 1990; Fallon et al., 1990; Brown et al., 1990). Therefore, whether EGF is expressed in brain in addition to TGF-α remains unclear.

At present, Rall et al (1985) provide the only evidence for preproEGF mRNA expression in the CNS, having performed dot-blot hybridization on RNA isolated from whole adult mouse brain. However, their report acknowledged that some cross-hybridization between their probe and other EGF-like mRNA sequences was possible. We have therefore chosen to examine EGF and TGF-α mRNA expression in brain by a ribonuclease protection assay in order to clarify whether EGF mRNA is in fact synthesized in the CNS and if so, how EGF gene expression in brain might relate to that of its structural homolog TGF-α. The advantage of using this technique over other commonly chosen methods (including dot-blot and Northern-blot hybridizations) is that it affords one the ability to detect and quantitate extremely low levels of mRNA and further, allows

one to distinguish between similar mRNA sequences expressed in the same tissue. Exploiting these methodological advantages then, our findings on the *i)* regional distribution, *ii)* expression between sexes and *iii)* developmental profiles of EGF and TGF-α mRNAs in mouse brain suggest that both EGF-R ligands may play a role in the development and support of the mammalian central nervous system.

Materials and Methods

Tissue collection

Anatomically-defined brain dissections were performed on 60 male and 30 female adult Swiss-Webster mice (86-88 days) as diagrammatically represented in Figure 1. Individual animals, sacrificed by cervical dislocation, were decapitated, their olfactory tracts transected *in situ* (corresponding to dissection 1 of Fig. 1), and the remaining intact brain transferred to an ice-cooled glass plate. After dislodging olfactory bulbs and whole pituitary from their bony fossae, transection of the brainstem (midbrain-pons-medulla) was performed at the medullary-spinal junction (dissection 2) and in a plane passing rostral to the superior colliculi and caudal to the mammillary nuclei (dissection 3). Cerebellum was subsequently isolated by severing the cerebellar peduncles (dissection 4). From the ventral surface, dissection of the basal hypothalamus was achieved by surrounding its protruding surface, delineated by the optic tracts, and cutting rostrally to reach the optic chiasm (dissection 5). The cerebral hemispheres, sliced in the mid-sagittal plane, were subsequently positioned medial surface-down and the olfactory tubercles (outlined by the myelinated lateral olfactory tracts) skimmed from the cortical surface (dissection 6). Following the dorso-lateral contours of the thalamus beneath the fornix (arrow 7), this diencephalic region (consisting primarily of thalamic structures)

was detached from laterally-bordering fibers of the internal capsule, and removed ventro-medially with anterior commissure and optic chiasm serving as rostral delimitations (dissection 8). The hippocampus, now medially exposed, was removed by elevation of its fimbrial border and subsequent cortical detachment. Dissection of the striatum (caudate-putamen-globus pallidus), approached from the medial surface of the hemisected brain through the lateral ventricle, was anatomically defined by encapsulating fibers of the corpus callosum and the internal capsule. With hippocampus and striatum removed, sampling of fronto-parietal cortex was permitted. All brain tissues, having been frozen over dry ice upon dissection, were ultimately stored at -80 ^{o}C until processed for the isolation of total cytoplasmic RNA. Submaxillary gland, serving as an EGF assay control tissue, and anterior pituitary (microscopically dissected from 20 of the male whole pituitaries), providing a TGF-α control, were similarly collected and stored.

For the developmental analysis of growth factor mRNA expression, twelve pregnant Swiss-Webster mice were commercially obtained with vaginal plug dates reported as embryonic day 1. At 14, 15, 16 and 17 days of gestation, pairs of female mice were killed by cervical dislocation and their embryos dissected from uteri. Whole embryonic brains were subsequently removed with the aid of a dissecting microscope, frozen on dry ice and stored at -80 ^{o}C until processed for RNA isolation. Two neonatal litters each of 1 and 10 day old Swiss-Webster pups were obtained (day of birth designated as postnatal day 0) and following their decapitation, whole brains were collected and frozen.

Isolation of cytoplasmic RNA

Total cytoplasmic RNA was prepared from dissected brain, pituitary and submaxillary gland tissue as described (Jakubowski and Roberts, 1992).

Briefly, individual tissues were removed from -80 °C storage, placed into the barrel of a 1 ml tuberculin syringe (serving as a disposable homogenizer) and briefly thawed. Ice-cold ribonuclease-free lysis buffer (10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 0.5 % Nonidet P-40, 0.25 % sodium deoxycholic acid, 0.3 M sucrose) was subsequently drawn into the syringe through a 22 gauge needle. By repeatedly expelling lysis buffer and tissue through the needle (~8-10 strokes), complete homogenization of tissue without the disruption of nuclei was achieved. A ratio of ~100 mg wet tissue weight per milliliter of lysis buffer was maintained. Lysates were subsequently layered over a sucrose cushion (10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 0.4 M sucrose) and centrifuged ~5000 x g for 10 minutes at 4 °C. Supernatants consisting of cytoplasmic fractions were recovered and treated with 0.2 mg/ml proteinase K, 0.03 volumes 5 M NaCl and 0.1 volume 10 x SET (1 x SET: 1 % SDS, 5 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0) for 1 hour at 45 °C. Following phenol-chloroform and chloroform extractions, cytoplasmic RNA fractions were precipitated at -20 °C with two volumes 4.5 M sodium acetate pH 7.

RNA samples to be assayed were recovered by centrifugation at ~16,000 x g for 20 minutes at 4 °C. The resulting pellets were washed with 70 % ice-cold (-20 °C) ethanol, dried and resuspended in diethylpyrocarbonate-treated deionized water. RNA concentrations were subsequently determined by A_{260} readings. Quantitated samples, prepared from individual animals, were ultimately pooled such that EGF and TGF-α assay samples (40-100 µg and 10-100 µg, respectively) could be drawn from the same RNA preparations. Agarose gel electrophoresis confirmed that the RNA was undegraded and free of DNA. In order to standardize the total quantity of RNA in all samples of a given assay, total yeast RNA was added where necessary. Aliquoted samples were ultimately dried, resuspended in 30 µl freshly prepared hybridization

buffer (80% formamide, 40 mM PIPES pH 6.7, 1 mM EDTA pH 8.0, 0.4 M NaCl) and stored at -80 ^{o}C until assayed.

Preparation of DNA templates for in vitro transcription of RNA standards and ^{32}P-labelled anti-sense riboprobes

Plasmid pmEGF 344, consisting of a 344 base pair (bp) mouse preproEGF cDNA insert flanked by T3 and T7 RNA polymerase promoters, was constructed by subcloning the 3' Pst I fragment of clone pmEGF-26F12, kindly provided by Dr. Axel Ullrich, into the Pst I site of the Bluescript II/KS(+) vector (Stratagene). As shown in Figure 2A, this cDNA insert (spanning bp 3023-3366, Gray et al., 1983) included nearly the entire coding sequence for the mature EGF peptide. Plasmid pmTGF-α 373, similarly constructed with the Bluescript II/KS(+) vector, contained a 373 bp BamHI-Ava I fragment (Fig. 2B) obtained from the 5' end of a 1400 bp TGF-α genomic Sau 3AI sequence, generously provided by Dr. Rik Derynck (Wilcox and Derynck, 1988).

In preparation for *in vitro* transcription reactions, EGF and TGF-α plasmid recombinants were enzymatically restricted either 5' (for anti-sense riboprobe) or 3' (for sense RNA standards) to their specific DNA insert sequences, purified by agarose gel electrophoresis and recovered using the Geneclean DNA isolation technique (Bio 101). ^{32}P-UTP-labelled anti-sense riboprobes (1-2 x 10^{9} cpm per μg) or unlabelled RNA standards were subsequently generated by respective T3- or T7-promoted *in vitro* transcription reactions performed as essentially described (Blum, 1989). Purification of probe or RNA standards from unincorporated nucleotides was achieved by ethanol precipitation at -20 ^{o}C in the presence of 2.5 M ammonium acetate.

Solution hybridization ribonuclease protection assay

Solution hybridization ribonuclease protection assays were performed as described (Sambrook et al.,1989). RNA standard tubes were prepared with increasing quantities of *in vitro* synthesized sense-strand RNA and aliquots of total yeast RNA, equalizing the total quantity of RNA in all tubes of a given assay. EGF-specific standards ranged from 50 fg to 4 pg while TGF-a standards ranged from 100 fg to 16 pg (zero tubes contained total yeast RNA only). All standards were in a final volume of 30 µl hybridization buffer (see above). Following the addition of excess radiolabelled riboprobe (1 µl of ~100-200 pg/µl), samples were heat denatured at 85 ^{o}C for 10 minutes and transferred to a 45 ^{o}C water bath for solution hybridization overnight (16-18 hours). Samples were then incubated with 300 µl RNase digestion mixture (40 µg/ml RNase A, 2 µg/ml RNase T1, 10 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 300 mM NaCl) for 1 hour at 30 ^{o}C, briefly treated with 0.1 mg/ml proteinase K (15 minutes at 37 ^{o}C in the presence of 1 % SDS), extracted with phenol-chloroform and ethanol precipitated with the addition of 15 µg total yeast RNA. RNA:RNA hybrid pellets were recovered by centrifugation, washed with 70 % ethanol, dried, resuspended in 7 µl gel-loading buffer and analyzed on a 4 % non-denaturing acrylamide gel. After drying and exposing the gel to x-ray film, full-length protected gel bands were cut out and quantitated by liquid scintillation counting. Using regression lines generated by plotting known amounts of RNA standards against the observed counts per minute (cpm) in their protected bands, sample cpm values were transformed to mass quantities. A mathematical correction was made to account for the slight difference in length between RNA standards (possessing short hybridizing spans of vector sequences) and the protected length of sample hybrids. Values were ultimately

adjusted to reflect the levels of full-length mRNA [4900 bp for mouse preproEGF (Rall et al., 1985) and 5000 bp for mouse TGF-α (Wilcox and Derynck, 1988)].

Results

Detection of EGF- and TGF-α-specific mRNAs

Solution hybridization ribonuclease protection assays were developed for the detection and quantitation of epidermal growth factor and transforming growth factor-α mRNA in mouse tissue. Representative autoradiographs demonstrating protected hybrids of EGF and TGF-α RNA are shown in Figure 3. The span of EGF-specific standards, ranging from 50 fg to 4 pg RNA, was determined such that the levels present in cytoplasmic RNA extracts fell between the lowest and highest non-zero standard values. Similarly, a typical TGF-α RNA standard curve, covering a range of values from 100 fg to 16 pg, was chosen so that all detected levels were within this defined range. Linear regression correlation coefficients (r^2) of all assay standard curves were $\geq$0.990.

Adult male submaxillary gland (SG) RNA, employed as a positive EGF mRNA assay control (Rall et al., 1985), was observed to protect the anti-sense EGF RNA probe (Fig. 3, upper left panel), providing the complementary (sense) EGF mRNA required for full-length hybrid formation and ribonuclease resistance. Similarly, cytoplasmic RNA isolated from the anterior lobe of adult pituitary, known to express TGF-α and its mRNA (Samsoondar et al., 1986; Kobrin et al., 1986, 1987; Borgundvaag et al., 1990), was observed to protect the anti-sense TGF-α RNA probe (Fig. 3, upper right panel) and was subsequently used as a positive TGF-α mRNA control.

Total yeast RNA, serving as a negative control for both EGF and TGF-α solution hybridization assays, was tested for its inability to protect EGF- or TGF-

α-specific RNA probes from ribonuclease digestion. As shown in the zero lanes of their respective standard curve autoradiographs (Fig. 3, upper panels), EGF and TGF-α protected bands were absent in the presence of total yeast RNA alone, thereby confirming the complete removal of all unhybridized radiolabelled probe.

Regional distribution of EGF and TGF-α mRNA in the mature murine CNS

EGF mRNA was detected in all adult male brain regions examined as well as whole pituitary, with autoradiographic confirmation of discrete, full-length protected bands (Fig. 4). In the CNS, EGF mRNA (Fig. 5, upper graph) was most abundant in the olfactory bulbs (36 ± 3 fg/μg) and basal hypothalamus (30 ± 3 fg/μg) while lower levels were detected in cerebellum (24 ± 4 fg/μg), olfactory tubercle (21 ± 1 fg/μg), brainstem (20 ± 3 fg/μg), striatum (17 ± 3 fg/μg), hippocampus (14 ± 1 fg/μg), thalamus (12 ± 2 fg/μg) and cerebral cortex (11 ± 2 fg/μg). Adult pituitary, a region derived in part from the developing CNS but shown to express EGF within its anterior lobe (Kasselberg et al., 1985), contained 234 ± 10 fg EGF mRNA per μg, 6-20 times the levels present in regions of brain (Fig. 5, upper graph). Adult male submaxillary gland contained 432 ± 3 pg EGF mRNA per μg, approximately 2,000 times the levels in male pituitary and four orders of magnitude greater than the levels present in adult brain (Fig. 6).

TGF-α mRNA levels in brain were 15-170 times greater than the regional levels quantitated for EGF (Fig. 5, lower graph). While clearly highest in the striatum (2.93 ± 0.22 pg/μg), a relative abundance of TGF-α mRNA was also noted for the olfactory tubercle (1.71 ± 0.05 pg/μg) and olfactory bulb (1.28 ± 0.09 pg/μg) (Fig. 5, lower graph). Slightly lower levels were observed in brainstem (0.84 ± 0.11 pg/μg), hippocampus (0.82 ± 0.09 pg/μg), thalamus

(0.72 ± 0.10 pg/μg) and hypothalamus (0.67 ± 0.13 pg/μg), while the concentrations in cerebral cortex and cerebellum (each 0.39 pg/μg) were the lowest among all CNS regions examined. TGF-α mRNA in adult male pituitary (215 ± 12 fg/μg), less than all levels observed in brain, were nearly equal to the pituitary levels of EGF mRNA (Fig. 5, 6C). No TGF-α mRNA was detected in cytoplasmic RNA isolated from adult male submaxillary gland.

Sex differences in EGF and TGF-α mRNA levels

Consistent with the order-of-magnitude differences reported between sexes for both EGF protein and preproEGF mRNA (Byyny et al., 1972; Rall et al., 1985), EGF mRNA levels in the adult male submaxillary gland (432 ± 3 pg/μg) were significantly higher than the levels detected in mature females (15 ± 1 pg/μg) (Fig. 6A). In adult pituitary (Fig. 6C), the level of EGF mRNA in males (0.23 ± 0.01 pg/μg) was also significantly higher than in females (0.17 ± 0.01 pg/μg), as were TGF-α mRNA levels between adult males (0.22 ± 0.01 pg/μg) and females (0.12 ± 0.02 pg/μg). Male-female pituitary mRNA levels compared between growth factors, however, revealed a greater sex difference for TGF-α mRNA, with female values 54 % below the levels detected in males; EGF mRNA in the adult female pituitary was only 26 % below its respective male value. Despite sexual differences in glandular levels of growth factor mRNA transcripts, no significant differences between sexes were observed in regions of adult brain for either EGF (Fig. 6B) or TGF-α mRNA (data not shown).

Developmental expression of EGF and TGF-α mRNA

EGF mRNA in the developing brain was detectable as early as embryonic day 14 (E14) (earliest time point studied) (Fig. 7). While levels appeared relatively stable into the early postnatal period (averaging 47 fg/μg

total RNA between E14 and PN1), the expression of EGF mRNA by postnatal day 10 (PN10) dropped by more than half the levels observed one day after birth (Fig. 8A, upper graph). By contrast, TGF-α mRNA levels rose between E14 and PN1 (reaching ~3 pg per μg total RNA) and subsequently, fell by ~55 % by PN10 (Fig. 8A, lower graph).

