Mitogenic Growth Factors and Nerve Dependence of Limb Regeneration

Jeremy P. Brockes

Summary. Regeneration of the amphibian limb after amputation depends on division of blastemal cells, the progenitor cells of the regenerate. This division is controlled, at least in the early stages of regeneration, by the nerve supply to the blastema. A monoclonal antibody to newt blastema cells has provided evidence that Schwann cells and muscle fibers contribute to the blastema, and identifies blastemal cells whose division is persistently dependent on the nerve. Glial growth factor, a molecule identified by its action on rat Schwann cells, is present in the newt blastema and is lost on denervation.

One outcome of the study of animal cell division in culture has been an emphasis on the importance of mitogenic growth factors. These molecules act at hormonal concentrations to promote transit through the cell cycle and hence to stimulate DNA synthesis and mitosis of cultured cells. Although considerable activity has been directed at understanding the mechanism of growth factors in vitro, there has been little progress in understanding how they might act in vivo to regulate cell division during development and regeneration. Progress in the methodology of gene cloning should facilitate the isolation of more growth factors, and the rate of appearance of new molecules may outstrip our ability to establish their physiological role (*1*). It is therefore worthwhile to study elementary cases in which a defined population of cells in a tissue proliferate in response to a signal from an identified source. One such case is the direct control of division by the nervous system.

Under some circumstances during development and regeneration, nerves appear to stimulate the division of nonneuronal cells. In the vertebrate peripheral nervous system, the principal glial element is the Schwann cell. This neural crest derivative is responsible for elaborating the myelin sheath and is also associated with unmyelinated axons. During development, Schwann cells divide in association with peripheral axons, and generation of the appropriate number appears to be regulated by the number of axons present (*2*). A more striking interaction is the effect of nerves on the division of nonneural cells during regeneration of appendages. A growth zone (blastema), composed of undifferentiated cells, arises at the site of amputation. The blastemal cells then divide and undergo morphogenesis to replace the missing part. In many organisms, such regeneration requires that nerve cells or their processes be present at the site of amputation (*3*). Nerve dependence is illustrated in vertebrates by the regeneration of the amphibian limb, the lizard tail, and the fish fin, and in invertebrates by the regeneration of caudal segments in annelid worms and of eyes in flatworms (*3*). The phenomenon can even be observed in the elementary nervous system of coelenterates. After amputation of the head in *Hydra*, cells in the nerve net release a peptide that stimulates division of the interstitial stem cells (*4*).

In amphibian limb regeneration, peripheral nerves appear to stimulate division of the blastemal cells. The use of a monoclonal antibody as a cell marker indicates that the nerve supply may also provide a source of cells for the blastema from the Schwann cells of the nerve sheath. Identification of the mitogenic growth factors involved is crucial to understanding the nerve-blastema interaction at the molecular level. The vertebrate nervous system contains several such growth factors, at least as defined by the stimulation of cultured cells. One such molecule, glial growth factor, was isolated by assaying for its action on the rat Schwann cell, a cell type that does not respond to several other known growth factors. The chemical characterization of this molecule, together with a relatively specific bioassay, has allowed certain tests of its relevance for proliferation in the blastema.

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Amphibian Limb Regeneration

The stages of limb regeneration in Urodele amphibians are illustrated by the two series of photographs in Fig. 1 (*5*). During the first 3 to 4 days after amputation, the wound surface is healed by epidermal cells that migrate over it and form the apical cap. Two processes underlie the early events of regeneration: (i) the appearance of blastemal cells at the site of amputation and (ii) their proliferation, which is stimulated by the nerve supply of the limb. Although division continues during the later stages, cells in the blastema progressively undergo cytodifferentiation and morphogenesis and give rise to the internal tissues of the regenerate. These processes have been widely studied in newts (*Triturus, Notophthalmus, Pleurodeles* sp.), salamanders (*Ambystoma maculatum*) and axolotls (*Ambystoma mexicanum*). The system has become a central one for the study of pattern formation (*6*), but this article is concerned only with events that occur before the onset of significant cytodifferentiation. The controlling influence of the nervous system is illustrated by an experiment (*7*) in which a major peripheral nerve is cut and the proximal end is deflected into the skin around the base of the limb. This procedure evokes a supernumerary appendage.