Total brain RNA content between embryonic day 14 and postnatal day 10, an index of general brain growth, was 57 (E14), 91 (E15), 114 (E16), 143 (E17), 166 (PN1) and 585 (PN10) μg per brain (averaged from two litters of each age). When levels of EGF and TGF-α mRNA in the developing CNS were expressed per individual brain, the observed patterns of mRNA expression for both growth factors appeared similar with total EGF mRNA (Fig. 8B, upper graph) and total TGF-α mRNA (Fig. 8B, lower graph) increasing throughout perinatal development, though an order-of-magnitude difference between EGF (3 to 11 pg mRNA per brain) and TGF-α (39 to 691 pg per brain) mRNA values was observed. Whereas total brain RNA content increased 3.5 fold between postnatal days 1 and 10 (from an average of 166 μg total brain cytoplasmic RNA to 585 μg), EGF and TGF-α total mRNA in whole brain (Fig. 8B) increased by only 25 and 54 %, respectively. This decrease in relative abundance of EGF and TGF-α mRNA compared to total brain RNA content, then, was reflected by the lower EGF and TGF-α mRNA concentrations observed 10 days after birth (Fig. 8A).

Discussion

This study provides the first specific, quantitative evidence for the expression of EGF mRNA in brain and expands upon reported findings concerning TGF-α gene expression in the CNS. Our comparison of the regional distribution, difference between sexes and developmental profile of EGF and TGF-α mRNAs

in mouse brain suggest that both EGF and TGF-α may play a role in the growth and support of the mammalian central nervous system.

Regional co-distribution of EGF and TGF-α mRNAs in adult brain

Messenger RNAs for both EGF and TGF-α were detected in all regions of the mature CNS studied, including brainstem, cerebellum, cerebral cortex, hippocampus, basal hypothalamus, olfactory bulb, olfactory tubercle, striatum and thalamus. Despite this overlap in distribution, TGF-α mRNA levels, quantitated in the pg per μg range, were 15-170 greater than the fg per μg levels observed for EGF mRNA. Highest concentrations of EGF gene transcripts were in the olfactory bulb and basal hypothalamus, while TGF-α mRNA levels were highest in the striatum and olfactory tubercle. The cerebellum, containing a relative abundance of EGF mRNA, was among the regions expressing the lowest levels of TGF-α mRNA.

The presence of both EGF and TGF-α gene transcripts in brain, together with previous immunological data (Fallon et al., 1984; 1990; Lakshmanan et al., 1986; Fallon, 1987; Schaudies et al., 1989; Kudlow et al., 1989; Brown et al., 1990), provides evidence to support local synthesis of both EGF receptor ligands in the mammalian CNS. Recently, Ojeda et al (1990) suggested that the low EGF mRNA levels detected in brain reflected a limited ability on the part of the CNS to synthesize EGF. Our findings reflect levels of EGF and TGF-α mRNA within whole anatomic regions containing a diversity of cell types and subpopulations and therefore, it is possible that EGF mRNA synthesis within any given brain region is occurring in only a small number of cells. Interestingly, mRNA levels for nerve growth factor (NGF) in the CNS (Shelton and Reichardt, 1986) are comparable to those of EGF. From this standpoint, TGF-α gene expression itself is out of the range of what is traditionally

representative of a neurotrophic agent. The striking differences in mRNA levels, then, potentially reflect the functional specificities of EGF and TGF-α in the CNS.

While our identification of both EGF and TGF-α mRNAs in brain is consistent with local synthesis, the biological form(s) of their respective peptides in brain remain unknown. Previous examinations of the cDNAs encoding EGF and TGF-α indicate that the mature forms of these peptides are derived from larger precursor molecules with structural features common to transmembrane proteins (Scott et al., 1983; Gray et al., 1983; Derynck et al., 1984; Lee et al., 1985a; Bell et al., 1986). Since proteolytic processing of the EGF and TGF-α precursors may not be required for biological activity, it is possible that the membrane-anchored, unprocessed forms of these proteins serve as active forms of EGF and TGF-α in brain (Brachmann et al., 1989; Wong et al., 1989; Mroczkowski et al., 1989; Breyer and Cohen, 1990). In kidney, in which the unprocessed EGF precursor molecule is reported to accumulate (Rall et al., 1985), it has been suggested that the proEGF precursor molecule might serve as a cell surface receptor in addition to generating mature EGF (Pfeffer and Ullrich, 1985). If unprocessed EGF were the predominant form present in the CNS, this could explain why some investigators have not detected EGF immunoreactive material in brain (Probstmeier and Schachner, 1986; Beerstecher et al., 1988) while others have (Fallon et al., 1984; Lakshmanan et al., 1986). More specifically, with EGF antibodies routinely raised against the 53 amino acid processed form of the mature EGF peptide, differences in antigenic determinants between processed and unprocessed EGF could result in a lack of EGF immunostaining for CNS tissue. Unlike the TGF-α precursor protein, however, which codes only for the mature TGF-α peptide, the EGF precursor also contains 7-9 EGF-like peptide sequences (Fig. 2), each sharing varying degrees of homology with EGF itself. Although conflicting opinions

have been offered regarding the potential for proteolytic release of these peptides from the EGF precursor (Gray et al., 1983; Scott et al., 1983; Doolittle et al., 1984), the presence of multiple EGF-like immunoreactive species in rat brain (Schaudies et al., 1989) potentially reflects differential processing of the full-length precursor in the CNS. Thus, the presence of EGF and TGF-α gene transcripts in brain provides a biochemical basis for multiple EGF-R ligands to be endogenously expressed in the mammalian central nervous system.

Perhaps the most intriguing finding of the present study is the regional co-distribution of EGF and TGF-α mRNAs. As both EGF and TGF-α are believed to utilize a common EGF receptor *in vivo* (Massagué, 1983; Carpenter et al., 1983; Marquardt et al., 1983, 1984), the mechanism by which either peptide might achieve different biological effects in any particular region of the nervous system remains unclear. Currently, there is no experimental evidence to support the existence of EGF receptor subtypes *in vivo.* However, differences in the biological potencies or activities of EGF and TGF-α have been observed (Ibbotson et al., 1986; Schreiber et al., 1986; Barrandon and Green, 1987; Gan et al., 1987). Winkler et al (1989) recently proposed that differences in EGF- and TGF-α-mediated effects might result from *i)* differences in growth factor binding sites in the EGF-R or *ii)* differences in receptor conformations induced upon binding. Decker (1990) has suggested that differential processing of the EGF-R following EGF or TGF-α binding could account for differences in growth factor-specific potencies. It is possible, however, that in addition to receptor mechanisms mediating potential differences in EGF and TGF-α activities in brain, micro-regional differences in the distribution of EGF and TGF-α in the CNS may restrict their local availability for binding to cell-specific EGF receptors.

Perhaps one of the most favored hypotheses concerning potential differences in activities of EGF and TGF-α in brain is that one peptide originates in glia while the other is generated by neurons. At present, the glial and/or neuronal sites of EGF and TGF-α mRNA synthesis in the CNS remain unknown. While data obtained from immunological studies tend to suggest a neuronal source for EGF (Fallon et al., 1984; Lakshmanan et al., 1986), TGF-α immunoreactive material has been observed in both neurons (Fallon, 1987; Kudlow et al., 1989) and glia (Fallon et al., 1990). A recent study using *in situ* hybridization histochemistry to localize TGF-α mRNA in mouse brain has revealed a predominantly neuronal distribution of labelled cells (Wilcox and Derynck, 1988). In view of the low regional concentrations of EGF mRNA present in mouse brain, an extremely sensitive *in situ* hybridization protocol will most likely be required to identify the glial or neuronal source of EGF gene expression in the mammalian CNS.

Sex differences in growth factor gene expression are tissue specific

Previous studies have reported sex differences in the levels of EGF or EGF mRNA in a variety of adult mouse tissues and fluids. For the submaxillary gland, EGF peptide and mRNA concentrations have been observed as significantly higher in males than in females (Byyny et al., 1972; Rall et al., 1985). Similarly, quantitation of EGF in blood serum and liver has revealed higher levels in males (Perheentupa et al., 1985a; Laborde et al; 1988). In contrast, both kidney and urine concentrations of EGF have been shown to be higher in females (Perheentupa et al., 1985b). In view of observed male-female differences in axonal sprouting following induced lesions in the CNS (Loy and Milner, 1980) and the ability of EGF to promote neurite outgrowth in culture (Morrison et al., 1987, 1988; Casper et al., 1991), we were interested in whether

the levels of EGF gene expression in brain might also show differences between sexes and if so, whether such differences might be paralleled by the expression of TGF-α mRNA.

From our studies, no significant sex differences were apparent in any region of the CNS for either EGF or TGF-α mRNAs. Even for the basal hypothalamus, in which androgen and estrogen receptors are relatively abundant, no significant male-female difference in growth factor gene expression was observed. However, since we did not use estrous-staged mice for our study, we do not rule out the possibility that female levels of EGF mRNA vary during the course of the estrous cycle.

In the adult pituitary, also known to be a rich source of gonadal steroid hormone receptors, significantly higher levels of EGF and TGF-α mRNAs were observed in males. Recently, Pascall and Brown (1988) located sequences in the 5' flanking region of the mouse EGF gene, homologous to sequences present in other genes known to be regulated by testosterone. In view of these findings, the sex difference in pituitary EGF mRNA levels could reflect androgen:receptor:DNA binding interactions that directly promote EGF transcriptional changes in this tissue. Alternatively, differences in levels of EGF mRNA between male and female pituitary might reflect sex differences in the numbers of each of the hormone-secreting cell types within the anterior lobe. Thus, with the expression of EGF apparently limited to the gonadotroph and thyrotroph cell populations (Kasselberg et al., 1985), the greater abundance of lactotrophs in female pituitary (Velkeniers et al., 1988) would lower the observed concentration of EGF mRNA in females below that of males. A similar argument, however, can not easily be made for the male-female difference in TGF-α mRNA levels as this peptide has previously been localized to the lactotroph cell population (Kobrin et al., 1987). Therefore, unless TGF-α were

sequestered within lactotrophs following its synthesis in some other cell type, the levels of TGF-α mRNA in female pituitary would be expected to be higher or at least equal to the levels detected in males. Further studies then are necessary to determine whether the sex difference in TGF-α mRNA expression may be due to differences in gonadal steroid hormone expression.

Co-expression of EGF and TGF-α mRNAs in fetal and early postnatal brain

This study provides the only evidence to date for the expression of EGF mRNA in fetal brain. The lack of information concerning EGF gene expression during embryogenesis has led to speculations that transforming growth factor-α is the fetal analog of EGF (Proper and Moses, 1981; Lee et al., 1985b; Twardzik, 1985; Popliker et al., 1987). Our findings now suggest that both EGF and TGF-α may participate in normal CNS developmental processes. Consistent with our data, EGF receptors are present in mouse brain as early as embryonic day 15 and in a manner similar to the rise in TGF-α mRNA, EGF receptors increase in number throughout late embryonic brain development (Adamson and Meek, 1984).

At present, the role of EGF or TGF-α in brain development remains unknown. Studies performed in culture have shown that specific populations of neurons and glia derived from fetal brain are responsive to the effects of EGF (Simpson et al., 1982; Honegger and Guentert-Lauber, 1983; Morrison et al., 1987, 1988; Knusel et al., 1990; Casper et al., 1991). In a preliminary report by Reynolds et al (1990), EGF and TGF-α have been shown to stimulate the proliferation of striatal multipotential progenitor cells *in vitro*. Interestingly, the cellular morphologies induced by TGF-α were somewhat different than those produced by EGF (Reynolds et al., 1990). In view of these findings and the strikingly different levels of EGF and TGF-α mRNAs present in fetal brain, EGF

and TGF-α are likely to have unique roles in the development of the mammalian CNS.

Functional implications

The potential for EGF and TGF-α to utilize a common receptor *in vivo* underscores the difficulty in assigning physiological roles specific to each peptide based on anatomic characterizations alone. While it is still not clear which cells in the brain actually synthesize EGF or TGF-α, the potential for both ligands to compete locally for EGF-R binding suggests that a variety of mechanisms may be involved in regulating growth factor-specific activities in the CNS. In this light, studies addressing specific changes in growth factor gene expression during the course of normal brain development or in response to brain injury are likely to contribute to our understanding of why two EGF-R ligands are physiologically required in this tissue. Further studies in our laboratory using mutant mice exhibiting selective neurodegenerative losses of cells known to express EGF receptors have revealed unique patterns of gene expression for EGF and TGF-α during specific neuronal or glial events (Lazar and Blum, unpublished observations). In view of such differences, it is unlikely that these structurally related peptides simply reflect a biochemical redundancy in the normal functioning of the mammalian CNS. More likely, EGF and TGF-α in brain serve within a multifunctional growth factor system whose members interact via a common membrane receptor.

Figure 1. Diagrammatic representation of the regional dissection of adult mouse brain. *A*, dorsal view; *B*, ventral view; *C*, mid-sagittal view. *Numbers 1-8* indicate the positions of cuts sequentially performed during dissection. See Materials and Methods for complete description. Dissection landmarks are abbreviated as follows: *S*, superior colliculi; *L*, lateral olfactory tract; *M*, mammillary nuclei; *CC*, corpus callosum; *F*, fornix; *AC*, anterior commissure.

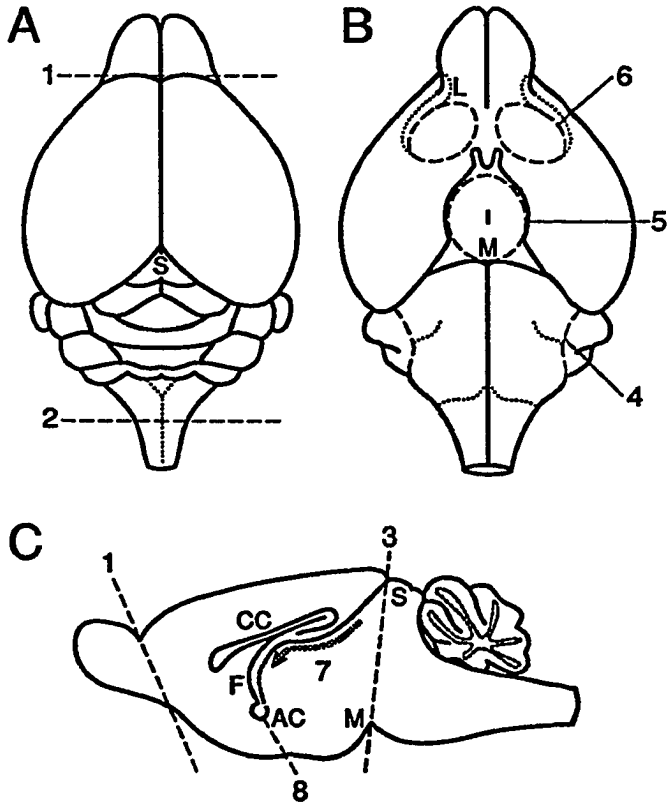


Figure 2. Schematic representation of EGF and TGF-α subclones used to generate RNA probes and standards. *A,* A 344 base pair (bp) mouse EGF cDNA sequence of plasmid *pmEGF 344,* shown in reference to the full-length preproEGF mRNA transcript (redrawn from Scott et al., 1983; Doolittle et al., 1984), was used as a template for the *in vitro* transcription of EGF-specific RNA probes and standards. Defining nucleotides 3023-3366 (Gray et al., 1983) of the preproEGF mRNA, this subclone fragment included sequences encoding the mature EGF peptide *(black box). Numbers 1-7* (Scott et al., 1983) or *letters a-i* (Doolittle et al., 1984), designated within the preproEGF mRNA transcript, indicate the EGF-like peptide sequences sharing varying degrees of homology with EGF. Untranslated 5' and 3' sequences are represented by lines; the open bar represents the protein-coding portion of the mRNA. Predicted signal sequence and transmembrane domains *(stippled regions)* are designated *s* and *t*, respectively. *B,* The TGF-α peptide coding region *(black box)*, predicted signal and transmembrane domains *(stippled areas)*, and untranslated 5' and 3' sequences *(extended lines)* of the TGF-α mRNA transcript are indicated (redrawn from Lee et al., 1985a). The 373 bp mouse genomic DNA fragment (contained within plasmid *pmTGF-α 373*), used in the generation of TGF-α-specific RNA probes and standards, defined the most upstream segment in the 3' untranslated region of the TGF-α mRNA transcript beginning 6 bases 3' to the translational stop codon (Wilcox and Derynck, 1988).

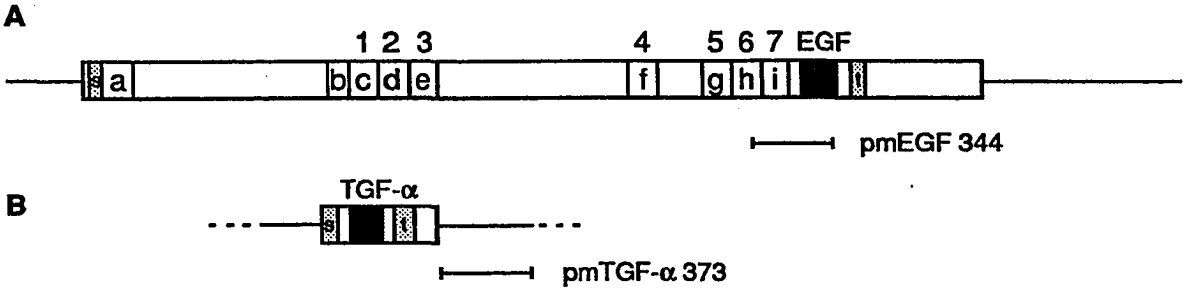


Figure 3. Standard curves of EGF and TGF-α ribonuclease protection assays. *Upper panels,* Representative autoradiographs of EGF and TGF-α standard curves are shown, demonstrating the linear relationship between increasing quantities of *in vitro*-transcribed RNA standards and the intensity of their full-length protected bands. Adult male mouse submaxillary gland *(SG)* RNA and anterior pituitary *(Pit)* RNA were used as positive control samples in all EGF and TGF-α assays, respectively. The difference in size between tissue RNA bands *(SG and Pit)* and RNA standards, shown in reference to ^{35}S-labelled Msp I-pBR322 DNA markers *(M)*, was a result of short vector sequences transcribed during the *in vitro* synthesis of RNA probes and standards. These sequences were not complementary to tissue mRNA sequences and consequently, were trimmed away during ribonuclease digestion. No protected bands were observed in zero lanes containing total yeast RNA alone. Autoradiographic exposure: 14 hours at -80 °C with an intensifying screen. (Note: Protected bands were not visible in the zero lanes of autoradiographs even after exposing gels for as long as 70 hours.) *Lower panels,* Following the exposure of dried gels to x-ray film, full-length protected gel bands were cut out (using the autoradiograph as a guide) and quantitated by liquid scintillation counting. Linear regression analysis of the relationship between known quantities of RNA standards and the counts per minute (cpm) present in their protected bands (shown graphically) provided a mathematical equation for transforming tissue RNA cpm values to mass quantities.

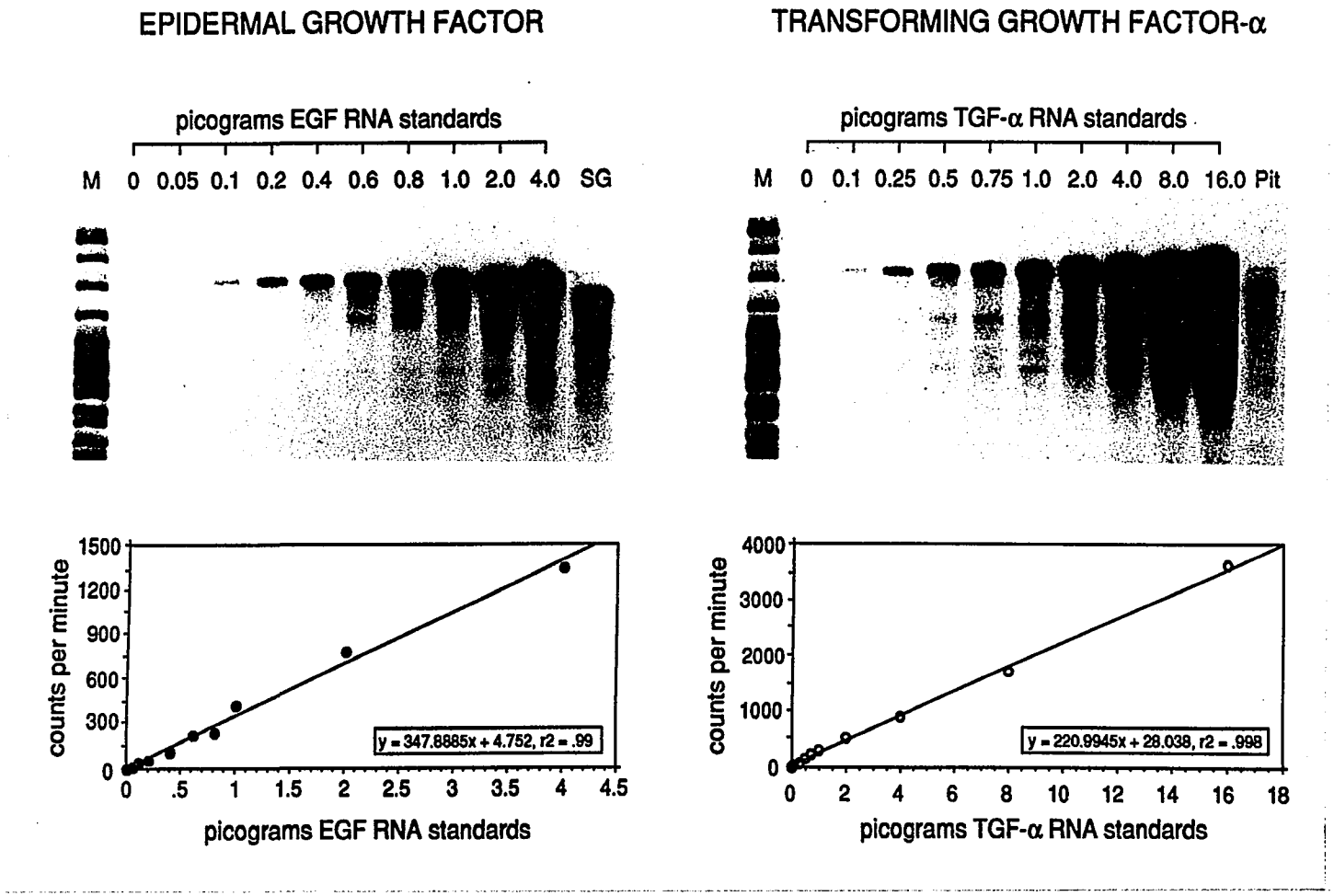


Figure 4. Ribonuclease protection analysis of EGF mRNA in defined regions of the adult male mouse CNS and pituitary. Total cytoplasmic RNA isolated from brain regions and whole pituitary were assayed for the presence of mRNA complementary to the 344 nucleotide EGF-specific riboprobe sequence. Full-length protected RNA bands, shown in reference to Msp I-digested pBR322 DNA markers, were evident in all sample lanes. Quantities of total cytoplasmic RNA assayed were as follows: 100 µg for brainstem, cerebellum, cerebral cortex, thalamus, hippocampus, olfactory bulb and striatum; 75 µg for whole pituitary; and 50 µg for basal hypothalamus and olfactory tubercle; all samples were standardized to a final quantity of 100 µg RNA by the addition of total yeast RNA. The autoradiographic image represents a 24 hour exposure at -80 ^{o}C with an intensifying screen.

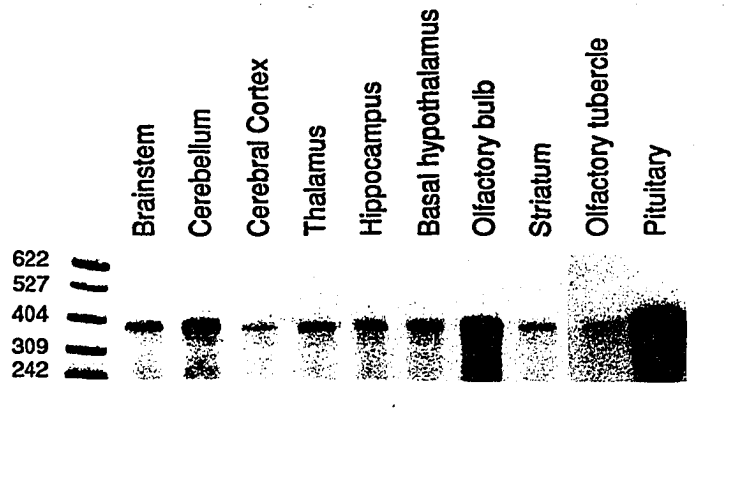


Figure 5. EGF and TGF-α mRNA levels in adult male mouse CNS and pituitary. Total cytoplasmic RNA isolated from anatomically-defined brain regions and whole pituitary were assayed for the quantitation of EGF and TGF-α mRNA (see Materials and Methods for quantitative procedures). The number of independent assay determinations (n) performed for each region were as follows: n=6 for brainstem, cerebellum, cerebral cortex, hippocampus, olfactory bulb and thalamus; n=3 for basal hypothalamus, olfactory tubercle and whole pituitary; n=2 for striatum. Values represent the mean ± SEM for each region.

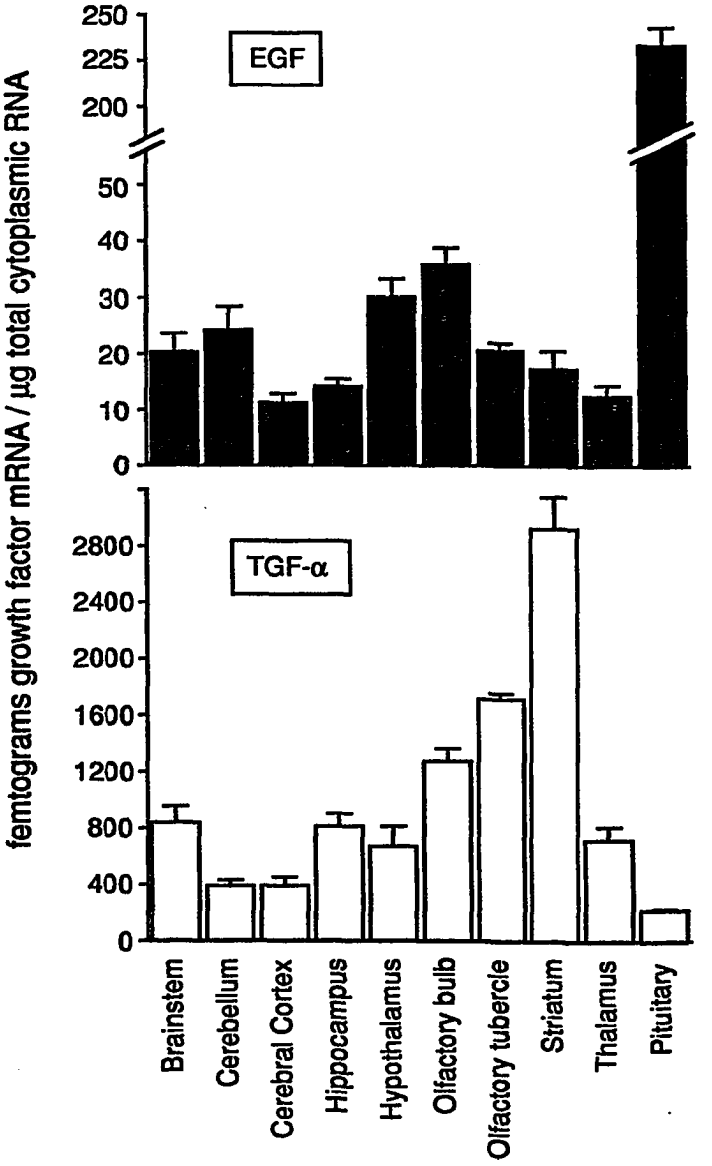


Figure 6. Comparison of EGF and TGF-α mRNA levels between sexes. Total cytoplasmic RNA isolated from male and female adult mouse submaxillary gland, brain regions and whole pituitary were assayed for the quantitation of EGF and TGF-α mRNA. Tissue collected from female mice were taken at random stages of the estrous cycle. To minimize the variability introduced during the handling of samples, RNA extractions and assay analyses for tissues matched by sex were performed simultaneously. The analysis of striatum was excluded due to the prohibitive number of animals required for adequate quantities of total RNA. Values represent the mean ± SEM of three independent determinations. *A,* EGF mRNA levels in adult submaxillary gland revealed significantly higher levels in males compared to females (**$p<0.0001$, Student's *t*-test). *B,* No significant difference between sexes was observed for the expression of EGF mRNA in adult brain regions ($p=0.2$, ANOVA). *C,* EGF and TGF-α mRNA levels present in adult male pituitary were significantly greater than their respective female values (*$p=0.01$, Student's *t*-test).

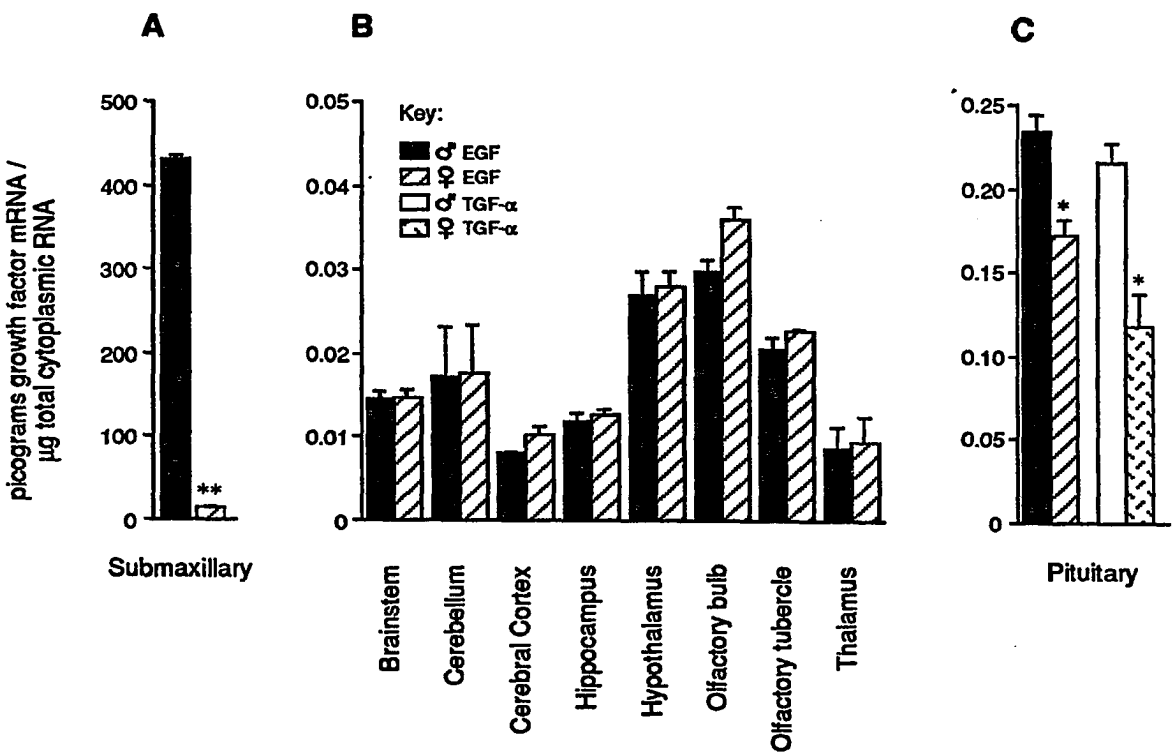


Figure 7. Ribonuclease protection analysis of EGF mRNA expression in prenatal and early postnatal mouse brain. *M,* ^{35}S-labelled Msp I/pBR322 DNA marker; *E14 - E17,* analysis of embryonic (E) brain cytoplasmic RNA derived from 14, 15, 16 and 17 day fetuses; *PN 1 - 10,* analysis of postnatal (PN) brain cytoplasmic RNA derived from 1 and 10 day old pups. All samples contained 75 μg total cytoplasmic RNA derived from brains of individual litters. The autoradiographic image represents a 24 hour exposure at -80 ^{o}C with an intensifying screen.