That denervation of the limb prevents regeneration has been recognized for many years, but our current understanding of the role of the nerve is particularly associated with Singer (*8*), who made a quantitative study of regeneration of the newt forelimb and its density of innervation. The forelimb is innervated by spinal nerves 3, 4, and 5, which merge at the level of the brachial plexus and then divide before entering the limb. The density of innervation to the regenerate can be varied by interrupting the three nerves at various levels. Such studies show, with considerable precision, a correlation between the frequency of regeneration and the density of severed nerve fibers at the amputation surface (*9*).

There is thus a need for an adequate quantity of innervation—sometimes expressed as the fractional area of axoplasm at the amputation surface rather than by the density of nerve fibers, to allow comparisons between species whose axon caliber differs markedly (*10*). This quantitative requirement can be met by either motor or sensory axons. It is possible with appropriate surgery to derive limbs with purely motor or sensory innervation; both are capable of supporting regeneration (*11, 12*).

Although the nerve has many effects on the general metabolism of the blastema, it is appropriate to focus on the stimulation of proliferation. Denervation before amputation or soon after amputation leads to a decrease in the mitotic index of the blastema (*13*) and in the [^{3}H]thymidine labeling index (*14*). While the mitotic index briefly increases immediately after denervation, perhaps owing to release of components from the degenerating axons, it then rapidly falls below the control level (*13*). Moreover, the consequences of denervation are dependent on the stage at which it is performed (*13*). If denervation occurs in the adult newt up to approximately 14 days after amputation, the mitotic index falls well below the control, and regeneration does not proceed. At this stage the blastema still has the appearance of a mound of undifferentiated cells. After 14 days, differentiation begins, and denervation produces a smaller decrease in the mitotic index. Furthermore, regeneration of such a denervated limb produces a limb that is smaller than normal but well formed (*13*). It seems possible from these results that a critical number of blastemal cells is needed to initiate differentiation and morphogenesis and that early denervation prevents this number from being attained.

The nerve dependence of regeneration is thus observed in the adult newt during the first 2 weeks after amputation, when the undifferentiated blastemal cells are stimulated to divide. It is not clear, however, whether this population of blastemal cells is homogeneous or whether it is made up of distinct populations that respond to different mitogenic influences. Before considering how the nerve might act at the molecular level to regulate cell division, it is appropriate to ask if blastemal cells are homogeneous or if they differ, possibly as a consequence of their derivation from different mature tissues (*15*). Evidence about the origin, identity, and proliferation of blastemal cells has come from the recent use of monoclonal antibodies as cell markers (*16–18*).

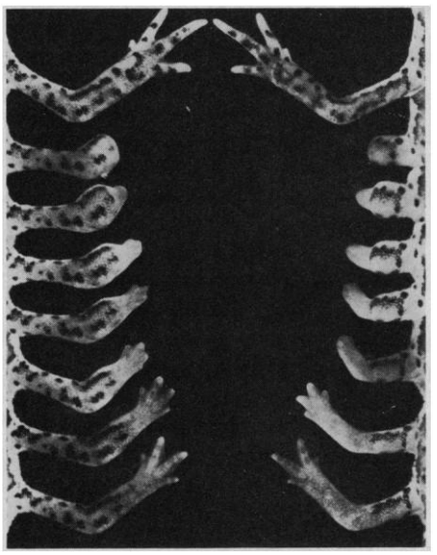

Fig. 1. Photograph showing successive stages in the regeneration of opposite limbs in an adult newt after amputation of lower (left) and upper arms. They are taken at 7, 21, 25, 32, 42, and 70 days after amputation. [Reprinted with permission of Academic Press]

A monoclonal antibody to blastemal cells. The origin of blastemal cells after amputation is an interesting and sometimes controversial problem. Anatomical studies of the regenerating limb with light microscopy (*15, 19*) and electron microscopy (*20*) have led to the general conclusion that blastemal cells arise by dedifferentiation from tissues at the site of amputation—including muscle, cartilage, Schwann cells, and connective tissue. A more actively experimental approach has been to graft marked tissue into normal or irradiated blastemas and then observe its fate; interpretation of these experiments, however, is often complicated by cellular heterogeneity and other factors (*21*). The multinucleate myofiber has been a particularly controversial source of blastemal cells, because muscle regeneration in vertebrates is generally considered to proceed by the intermediary of reserve satellite cells (*22*). Further insight into these issues has been gained by use of a monoclonal antibody to an intracellular antigen specific for a subpopulation of blastemal cells and appearing locally in Schwann cells and myofibers at the plane of amputation.