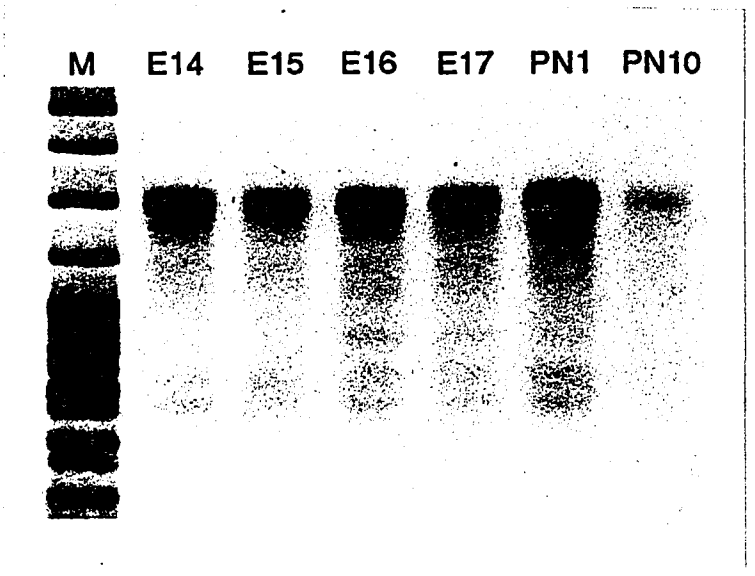
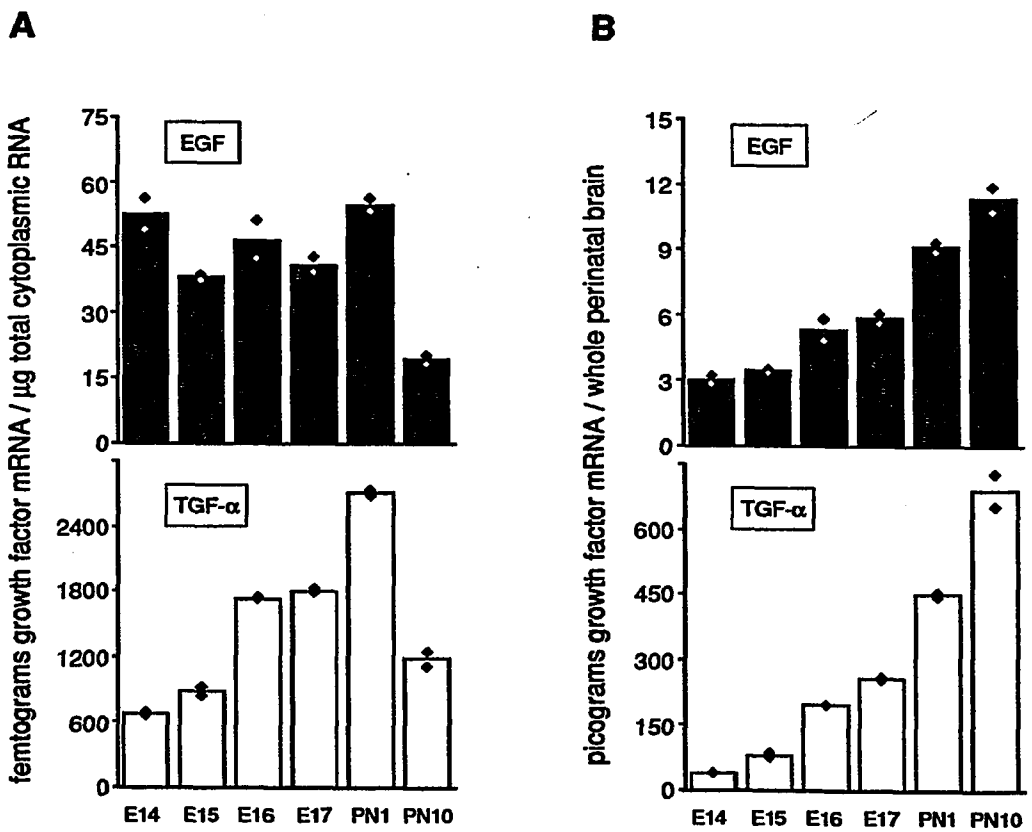


Figure 8. Expression of EGF and TGF-α mRNA in the developing CNS. Embryonic *(E)* whole brain from *14*, *15*, *16* and *17* day mouse fetuses and postnatal *(PN)* whole brain from *1* and *10* day old pups were processed for the isolation of total cytoplasmic RNA and assayed by ribonuclease protection analysis. Levels of EGF and TGF-α mRNA are expressed per microgram total RNA *(A)* or per whole perinatal brain *(B)*. Bars represent the average of two independent assay determinations (each indicated with a diamond).



CHAPTER 2

Epidermal Growth Factor and Transforming Growth Factor-α mRNA Expression in Purkinje Cell Degeneration (Pcd) and Weaver Mutant Mice

Abstract

Epidermal growth factor (EGF) and its structural homolog, transforming growth factor-α, are members of a growth factor family believed to play a role in the growth, development and support of the mammalian central nervous system. In order to gain insight into their biological potentials *in vivo*, we have characterized the developmental expression of their respective mRNAs in the cerebellum and olfactory bulbs of both normal and neurodegenerative mutant mice using solution-hybridization ribonuclease-protection analysis. Growth factor gene expression in normal (genotype *+/+*) developing cerebellum and olfactory bulbs was highest for both EGF and TGF-α mRNAs during the second postnatal week (postnatal day 9 (P9) the earliest timepoint studied). By approximately P21, levels of growth factor mRNAs were not significantly above the levels observed in fully mature adults (10 weeks). An examination of EGF gene expression in Pcd mutant (*pcd/pcd*) cerebellum, characterized by a complete loss of Purkinje neurons (Mullen et al., 1976), revealed significantly higher concentrations than unaffected littermates beginning P39. Between P39 and P62, EGF mRNA content in Pcd mutant cerebellum transiently increased. For TGF-α, Pcd mutant cerebellar mRNA concentrations and content were higher than controls after postnatal day 65. In Pcd olfactory bulbs, in which degenerating nerve fibers are present at ~13 postnatal weeks (Mullen et al., 1976), TGF-α mRNA concentrations and content at ~P65 were transiently below those of unaffected mice, subsequently returning to near-control values, while EGF mRNA concentrations began to increase between weeks 11 and 12. In the weaver mutant (*wv/wv*) cerebellum, characterized by severe granule cell losses (Rakic and Sidman, 1973), both EGF and TGF-α mRNA concentrations did not differ from non-ataxic homozygous (*+/+*) or heterozygous (*+/wv*) mice. Weaver mutant cerebellar levels of total EGF or TGF-α mRNA, however, were

consistently below the levels present in *+/+* and *+/wv* cerebellum. In view of the differences in EGF and TGF-α gene expression resulting from abnormalities in genetically-determined neurodevelopmental programs, these putative EGF receptor ligands are likely to have distinct physiologic roles both in normal development and in response to injury in the mammalian CNS.

Introduction

Epidermal growth factor (EGF) and its structural homolog, transforming growth factor-α (TGF-α) are members of a growth factor family present in the CNS which utilize a common EGF membrane receptor (EGF-R) (Massagué, 1983; Carpenter et al., 1983; Marquardt et al., 1983, 1984; Fallon et al., 1984; Rall et al., 1985; Lee et al., 1985a; Lakshmanan et al., 1986; Fallon, 1987; Wilcox and Derynck, 1988; Kudlow et al., 1989; Schaudies et al., 1989; Ojeda et al., 1990; Fallon et al., 1990; Brown et al., 1990; Carpenter and Wahl, 1990; Fisher and Lakshmanan, 1990). Based on *in vitro* systems, EGF has been shown to stimulate astroglial proliferation and differentiation and to promote neurite outgrowth and survival of specific neuronal populations (Leutz and Schachner, 1981, 1982; Simpson et al., 1982; Raff et al., 1983; Guentert-Lauber and Honegger, 1983; Honegger and Guentert-Lauber, 1983; Almazan et al., 1985; Morrison et al., 1987, 1988; Knusel et al., 1990; Casper et al., 1991). It has therefore been suggested that the activities of EGF and/or TGF-α *in vivo* may be related to the normal development and maintenance of subsets of neurons and glia (Araujo et al., 1990; Plata-Salamán, 1991). In support of this general hypothesis are the reported observations that EGF receptors are present in developing and mature brain and that they are expressed on both neurons and astroglia at different times during development (Adamson and Meek, 1984;

Hiramatsu et al., 1988; Gómez-Pinilla et al., 1988; Werner et al., 1988; Chabot et al., 1988).

In the present study, we examined temporal patterns of expression of EGF and TGF-α mRNAs in the normal mouse CNS, focusing on the cerebellum and olfactory bulbs, in an effort to correlate potential changes in growth factor mRNA levels with well-characterized neurodevelopmental events. Advantages to studying these brain regions included the relatively simple, stereotyped nature of cerebellar and olfactory bulb cytoarchitecture and the fact that, in rodents, the development of these areas continues well into the postnatal period (Fujita, 1969; Meller and Glees, 1969; Larramendi, 1969; Hinds, 1968a, 1968b; Hinds and Hinds, 1976). With the availability of mutant mice demonstrating specific losses of cerebellar and olfactory bulb neurons, we were further able to study the effects of abnormal developmental programs on growth factor gene expression, thereby gaining insight into the potential roles played by these peptides in the normal brain. Two cerebellar mutant strains were examined, the Purkinje cell degeneration (Pcd) (Mullen et al., 1976) and the weaver mouse (Rakic and Sidman, 1973). For Pcd mutant mice, cerebellar Purkinje cells are typically lost beginning around postnatal day 18 and by the fifth postnatal week, very few Purkinje neurons remain (Landis and Mullen, 1978). In Pcd olfactory bulbs, a relatively slow, progressive neurodegeneration occurs so that it is only by the thirteenth postnatal week that degenerating fibers are observed (Mullen et al., 1976). Therefore, a comparison between Pcd mutant cerebellum and olfactory bulbs provided an opportunity to determine whether potential changes in growth factor gene expression occur in conjunction with or prior to the onset of the neurodegenerative process. In weaver cerebellar mutant mice, there is a rapid degenerative loss of granule neurons in the vermis (midline region) during the first postnatal month (Rakic

and Sidman, 1973). We therefore compared the developmental patterns of growth factor mRNAs in the cerebellum of Pcd and weaver mutant mice to address the effects of specific neuronal losses on the expression of EGF and TGF-α mRNAs. Finally, in order to follow potential changes in astroglial activity that normally accompany CNS injury (Duffy, 1983; Lindsay, 1986; Takamiya et al., 1988) and which reportedly occur in the molecular layer of the Pcd mutant cerebellum (Mullen et al., 1976), glial fibrillary acidic protein (GFAP) mRNA was examined. Since astrocytes have previously been suggested as a source of TGF-α in brain (Fallon et al., 1990), this approach was valuable in that it allowed us to assess a potential relationship between TGF-α and GFAP mRNAs in the mammalian CNS.

Materials and Methods

Animal Breeding

Colonies of mice were established at Mount Sinai Medical Center from breeder pairs commercially obtained from the Jackson Laboratory (Bar Harbor, Maine). Normal F_1 hybrid B6C3H mice (genotype *+/+*) were obtained by intercrossing inbred C57Bl/6J females with C3HeB/FeJLe-a/a males. Purkinje cell degeneration (Pcd) mutant mice (*pcd/pcd*) and their non-ataxic littermates (*+/?*), also of the B6C3H hybrid background, were derived from heterozygous crosses (*+/pcd* x *+/pcd*). Mice heterozygous for the weaver mutant allele (*+/wv*) on the B6CBA-A^{w-J}/A hybrid background were intercrossed (*+/wv x +/wv*) to generate *wv/wv*, *+/wv* and *+/+* mice.

Tissue collection

At selected ages, mice were sacrificed by cervical dislocation and decapitated. Their olfactory bulbs and cerebellum were subsequently removed,

frozen over dry ice and weighed. For the normal developmental studies in B6C3H mice, tissues were collected from both males and females. For Pcd studies, weaned mice were observed beginning ~4 weeks of age for the onset of ataxia and following the identification of mutant (*pcd/pcd*) animals, same-sex non-ataxic (*+/?*) littermates were randomly selected as controls. Three independent sets of Pcd sex-matched mutant-control pairs were subsequently sacrificed at selected ages between 30 and 115 days of age for the collection of both cerebellum and olfactory bulbs. Weaver litters were observed ~12-14 days postnatally for the identification of mutant mice (*wv/wv*) exhibiting the characteristic ataxia of gait and fine tremor. At selected ages between 14 and 24 days, whole litters were sacrificed and individual cerebella collected. The cerebella of non-ataxic weaver littermates (*+/+* and *+/wv*) were hemisected in the mid-sagittal plane and subsequently, one half (randomly chosen) was frozen for RNA extraction and the other half taken for histology.

Histology of weaver cerebellum

With the ability to distinguish *+/wv* and *+/+* mice on the basis of tissue morphology, cerebellar halves of non-ataxic weaver littermates were prepared for histologic examination by embedding in O.C.T. medium, freezing over dry ice and sectioning on a -20°C cryostat. Semithin (10-15 μm) sections were thaw-mounted onto slides and immersion fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4). Sections were ultimately stained with either hematoxylin and eosin (H & E) or thionin (Nissl) for light microscopic examination.

Solution hybridization ribonuclease protection analysis

The preparation of mouse brain total cytoplasmic RNA and its analysis by EGF and TGF-α ribonuclease protection assays has been described (Chapter 1). For the analysis of GFAP mRNA expression in mouse cerebellum and olfactory bulbs, we employed a mouse GFAP cDNA recombinant plasmid as template for the *in vitro* transcription of radiolabelled anti-sense riboprobe (1-2 x 10^9 cpm/μg) and unlabelled sense RNA standards, essentially performed as described (Blum, 1989). The recombinant plasmid pmGFAP, constructed by R. Hellendall, consisted of a 299 base pair (bp) cDNA sequence inserted into the Hind III and Pst I multiple cloning sites (MCS) of the Bluescript KS+ vector (Stratagene). This insert fragment corresponds to bp 951-1250 in the mouse GFAP cDNA coding domain (Lewis et al., 1984). As the mature GFAP mRNA transcript in its full length is 2700 bp, as determined by Northern analysis (Lewis et al., 1984), mass values of GFAP mRNA levels in cerebellar and olfactory bulb samples determined by linear regression analysis of 299 bp GFAP RNA standards (non-zero values ranging from 0.2 to 16 pg) required a correction factor of 9 in order to reflect full-length mRNA levels present in tissue. All samples analyzed for the expression of GFAP mRNA (~1-4 μg) were drawn from cytoplasmic RNA samples to be assayed for the presence of TGF-α mRNA.

Statistical Analysis

In order to determine whether cerebellar mutant and control mRNA levels were significantly different, a 2-factor Analysis of Variance (ANOVA) was performed (for genotype and age) (Zar,1984). Fisher Protected Least Significant Difference tests (Fisher PLSD) were subsequently used to identify the ages at which significant differences were occurring. Significance levels used in the Fisher PLSD included 95 and 99 %.

Results

Growth factor gene expression in the developing cerebellum and olfactory bulbs of normal (+/+) B6C3H mice

Postnatal growth curves for the cerebellum and olfactory bulbs, as indexed by tissue weight (mg) and total RNA content (μg), are given in Fig. 1. In general, both regions demonstrated rapid increases in weight during the second and third postnatal weeks (postnatal day (P) 9 through P21), while tissue weight gains at later ages were proportionally less. With respect to total RNA, a pronounced increase was observed for the cerebellum between P9 and P12, paralleling a rapid gain in tissue weight (Fig. 1A). However, an examination of cerebellar tissue RNA content at subsequent ages revealed a progressive decrease in total RNA content, such that an inverse relationship between tissue weight and tissue RNA was observed. For the olfactory bulb, highest levels of total RNA were also apparent around postnatal day 12 (Fig. 1B). Yet, the rate of decline in total RNA content following P12 for this region was generally slower and of smaller proportion than that observed for the cerebellum.

The levels of EGF, TGF-α and GFAP mRNA expression in normally developing cerebellum and olfactory bulbs were determined by ribonuclease protection assay and are shown in Fig. 2 and 3, respectively. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and total mRNA content (expressed as pg or ng mRNA / brain region) were determined for each gene transcript. A comparison between sexes for EGF and TGF-α mRNA concentrations at defined ages did not reveal any significant male-female differences for either brain region and therefore, growth factor mRNA concentrations represent combined means (± SEM) of age-matched male and female data (Fig. 2A, 2B; Fig. 3A, 3B). Similarly, no significant differences were

observed between male and female total EGF mRNA in the cerebellum or total TGF-α mRNA content in either the cerebellum or the olfactory bulbs and subsequently, male and female data were combined (Fig. 2A, 2B; Fig. 3B). However, an examination of total EGF mRNA levels in the developing olfactory bulbs revealed a difference between male and female mean values at postnatal day 54 (Fig 3A). We have therefore represented these latter values separately, combining all other male and female EGF mRNA content data by age. GFAP mRNA concentrations and total mRNA content in the developing cerebellum and olfactory bulbs were determined only for males and are shown in Fig. 2C and 3C, respectively.

Growth factor gene expression in the cerebellum of Purkinje cell degeneration mice

A temporal comparison between changes in cerebellar weights (mg) and total RNA content (μg) in Pcd mutant mice (*pcd/pcd*) and their sex-matched littermate controls (*+/?*) are shown in Fig. 4A and 4B. With respect to growth factor gene expression (Fig. 5), significantly higher concentrations of EGF mRNA in *pcd/pcd* mice compared to controls were observed from postnatal day 39 onward (Fig. 5A). A corresponding increase in total EGF mRNA content was apparent between postnatal days 39 and 62 (Fig. 5B). For TGF-α, mRNA concentrations were observed to significantly increase in *pcd/pcd* mice after postnatal day 65 (Fig. 5C). However, while TGF-α mRNA concentrations continued to rise, TGF-α mRNA content was only transiently elevated between postnatal days 70 and 99 (Fig. 5D). GFAP mRNA levels, both in content and concentration, were highest during the final stages of Purkinje neurodegeneration (~P45) and thereafter, declined to relatively stable levels that remained significantly above those of controls (Fig. 5E and 5F). These

latter findings are consistent with the occurrence of a severe astrocyte reaction in *pcd/pcd* cerebellar cortex between 30 and 128 days of age (Ghetti et al., 1981).

Growth factor gene expression in the cerebellum of weaver mice

With the ability to distinguish *+/wv* and *+/+* cerebellum on the basis of light microscopic examination (Fig. 6), we compared mutant (*wv/wv*) levels of growth factor and GFAP gene expression with both *+/wv* and *+/+* mice. A temporal comparison between changes in weight (mg) and total RNA content (μg) in weaver mutant (*wv/wv*), heterozygous (*+/wv*) and wild-type (*+/+*) cerebellum are shown in Fig. 4E and 4F, respectively. In the mutant (*wv/wv*) cerebellum, both EGF and TGF-α mRNA concentrations did not appear to differ from non-ataxic homozygous (*+/+*) or heterozygous (*+/wv*) mice (Fig. 7A and 7C). Mutant cerebellar levels of total EGF or TGF-α mRNA, however, were consistently below the levels present in *+/+* and *+/wv* cerebellum (Fig 7B and 7D). For GFAP mRNA, concentrations in *wv/wv* mice remained well above the values observed in *+/+* littermates and at ~P16, a marked elevation in concentration was observed. In contrast, total GFAP mRNA content in *wv/wv* cerebellum was only slightly above *+/+* levels.

Growth factor gene expression in the olfactory bulbs of Purkinje cell degeneration mice

Temporal comparisons of the changes in olfactory bulb weight (mg) and total RNA content (μg) for Pcd mutant mice (*pcd/pcd*) and their sex-matched littermate controls (+/?) are shown in Fig. 4C and 4D. With respect to growth factor and GFAP gene expression, relatively few differences were observed between mutant (*pcd/pcd*) and control (*+/?*) mice until ~P90, corresponding with

the onset of fiber degeneration in *pcd/pcd* olfactory bulbs (Mullen et al., 1976). For EGF mRNA, concentrations in *pcd/pcd* olfactory bulbs were significantly greater than their littermate controls (*+/?*) after P87 (Fig. 8A). In contrast, no significant differences in EGF mRNA content were observed (Fig 8B). For TGF-α, mRNA concentrations and content did not significantly differ throughout most of the ages examined (Fig. 8C and 8D). However, at ~P65, TGF-α mRNA levels in mutants, both in concentration and content, were significantly below those of controls, subsequently returning to normal values. In agreement with the presence olfactory bulb fiber degeneration at ~P90 (thirteen weeks), GFAP mRNA levels were highest around postnatal day 87.

Discussion

This study demonstrates that EGF and TGF-α gene expression in the cerebellum and olfactory bulb are differentially affected by abnormalities in the neurodevelopmental programs which occur in Purkinje cell degeneration (Pcd) and weaver mutant mice. Based on this data and the quantitative differences in the normal developmental profiles of EGF and TGF-α mRNAs, we present the first evidence that these putative EGF receptor ligands are likely to have distinct physiologic roles both in development and in response to injury in the mammalian CNS.

Growth factor gene expression during normal cerebellar and olfactory bulb development

Quantitation of the *in vivo* expression of EGF and TGF-α mRNAs in the normal cerebellum and olfactory bulb provides an opportunity to explore potential relationships between changes in growth factor mRNA levels and well-characterized neurodevelopmental events. From our studies, highest mRNA

levels for both growth factors were observed to coincide with the early period in postnatal development of these brain regions. In general, the types of events that normally occur within these areas during this interval include neuronal and glial proliferation and differentiation, cell migration, neurite outgrowth, and the formation of cell-to-cell contacts. In this context, it is not surprising that the numerous neurobiological effects attributed to EGF on the basis of observations in culture have included the promotion of glial proliferation (Leutz and Schachner, 1981, 1982; Simpson et al., 1982; Raff et al., 1983), glial differentiation (Guentert-Lauber and Honegger, 1983; Honegger and Guentert-Lauber, 1983; Almazan et al., 1985), and the enhancement of neuronal survival and process outgrowth (Morrison et al., 1987, 1988; Casper et al., 1991). While there are few descriptions of the effects of TGF-α on neuronal or glial cells in culture (Reynolds et al., 1990), EGF and TGF-α are believed to utilize a common membrane receptor *in vivo* (Massagué, 1983; Carpenter et al., 1983; Marquardt et al., 1983, 1984) and therefore, the effects observed for EGF could very well be shared by TGF-α. However, in view of the striking differences in absolute levels of growth factor mRNAs present in the developing cerebellum and olfactory bulbs, the physiologic roles of these agents are likely to be distinct. Therefore, to define the context in which EGF and TGF-α mRNA levels may be changing in the CNS, we begin with a brief review of the sequence of events occurring during normal murine cerebellar and olfactory bulb development.

In the mouse, Purkinje cells (Pc) originate from the ventricular matrix of the cerebellar anlage between embryonic days (E) 11 and 13 and are followed by Golgi neuron production (Fujita, 1969). By approximately E15, the formation of glial precursors begins. It is generally accepted that the radially-oriented Bergmann glia are derived from these outwardly migrating glioblasts (Fujita,

1969; Meller and Glees, 1969) and that they have attained their fully mature forms by birth (Meller and Glees, 1969). Between E13 and postnatal day (P) 1, the external matrix cells begin to differentiate forming the external granule cell layer (EGL). Basket and stellate neurons are subsequently generated beginning in the first postnatal week. Between postnatal day 4 and 20, the post-mitotic granule neurons of the EGL begin to migrate along radially-oriented fibers of the Bergmann glia to reach their final positions in the internal granule cell layer (IGL). It is during the first three postnatal weeks then (predominantly P7 through P14) that the majority of cerebellar cortical synapses are formed (Larramendi, 1969). For the olfactory bulb, cytoarchitectural arrangements proceed in a similarly stereotyped fashion. In general, larger neurons originate before smaller ones, neurons before glia (Hinds, 1968a). Thus, mitral cells (E11 to E13) and tufted cells (E13 to E18) are generated prenatally while granule neurons (E18 to P20) and glial precursors (E17 and P10) are produced into the early postnatal period. Following histogenesis, each cell type begins an outwardly migration away from the germinal ventricular zone such that tufted cells will migrate past the newly formed mitral cell layer, and granule neurons past both mitral and tufted cells (Hinds, 1968b). Synapses are first observed around E15 (glomerular), but continue to be formed well into the postnatal period (Hinds and Hinds, 1976).

In the present study, growth factor gene expression in the developing cerebellum and olfactory bulbs revealed highest levels of both EGF and TGF-α mRNAs during the second postnatal week (P9 being the earliest timepoint studied). By approximately P21, levels of growth factor mRNAs were not significantly above the levels observed in fully mature adults (10 weeks). In the most general sense, the period over which the highest levels of EGF and TGF-α mRNAs were observed (P9 through P21) coincides with the young stage of

brain development at which high rates of protein synthesis and RNA concentrations are observed (Dunlop et al., 1984). Indeed, high levels of growth factor mRNAs were observed around the same time that total tissue RNA levels were elevated, suggesting that EGF and TGF-α may play a role during periods of rapid CNS growth. Consistent with our data, EGF binding sites in whole brain preparations of rats are more abundant at 2 weeks of age and progressively decline into adulthood (Hiramatsu et al., 1988).

EGF has previously been shown to stimulate DNA synthesis in GFAP$^+$-astrocytes of primary mouse cerebellar cultures (Leutz and Schachner, 1981, 1982). In the developing rat brain, EGF receptor (EGF-R) immunoreactivity has been observed on astroglia at approximately P16, reaching maximal intensity at P19 and progressively declining to much weaker staining in the adult (Gómez-Pinilla et al., 1988). If we take into consideration that rat brain development proceeds along a similar, but slightly later developmental schedule than the mouse, we might expect to see EGF-R immunostaining on astroglia in the murine CNS as early as P14. In the present study, EGF, TGF-α and GFAP mRNA content in the developing cerebellum were determined to be highest around P12 and subsequently, observed to decline to their respective adult levels. Therefore, if EGF or TGF-α mRNAs are taken as indicators of the expression of their respective peptides, then the expression of EGF or TGF-α in the developing cerebellum appears to coincide with the appearance of EGF receptors on astroglia and to decrease in a parallel fashion. Therefore, changes in growth factor mRNA levels may reflect a direct effect on the proliferation or maturation of cerebellar astrocytes during early postnatal development.

Morrison et al (1988) recently reported that EGF enhances the survival and process outgrowth of cerebellar neurons grown in culture and that the

identity of these cells are likely to be of the Purkinje or Golgi cell type. Consistent with their observations, EGF-R immunoreactivity is observed on cerebellar Purkinje cells in rat brain as early as P11 and remains throughout late postnatal development (Gómez-Pinilla et al., 1988). Since the appearance of EGF-R expression on Purkinje cells coincides with the relatively early period of high levels of both EGF and TGF-α mRNAs, EGF and/or TGF-α may directly influence the normal development of Purkinje cells *in vivo* during this early postnatal period. In the context of the Purkinje cell developmental timetable, the period of elevated growth factor mRNA levels coincides with Purkinje cell dendritic synaptogenesis.

At present, there is a lack of information concerning possible cell target(s) for EGF/TGF-α activities in the developing olfactory bulb. However, potential sources of ligand in this region have been revealed. On the basis of *in situ* hybridization histochemistry, TGF-α mRNA has been identified in both mitral and periglomerular neurons of the main olfactory formation, in the accessory olfactory bulb and in anterior olfactory nuclei, suggesting a role for TGF-α in olfactory processes (Wilcox and Derynck, 1988). A somewhat surprising finding of the present study was a difference in EGF mRNA content for male and female olfactory bulbs at postnatal day 54. Since there is a significant increase in the size of the accessory olfactory bulb in male rats between P40 and P60 (Roos et al., 1988), it is possible that EGF plays a role in promoting sexually-dimorphic growth of this structure during late postnatal development.

Altered patterns of growth factor gene expression in Purkinje cell degeneration and weaver mutant mice

An examination of EGF gene expression in Pcd mutant (*pcd/pcd*) and control (*+/+* and *+/pcd*) cerebellum revealed significantly higher concentrations of EGF mRNA in affected mutants from postnatal day 39 onward. A corresponding increase in total EGF mRNA content was observed between P39 and P62. In contrast, TGF-α gene expression in mutant cerebellum began to increase after postnatal day 65. However, while TGF-α mRNA concentrations continued to rise, TGF-α mRNA content was only transiently elevated (P70 to P99). In the weaver mutant (*wv/wv*) cerebellum, both EGF and TGF-α mRNA concentrations did not differ from non-ataxic homozygous (*+/+*) or heterozygous (*+/wv*) mice. Mutant cerebellar levels of total EGF or TGF-α mRNA, however, were consistently below the levels present in *+/+* and *+/wv* cerebellum. In view of the differences in EGF and TGF-α gene expression resulting from abnormalities in genetically-determined neurodevelopmental programs, these putative EGF receptor ligands are likely to have distinct physiologic roles in the CNS.

One of the advantages of examining brain tissues in which selective neuronal losses occur is that they provide insight into possible cell sources of growth factor synthesis in the CNS. Thus, considering that Pcd mutant (*pcd/pcd*) cerebella lose the majority of their Purkinje cells (Pc) during the first postnatal month, with virtually all Purkinje neurons depleted by the seventh week (Mullen et al., 1976), it seems reasonable to assume that Pc are not a significant source of EGF or TGF-α mRNAs in the developing cerebellum. For both gene transcripts, mRNA concentrations and total mRNA content in mutants (*pcd/pcd*) were always greater or equal to their respective Pcd littermate controls (*+/?*). Therefore, loss of growth factor gene expression does not

appear to accompany Pc loss. Perhaps most intriguing is our observation that absolute levels of EGF and TGF-α mRNA increase in Pcd mutant cerebellum. Therefore, we suggest that the changes in growth factor gene expression are not simply do to the loss of cells, but to increases in either number of cells expressing these mRNAs or to increases in growth factor gene expression within cells.