After mice were immunized with newt forelimb blastemas (mostly at 16 days after amputation), about 1500 antibody-secreting clones were screened by indirect immunofluorescence on sections of the early regenerating limb. Another 1000 clones were screened after immunization with mature tissues (*17*). Many clones showing specificity for differentiated cell types or extracellular components were discarded. One clone, termed 22/18, stains blastemal cells but not differentiated tissue. Although the identity of the antigen is not known, it is apparently intracellular as evidenced by the staining properties of blastemal cells in culture (*23*).

Antibody 22/18 produces no detectable staining of sections of the normal newt limb. Immunoreactivity first appears, 4 to 5 days after amputation, in Schwann cells of the nerve sheath at the plane of amputation. The staining is not present in axons, or in fibroblasts, as evidenced by double staining with antibody 22/31, a reagent that stains fibroblasts and some cells in the early blastema that do not react with 22/18 (*17, 18*). Furthermore, some of the cells in the sheath that stain with 22/18 also stain with monoclonal antibodies to newt Po, the major protein in peripheral myelin (Fig. 2, A to C). Schwann cells have been viewed in anatomical studies as contributing to the blastema after dedifferentiation (*19, 24*) and also in some experimental cases where nerves appear to support regeneration of an irradiated blastema (*25*). Other mature tissues, such as muscle and cartilage, do not stain detectably during this early period after amputation. During the 2 weeks after amputation, most of the cells in the blastema react strongly with antibody 22/18 (Fig. 2, D and E), but during the third week the number of cells that react with the antibody declines, although the population differentiating into skeletal muscle apparently still stains with antibody 22/18 (see below). In the later regenerate, 22/18 staining is lost from all cell types in the limb.

It is a prediction of the dedifferentiation hypothesis that at an appropriate time in regeneration, cells with blastemal properties should also temporarily express properties of the differentiated parent cells. In the case of muscle, this has been tested by searching for mononucleate cells that stain with both 22/18 and a monoclonal antibody specific for myofibers (*16*). Such double-labeled cells are found in an appropriate location between 12 and 15 days after amputation (*16*) and can also be observed in populations enzymatically dissociated from the blastema. Two observations support the hypothesis that these cells are derived by dedifferentiation: (i) they are not observed at earlier stages despite the close apposition of myofibers and blastemal cells that react with antibody 22/18, and (ii) the association between the two markers in the double-labeled cells is specific in that the 22/31 blastemal marker does not show this association. Fur-

thermore, the appearance of the 22/18 antigen in multinucleate myofibers at this time strongly suggests that such cells are an intermediate in dedifferentiation, as is the case with the Schwann cells that react with 22/18 soon after amputation. These observations are being extended by the use of in situ hybridization to detect muscle and Schwann cell–specific messenger RNA's in blastemal cells. The double-labeled cells again become detectable in the blastema about 3 weeks after amputation, when blastemal cells start to differentiate into muscle (*16*). It is not clear, however, whether these cells were derived originally from muscle or from another source such as Schwann cells.

This antibody has also been useful in identifying blastemal cells whose division is persistently dependent on the nerve (*17*). In experiments on newts described in the legend to Table 1, blastemas of the right forelimbs were denervated at two different times after amputation. At both the early and later stages the labeling index of the 22/18–positive population decreased by a factor of about 7 as a result of denervation 2 days earlier, whereas the 22/18–negative population decreased by a factor of about 3 at the early stage and by a factor of about 1.3 at the later stage. Furthermore, the proportion of 22/18–positive cells in the total thymidine-labeled population is significantly greater at the early time. Thus, while the proliferation of most of the blastemal cells at the later time is essentially independent of the nerve and is presumably in response to growth factors from other sources, a minority population retains the quantitative dependence exhibited earlier. It is not known, however, if they arise as the progeny of early 22/18–positive cells. These data are clearly significant in explaining the different consequences of denervation at the two times.

In summary, antibody 22/18 has provided new evidence about the origin and cellular composition of the blastema supports the idea that Schwann cells in the nerve sheath make an important contribution to the early blastema and that a decreasing proportion of cells in the blastema retain a marked dependence on the nerve for division. This is true even in the later stages when tissue pattern is emerging.