In contrast to the Purkinje cell loss in Pcd mutant cerebellum, weaver mutant mice (*wv/wv*) exhibit a rapid degenerative loss of granule neurons in the vermal (midline) region. Therefore, a comparison between Pcd and weaver cerebellum allowed us to address the effects of specific neuronal losses on the expression of EGF and TGF-α mRNAs. For behaviorally-normal heterozygotes (*+/wv*) of the weaver strain, granule cell loss also occurs but is relatively mild (Rakic and Sidman, 1973). Since histological examination provided an approach for distinguishing *+/wv* and *+/+* cerebellum, we were able to compare potential changes in *wv/wv* growth factor gene expression with both *+/wv* and *+/+* mice. From our studies, absolute levels of both EGF and TGF-α mRNAs in weaver mutants were below the levels present in *+/wv* and *+/+* cerebellum. Therefore, as a first approximation, it would appear that granule cells could be a source of both growth factor gene transcripts during early postnatal development. Initially, this interpretation might appear to conflict with our findings concerning the concentrations of growth factor mRNAs in mutant cerebellum. Indeed, EGF and TGF-α mRNA concentrations in *wv/wv*, *+/wv* and *+/+* cerebellum were very similar. Since the degeneration of granule neurons occurs primarily within the vermal region, it is possible that the loss in absolute numbers of granule cells within this midline area is relatively small in comparison to all remaining cells of the vermis and of the much larger lateral hemispheres. Therefore, growth factor mRNA concentrations, in comparison to

absolute content, might not be as severely affected. Consistent with this idea of an incomplete loss of cells expressing EGF and/or TGF-α mRNAs is the observation that growth factor gene expression does not completely disappear. Recently, Herrup and Trenkner (1987) reported that the medial half of *wv/wv* cerebellum exhibited a modest reduction (25-50%) in Purkinje neurons in addition to granule cell losses in the vermis. Based on their findings, we suggest that a partial loss of the Purkinje cell RNA pool in the context of granule cell RNA losses, the latter of which potentially including EGF and/or TGF-α mRNAs, effectively cancel one another so that growth factor mRNA concentrations appear relatively unaltered. Once again, the underlying assumption, based on our Pcd findings, is that Purkinje neurons are not a significant source of either growth factor mRNAs.

Further support for granule neurons as a potential source of EGF mRNA in the cerebellum is the observation that EGF mRNA concentrations in Pcd mutants remain above control values. Thus, with the Purkinje cell RNA pool removed in *pcd/pcd* mice, granule cell expression of EGF mRNA constitutes a greater proportion of the total cerebellar RNA as compared to controls. Therefore, mRNA concentrations appear elevated. Interestingly, during the final stages of Purkinje cell degeneration (~30-50 days), absolute levels of EGF mRNA in Pcd mutants were observed to increase above the levels present in controls, returning to control values at approximately postnatal day 60. While we acknowledge the possibility that multiple cell types may be contributing to the overall profiles of EGF and TGF-α mRNA changes in both Pcd and weaver cerebellum, one possible explanation relating specifically to granule cells for this transient rise in EGF mRNA content might be a reaction of granule neurons to the loss of their principal post-synaptic targets, the Purkinje cells. As shown by our normal studies, EGF mRNA levels during normal cerebellar development

are highest during a period of extensive synapse formation (~P7 through 14). Since EGF receptors have previously been localized to the dendrites of Purkinje neurons (Gómez-Pinilla et al., 1988), the expression of EGF mRNA, and thereby, EGF, by granule cells might provide a mechanism by which parallel fibers (granule cell axons) and Purkinje cell dendrites interact. In support of this model is the observation that EGF, in its transmembrane form, is biologically active (Mroczkowski et al., 1989; Breyer and Cohen, 1990). Alternatively, EGF may be released in its processed form from granule cell presynaptic terminals to play a neurotransmitter- or neuromodulatory-like role in cell-to-cell signaling between granule and Purkinje neurons, as supported by immunological localizations of EGF to nerve fibers and their terminals (Fallon et al., 1984; Lakshmanan et al., 1986).

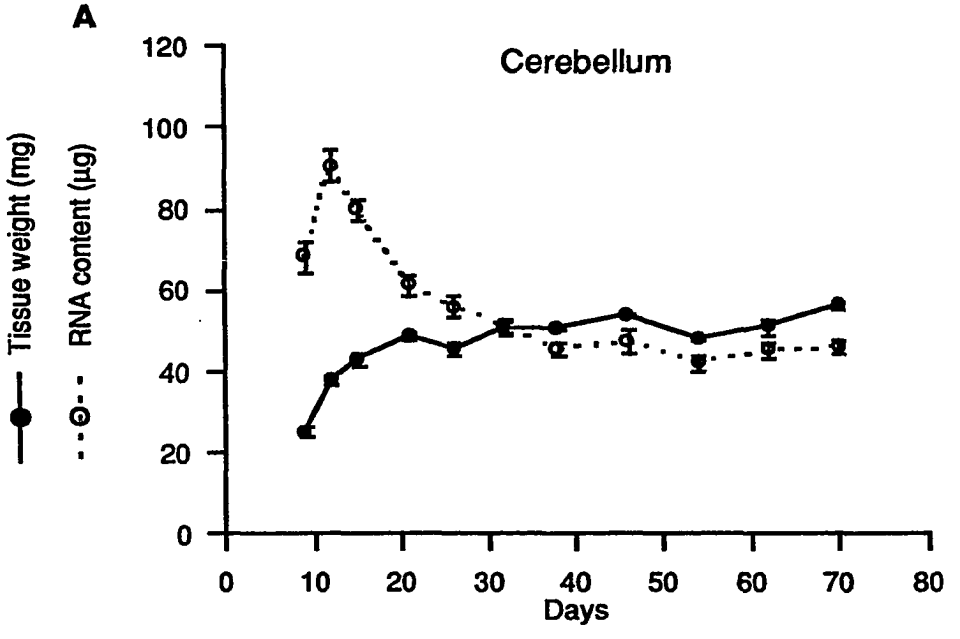
Recent immunological studies concerning the cellular localization of TGF-α within the mammalian CNS have suggested that astrocytes, in addition to neurons, might be a potential source of this peptide (Fallon, 1987; Kudlow et al., 1989; Fallon et al., 1990). We were therefore interested in whether potential changes in astrocyte-specific GFAP mRNA gene expression, reflecting potential changes in astrocyte number and/or activity, would correspond with altered patterns of TGF-α mRNA. In general, GFAP gene expression (both in content and concentration) in *pcd/pcd* cerebellum remained well above control levels. This observation is consistent with reported increases in GFAP immunostaining in astrocytes in response to CNS injury (Duffy, 1983; Lindsay, 1986). With respect to the temporal sequence of neurodegenerative events, levels of GFAP mRNA were highest during the final stages of Purkinje cell degeneration (~P40) and thereafter, rapidly decreased to a level that remained significantly above those of controls. For TGF-α, only transient elevations in mRNA content were observed (P70-99) while concentrations did not significantly differ from controls

until after postnatal day 65. Based on these findings, TGF-α mRNA levels do not appear to reflect the overall pattern of changes in GFAP mRNA in *pcd/pcd* mutant cerebellum. Interestingly, increases in TGF-α content and concentration between P70 and P99 coincided with small elevations in GFAP mRNA. While the relationship between such changes might be coincidental, we suggest that these potentially synchronous elevations in gene expression might reflect an influence of GFAP$^+$-astrocytes on neurons (possibly granule cells) expressing TGF-α mRNA. Conversely, increases in TGF-α mRNA in mutant cerebellum may reflect molecular changes occurring within specific GFAP$^+$ cells. The implications of this latter hypothesis are intriguing in that i) Bergmann glia are known to comprise a sub-population of GFAP$^+$-astrocytes in the cerebellum, ii) Bergmann glia are grossly abnormal in *wv/wv* cerebellum, iii) TGF-α mRNA levels are reduced in *wv/wv* cerebellum, iv) EGF receptors are present on astroglia and v) there is an intimate relationship between Bergmann glia, potentially expressing TGF-α mRNA and expressing EGF-R, and granule neurons, potentially expressing EGF mRNA, during normal cerebellar development. Therefore, as our initial working model, we propose that granule neuron expression of EGF, Bergmann glial expression of TGF-α and Purkinje/Bergmann expression of EGF receptors provide a molecular basis for cell-to-cell interactions that are essential for the development and proper maintenance of normal cerebellar function.

Taken together, it appears that both EGF and TGF-α may play a role in early postnatal development of both the cerebellum and the olfactory bulbs and that they may respond differentially to the neurodegenerative events occurring in these regions. From *in vitro* observations, EGF has been shown to be required for the trophic support of neurons and therefore, it has been suggested that they may play a role in the prevention of neuronal loss *in vivo* as well.

However, our observations suggest that alterations in EGF and TGF-α gene expression occur relatively late with respect to neurodegeneration. It appears then that a biological response of newly synthesized growth factor might be related to the removal of neuronal debris, formation of glial scarring or the maintenance of the functional integrity of the remaining neuronal circuits.

Figure 1. Tissue weight and total RNA content as indices of normal cerebellar and olfactory bulb development in B6C3H (*+/+*) mice. For each age, values represent the mean ± SEM for equal numbers of males and females (n=6 for weight; n=12 for total RNA; P12 represents only males: n=3 for weight, n=6 for total RNA).



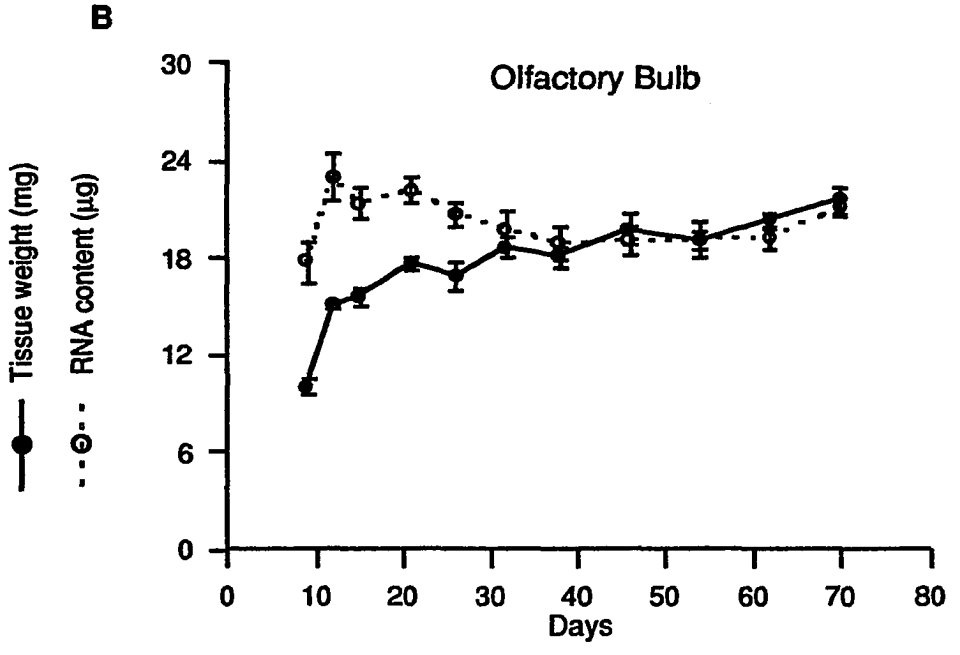


Figure 2. Developmental expression of EGF, TGF-α and GFAP mRNA in the cerebellum of B6C3H (*+/+*) mice. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and the total mRNA content (expressed as pg or ng mRNA / cerebellum) for each mRNA transcript are shown (given as means ± SEM). No significant differences between male and female concentrations or total mRNA content at matched ages were observed for either EGF or TGF-α mRNAs (2-factor ANOVA, $p > 0.67$) and therefore, male (n=2-3) and female (n=2-3) data were combined (n=4-6). GFAP mRNA levels were determined only for males (n=3). Postnatal day 12 values for all mRNA transcripts represent the mean ± SEM for 3 males.

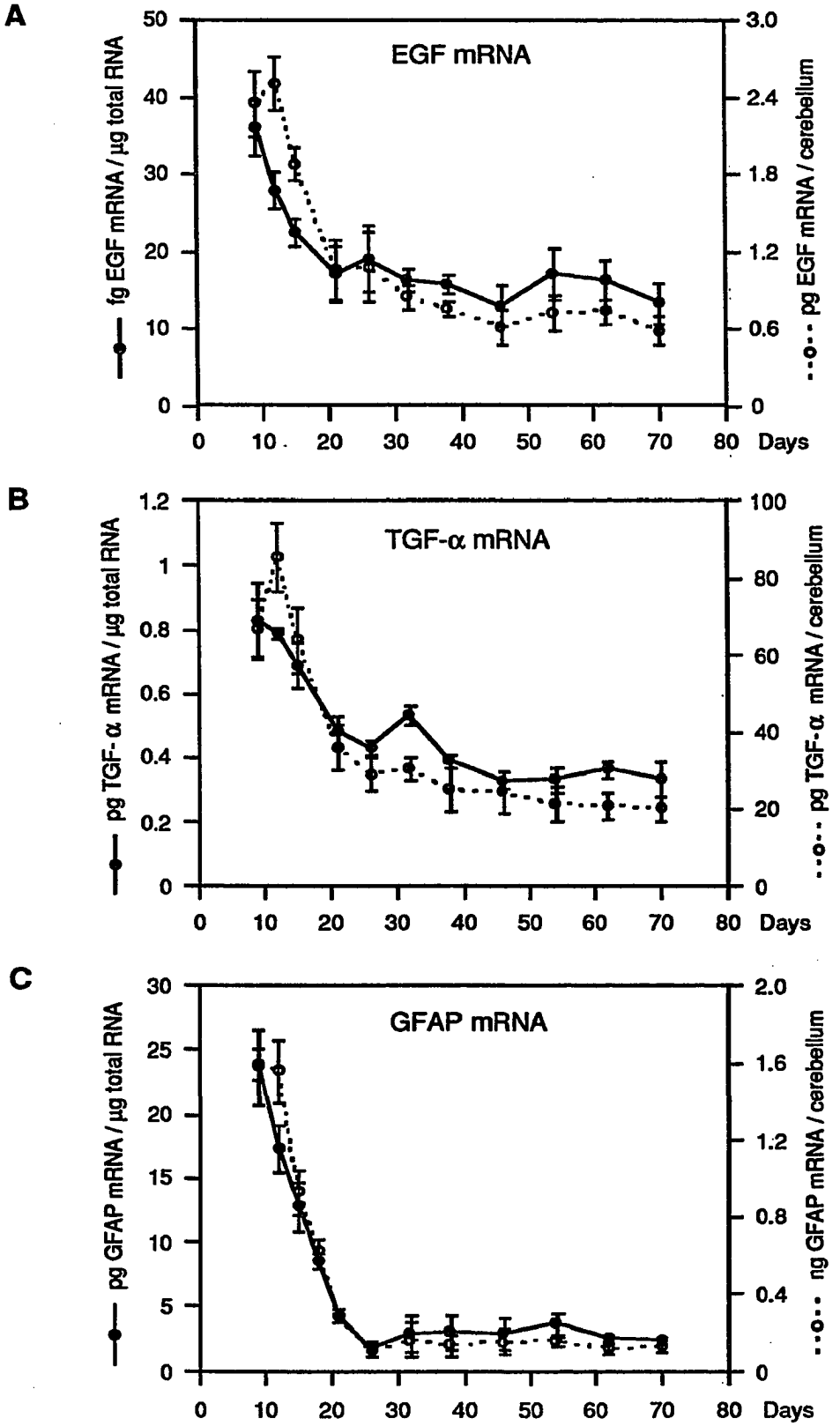


Figure 3. Developmental expression of EGF, TGF-α and GFAP mRNA in the olfactory bulbs of B6C3H (*+/+*) mice. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and the total mRNA content (expressed as pg mRNA / olfactory bulb pair) for each mRNA transcript are shown (given as means ± SEM). No significant differences between male and female concentrations at matched ages were observed for either EGF or TGF-α mRNAs (2-factor ANOVA, $p = 0.329$ and 0.996, respectively) and therefore, male (n=3) and female (n=3) data were combined (n=6). Similarly, male and female total mRNA levels for TGF-α at matched ages were not significantly different (2-factor ANOVA, $p = 0.791$) and were combined (n=6). However, a difference between male and female total EGF mRNA content at postnatal day 54 was observed (2-factor ANOVA, $p=0.074$) and therefore, male (*m*) and female (*f*) mean values at this age are represented separately. All other EGF mRNA content values represent the combined mean ± SEM for both male and female data (n=6). GFAP mRNA levels were determined only for males (n=3). Postnatal day 12 values for all mRNA transcripts represent the mean ± SEM for 3 males.

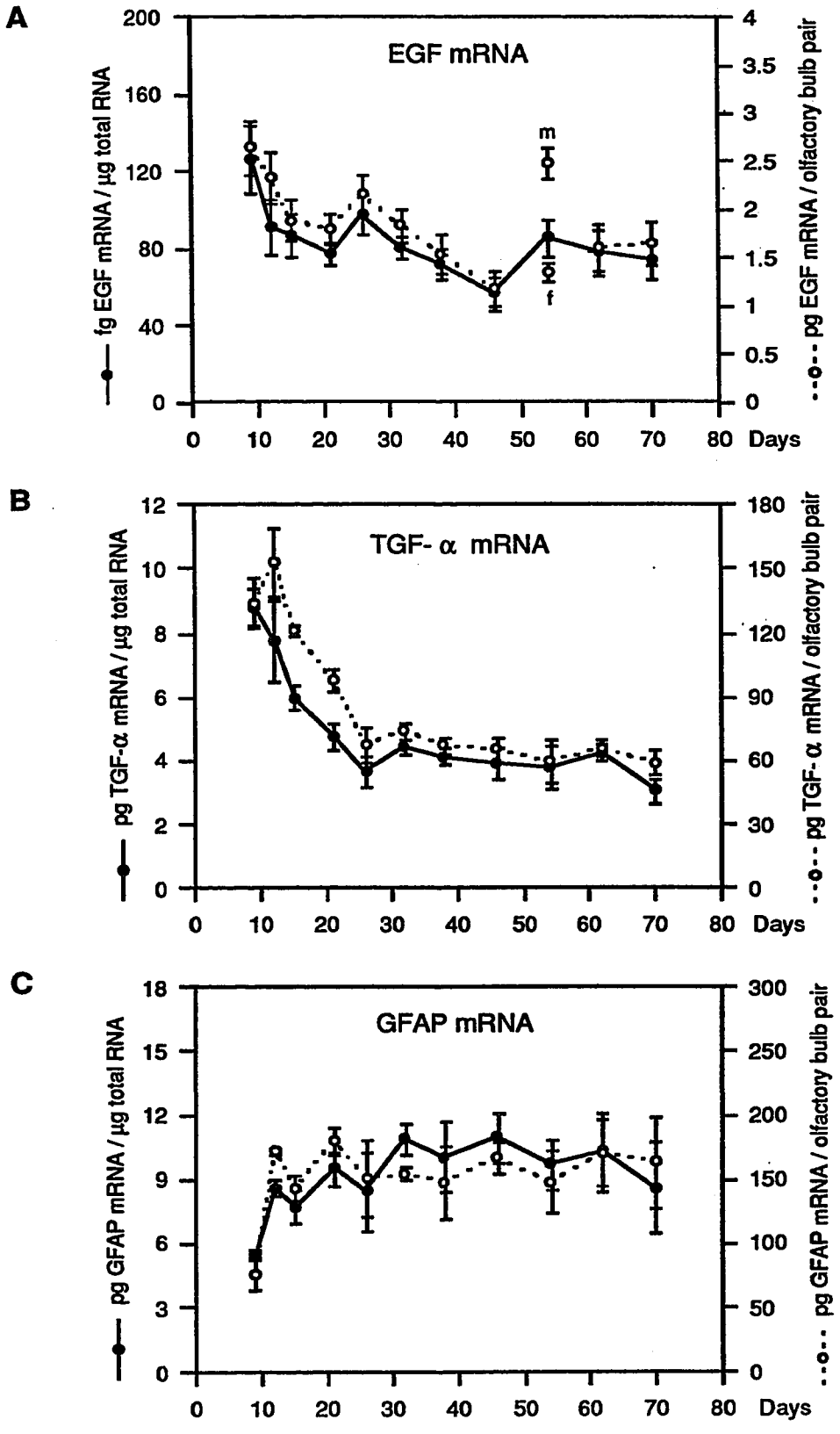


Figure 4. Tissue weight and total RNA content as indices of altered cerebellar and olfactory bulb development in Purkinje cell degeneration (Pcd) and weaver mutant mice. Comparisons are between Pcd mutant (*pcd/pcd*) and sex-matched littermate control (*+/?*) cerebellum (**A-B**) and olfactory bulbs (**C-D**); and weaver mutant (*wv/wv*), heterozygote (*+/wv*) and wild-type (*+/+*) cerebellum (**E-F**). All values represent the mean ± SEM. For Pcd mutant and control mice, n=3; for weaver *wv/wv* mice, n=3; for weaver *+/wv* and *+/+* mice, n=2.

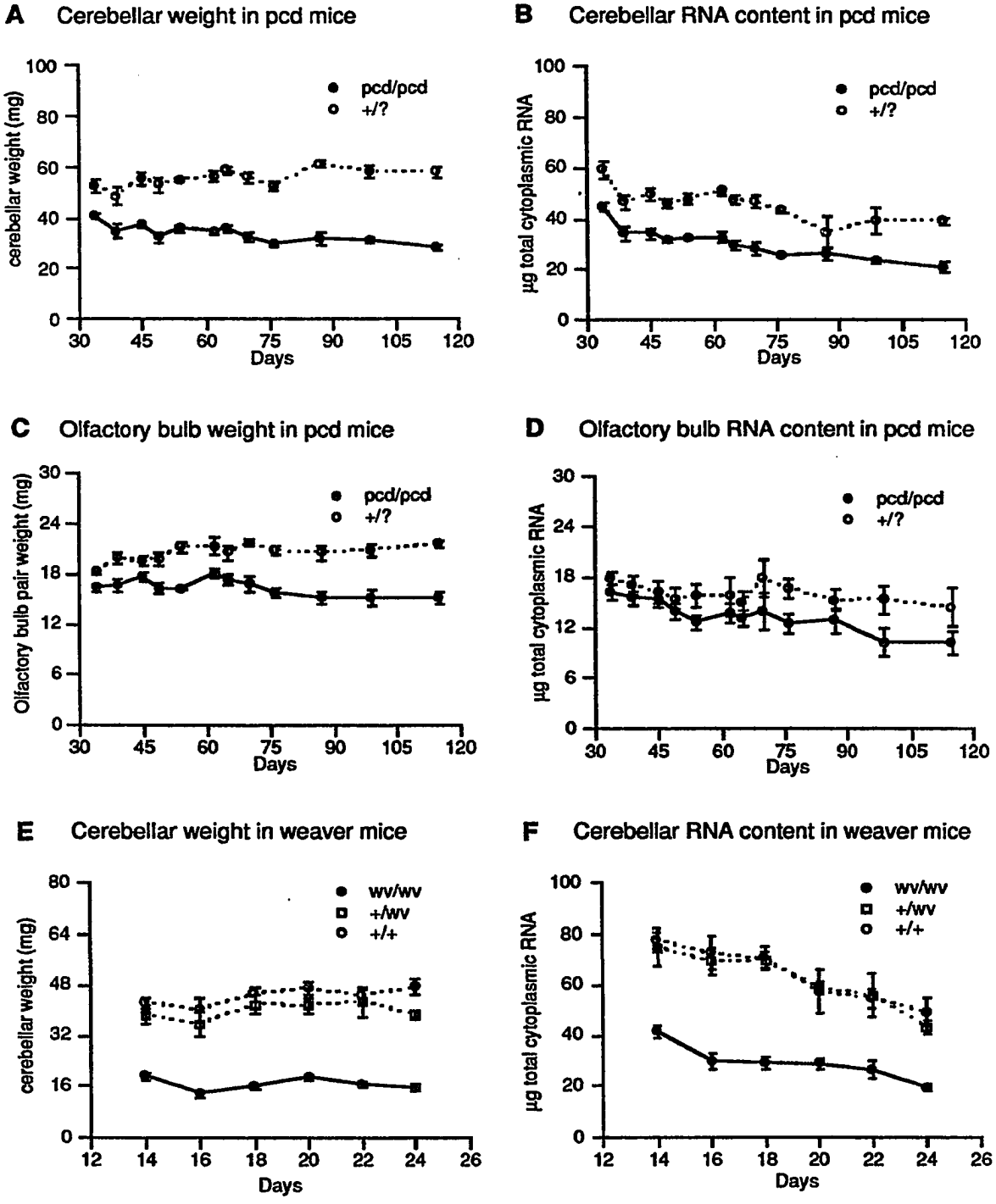


Figure 5. Ribonuclease protection analysis of EGF, TGF-α and GFAP mRNA expression in the cerebellum of Purkinje cell degeneration (Pcd) mice. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and the total mRNA content (expressed as pg or ng mRNA / cerebellum) for each mRNA transcript are shown. Closed circles represent mean values (± SEM) for Pcd mutant (*pcd/pcd*) mice; open circles represent mean values (± SEM) for non-ataxic (*+/?*) sex-matched littermates. All determinations were performed in triplicate. (*$p < 0.05$, **$p < 0.01$, ANOVA).

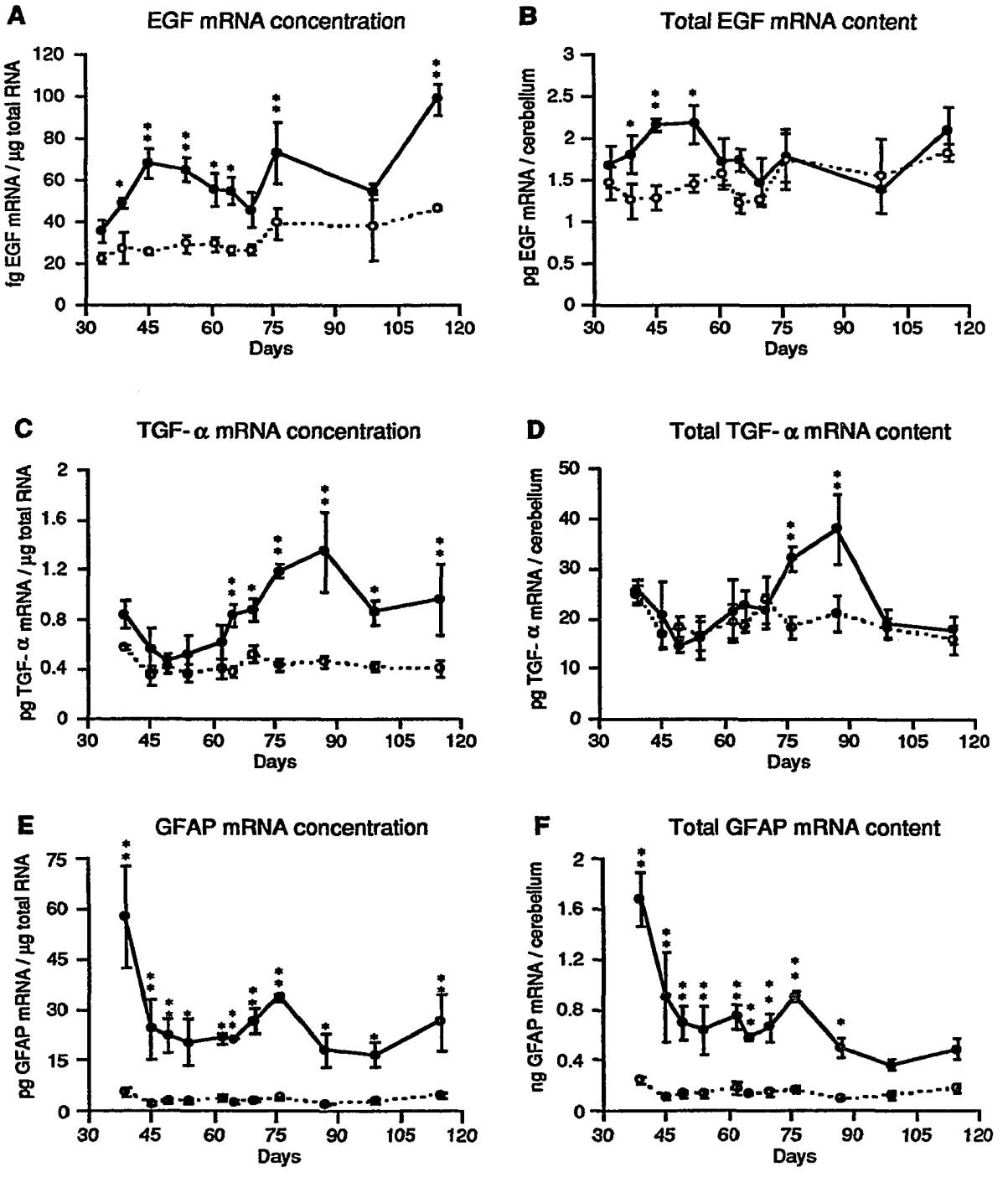
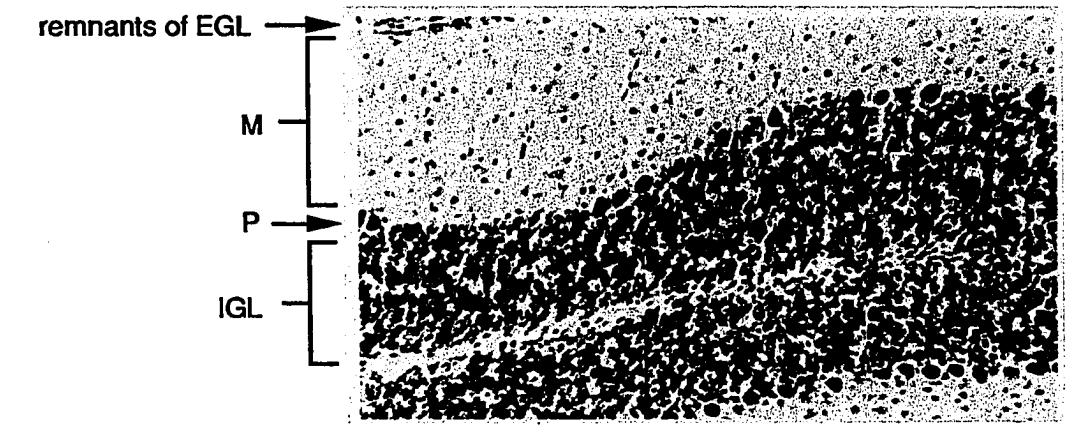
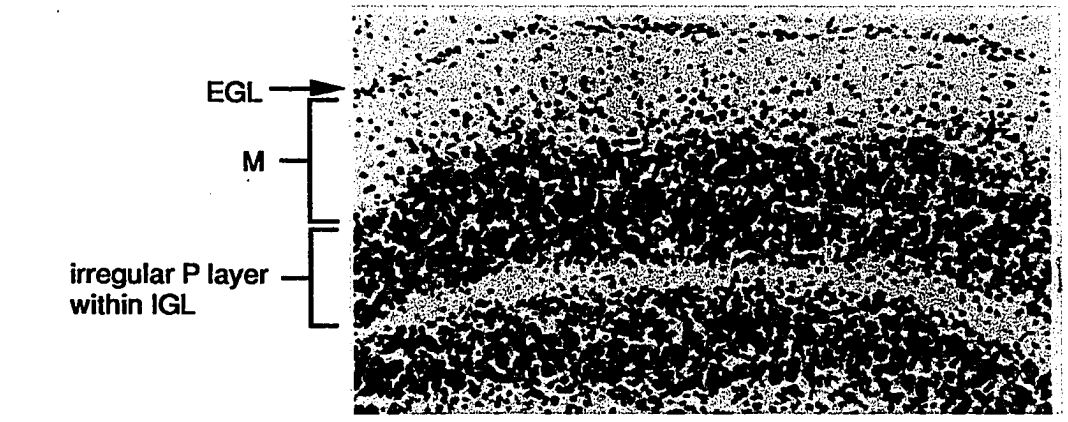


Figure 6. Histological determination of weaver genotypes. **A,** *+/+* genotype: The normal cerebellum displays a regular, trilaminar organization in cytoarchitecture (*M*, molecular layer; *P*, Purkinje cell layer; *IGL*, internal granule cell layer) with only remnants of an external granule cell layer (*EGL*) remaining. **B,** *+/wv* genotype: In the heterozygote, an external granule cell layer (*EGL*) is observed. In addition, the molecular layer appears hypercellular, Purkinje cell soma are irregularly aligned and the *IGL* is thinner. **C,** *wv/wv* genotype: In the weaver mutant cerebellum, a marked reduction in granule neurons is observed. In addition, Purkinje cells are reduced in number and appear to be randomly aligned.