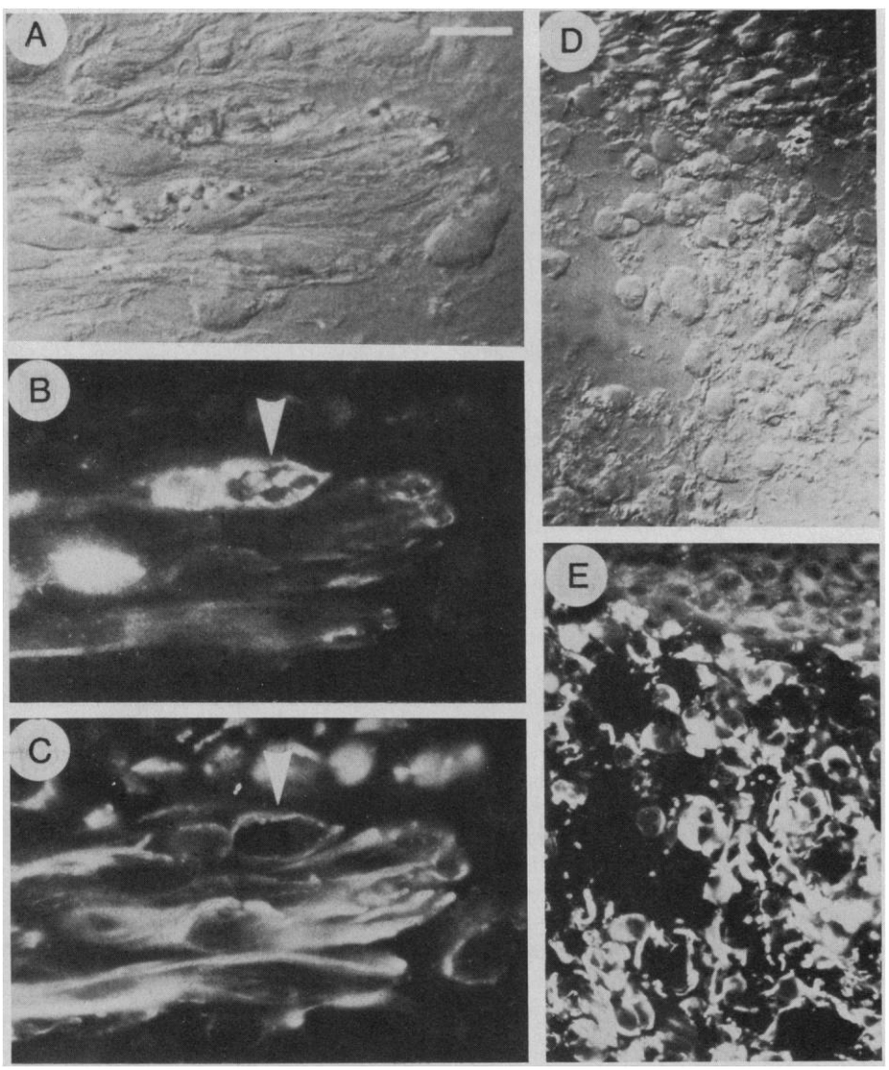

Fig. 2. Staining of the early bud blastema with 22/18 and monoclonal antibodies to newt Po. A longitudinal 10-μm section of an early forelimb blastema was stained with 22/18 and a mixture of monoclonal antibodies to newt Po (reagents 30/3, 30/14, and 30/26). The difference in immunoglobulin class between 22/18 (immunoglobulin M) and the Po monoclonal antibodies (all immunoglobulin G) was exploited to allow their separate detection on the same section with rhodamine and fluorescein reagents (*16*). (A) A region between the blastema and mature tissue shows Schwann cells in one of the brachial nerves and blastema cells photographed under Nomarski optics (scale bar, 20 micrometers). (B) Fluorescein optics to detect Po, and (C) rhodamine optics to detect 22/18. Arrowheads mark a cell expressing both antigens. (D) A region of blastema in a different section showing the wound epidermis under Nomarski optics (scale bar, 50 micrometers). (E) Rhodamine optics to show 22/18 staining of the early blastema. The staining of the epidermis is equivalent to control.

Assays for the Nerve-Blastema Interaction

The effect of the nerve on limb regeneration is usually attributed to the direct action of one or more mitogenic growth factors on the blastemal cells (*26*), possibly in conjunction with other signals from the wound epidermis (*27*). The agent responsible, sometimes called the neurotrophic factor (*28*), has been detected in a number of experimental systems.