A *+/+*



B *+/wv*



C *wv/wv*

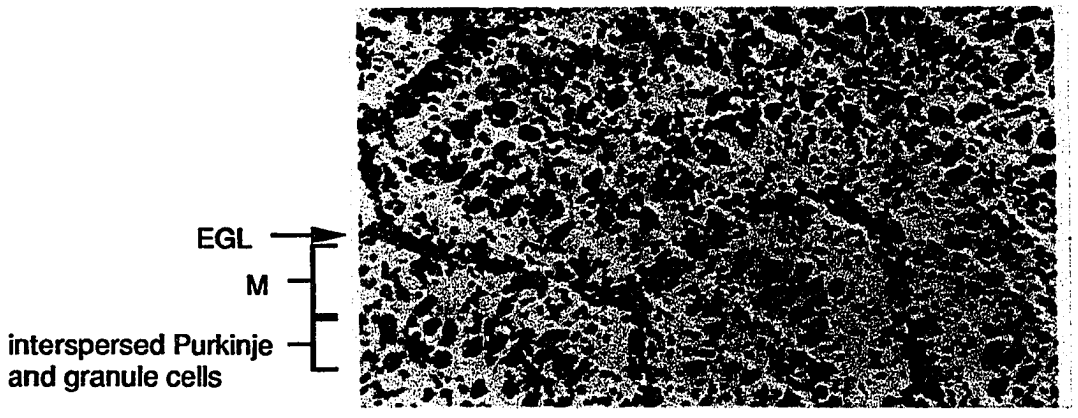


Figure 7. Ribonuclease protection analysis of EGF, TGF-α and GFAP mRNA expression in the cerebellum of weaver mice. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and the total mRNA content (expressed as pg or ng mRNA / cerebellum) for each mRNA transcript are shown. Values represent means ± SEM. For *wv/wv* cerebellum (closed circles), n=3. For the cerebellar genotypes *+/wv* (open squares) and *+/+* (open circles), n=2.

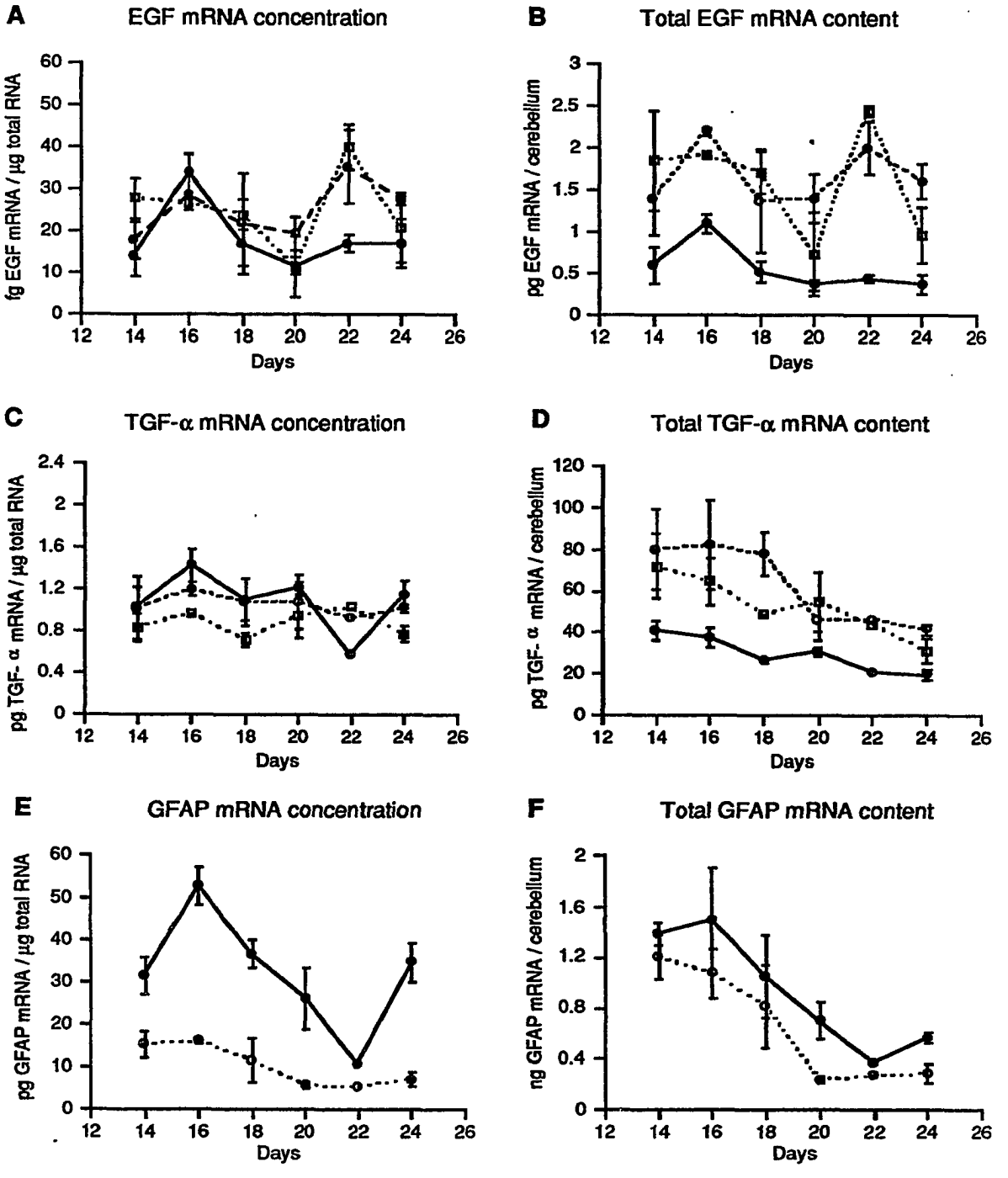
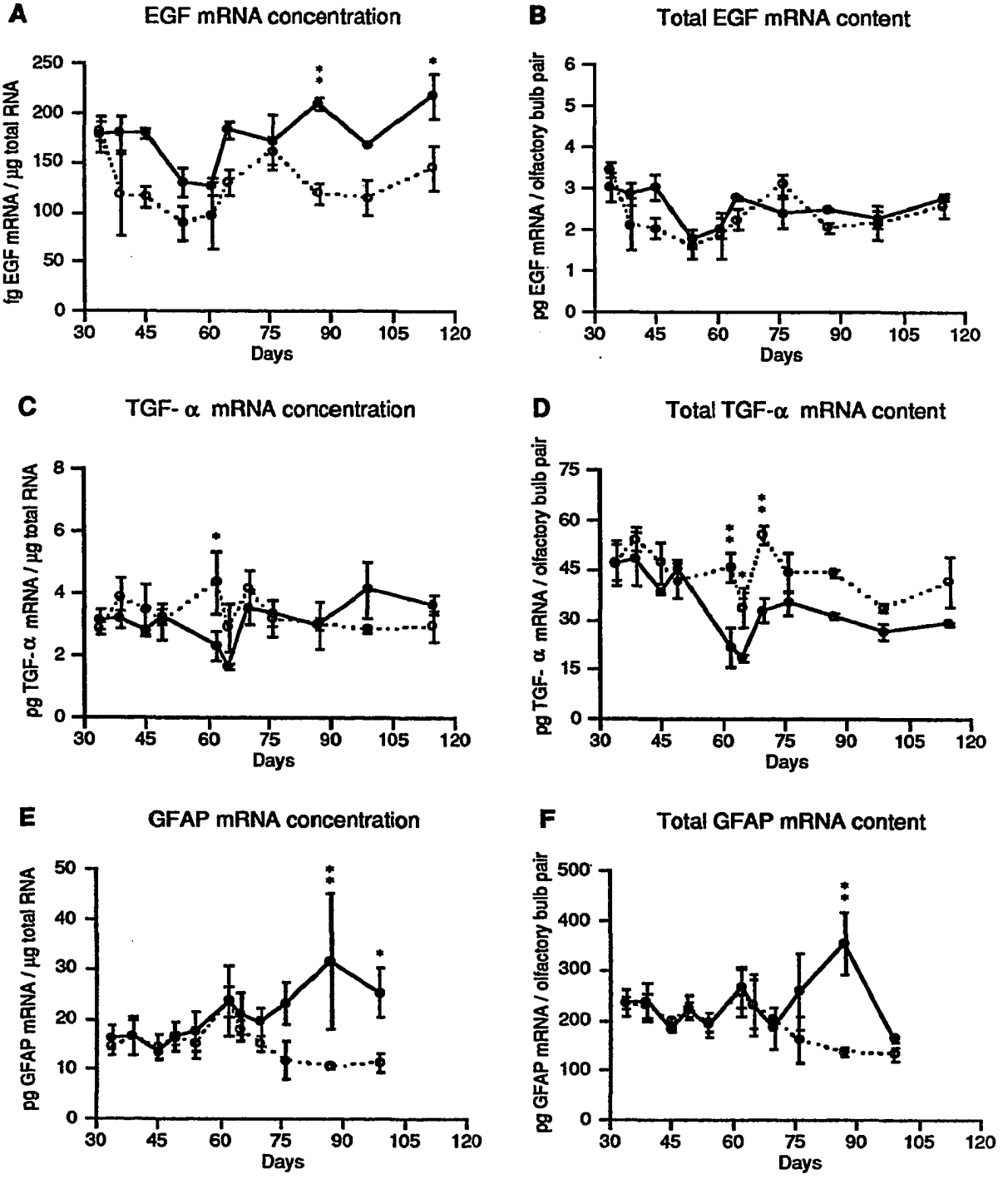


Figure 8. Ribonuclease protection analysis of EGF, TGF-α and GFAP mRNA expression in the olfactory bulbs of Purkinje cell degeneration (Pcd) mice. Both the concentration (expressed as fg or pg mRNA / μg total RNA) and the total mRNA content (expressed as pg mRNA / olfactory bulb pair) for each mRNA transcript are shown. Closed circles represent mean values (± SEM) for Pcd mutant (*pcd/pcd*) mice; open circles represent mean values (± SEM) for non-ataxic (*+/?*) sex-matched littermates. All determinations were performed in triplicate. (*$p < 0.05$, **$p < 0.01$, ANOVA).



General Discussion

It has long been known that following injury to the central nervous system in animal models, there is a rapid accumulation of specific substances at the lesion site which facilitate neuron survival and process outgrowth (Nieto-Sampedro et al., 1982). It appears that such neuronotrophic and neurite-promoting factors attempt to restore lost synaptic connections by inducing a compensatory sprouting of surviving terminals. It has also been realized that this process of synaptic terminal degeneration followed by neurite replacement reflects the normal synaptic turnover that occurs in the non-diseased state (Cotman and Nieto-Sampedro, 1984). Moreover, in the normal course of aging, the release of neurotrophic activities following a neurologic insult and the subsequent replacement of lost synaptic connections are both significantly less than that observed in younger animals (DeKosky et al., 1984).

Observations such as these have generated much interest in the identification of neurotrophic agents responsible for the development, maintenance and survival of distinct neuronal populations in the mammalian CNS. Perhaps one of the most thoroughly characterized, by virtue of its early discovery, is nerve growth factor (NGF). While it was first recognized for its role in the survival and maintenance of neurons in the sympathetic nervous system (Levi-Montalcini and Angeletti, 1963), it has since been shown to prevent the retrograde degeneration of transected cholinergic neurons in the basal forebrain (Montero and Hefti, 1988). In addition, NGF has been shown to ameliorate cholinergic atrophy and spatial memory loss in aged animal models (Fischer et al., 1987). This association of nerve growth factor with neuronal survival and lesion repair in the cholinergic pathways associated with memory

has suggested a potential role for NGF in Alzheimer's disease (Hefti and Werner, 1986).

Recently, it has been suggested that epidermal growth factor may have neurotrophic activities in the central nervous system as well. From observations in culture, EGF has been shown to enhance the survival and process outgrowth of cerebellar, pontine, septal, mesencephalic and subneocortical telencephalic neurons derived from the developing rodent brain (Morrison et al., 1987, 1988; Knusel et al., 1990; Cohen et al., 1990; Casper et al., 1991). Localization studies have revealed EGF-immunoreactive material in 'pallidal' structures of the brain directly involved in the extrapyramidal motor circuit known to degenerate in Parkinson's disease (Fallon et al., 1984). A further association of EGF with the neurodegenerative process has stemmed from the observation that patients suffering from amyotrophic lateral sclerosis (ALS) manifest reduced levels of EGF in cerebrospinal fluid (Cieslak et al., 1986). Moreover, increases in astrocyte EGF receptor immunoreactivity in the vicinity of lesions in the adult rat brain have been observed (Nieto-Sampedro et al., 1988). As transforming growth factor-α demonstrates a co-distribution with the enkephalin system in the brain, it is possible that this peptide plays a role in the survival of enkephalin-containing neurons in the CNS (Code et al., 1987). Thus, accumulating evidence suggests that the EGF/TGF-α system in brain may be involved in the maintenance and functional stability of specific neuronal populations.

Perhaps the normal developmental function of EGF in brain is reflected in the effects of overexpression of the EGF receptor gene detected in primary human intracranial tumors (Libermann et al., 1984, 1985). Indeed, the unregulated cell growth and division observed in the progression of these human gliogenous tumors might represent an exaggeration of the normal EGF

growth-promoting activity hypothesized to be present in human brain. An association between unregulated cell growth and abnormal EGF receptor function has been previously demonstrated for the v-*erb*-B1 oncogene product. This structurally altered EGF receptor-like protein, isolated from the acutely transforming avian erythroblastosis virus (AEV), has been shown to induce erythroleukemia in chickens and to transform cultures of both erythroblasts and fibroblasts (Downward et al., 1984; Stoscheck and King, 1986). As the EGF receptor has been shown to be the normal cellular counterpart (and by definition, the proto-oncogenic product) of this AEV-transforming protein, the EGF receptor and its ligands are likely to play a role in the regulatory functions of cell growth.

Therefore, we propose that the normal physiological function(s) of EGF and TGF-α in the mammalian central nervous system are related to those roles previously assigned to proto-oncogenic encoded proteins, including the control of development, growth and wound repair. Our studies support the involvement of both EGF and TGF-α during embryonic and postnatal development and suggest a role for these peptides following neurodegenerative injury to the CNS. Since these peptides appear to be independently regulated at the molecular level, the differential expression of these factors in brain may provide a basis for which EGF and TGF-α may share, in a co-operative manner, the same cell-surface receptor *in vivo*.

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