In one investigation, forelimb blastemas of adult newts were cultured with sensory ganglia on either the same or the opposite side of a small-pore filter (*29*). Ganglia could stimulate the mitotic index in the blastema across the filter and a stimulation of approximately three times control values was obtained under some circumstances. The highest mitotic index was observed closest to the nerve cells, suggesting a diffusible factor. In other experiments, the infusion or injection of extracts into a denervated blastema prevented the decrease in protein synthesis that is observed relative to an innervated one (*30*). This experimental design has also been used on cultured blastemas to assay for activities in brain homogenates (*31*). Although these procedures have supported the concept of the existence of relevant activities, the effects observed are too small (generally less than threefold) and the assays too cumbersome to guide extensive purification and rigorous

biochemical identification. Furthermore, although bioassays can detect the activity of mitogenic factors in the subnanomolar range, the level of these factors in tissue sources is often less than 1 part in 10^5. Thus the possibility of purifying such an activity from the blastema or amphibian brain seems remote at the moment.

The purification of mitogenic growth factors has depended on assays that measure the stimulation of DNA synthesis or the increase in the number of cultured cells. Tissue sources and cultured cells of higher vertebrates have been used in such assays to identify growth factors from the nervous system. The growth and division of animal cells in culture is dependent on the presence of growth factors and hormones, and it has been possible to maintain some cell types in a defined medium with an appropriate mixture of purified growth factors (*32*). Much of the work on proliferation has been done on cell lines—in particular the murine 3T3 cell (*33*). These cells are strongly stimulated by activities in animal sera such as the platelet-derived growth factor (PDGF). The cells can be arrested in the cell cycle by allowing them to deplete the medium, or by growth in low serum concentrations; cells arrested in this way are responsive to various mitogenic growth factors.

Table 1. Cells that react with antibody 22/18 are dependent on the nerve for division at early and late stages. Adult newts (*Notophthalmus viridescens*) were bilaterally amputated through the upper forelimb and unilaterally denervated at early or late stages of regeneration. Two days after denervation, animals received an intraperitoneal injection of 25 microcuries of [^{3}H]thymidine (78 curies per millimole). After 24 hours, the two forelimbs were fixed, mounted together and sectioned with a cryostat. Sections were collected on glass slides, stained for 22/18 and nuclei as described (*16*), dipped in emulsion, and developed after 3 days' exposure. The number of 22/18–positive and 22/18–negative cells with labeled nuclei were counted in four random fields from four different sections for each limb (about 1000 nuclei per limb). The data are expressed as mean ± standard error for the ratio of cells on the denervated side to those on the innervated side. The percentage of total thymidine-labeled nuclei that were 22/18–positive in the innervated limbs was 75 ± 9 and 27 ± 4 at early and late times, respectively.

Denervation		Newts (No.)	Ratio of thymidine-labeled nuclei in blastema (denervated/innervated)		
Stage	Time (days)		Total cells	22/18–positive cells	22/18–negative cells
Early bud	10	4	0.20 ± 0.03	0.13 ± 0.02	0.35 ± 0.06
Palette	25 to 30	8	0.52 ± 0.15	0.14 ± 0.03	0.77 ± 0.21

Mitogenic Growth Factors of the Nervous System

Mitogenic growth factors of the nervous system include fibroblast growth factors (FGF's), neuropeptides, astrocyte growth factors, and growth factors for Schwann cells.

Fibroblast growth factor. In a survey of adult bovine tissues, the brain and pituitary yielded extracts with significant mitogenic activity for arrested 3T3 cells (*34*). The subsequent characterization of the FGF activities in the brain and pituitary illustrates some of the problems of purifying rare molecules from large quantities of tissue. Brain FGF was originally described as a basic protein of molecular size 13,000 that acted on various cells of mesodermal origin (*35*). The brain FGF preparation was separated into three forms that were identified as different fragments of myelin basic protein—a product of oligodendrocytes—and the mitogenic activity was attributed to these fragments (*36*). This identification has been contested on several grounds (*37*), and it has been reported that the activity in brain FGF fractions resides in at least two different mitogens, one acidic and one basic. The basic activity is similar to that in pituitary FGF preparations but distinct from fragments of myelin basic protein. The acidic activity has recently been purified by reversed-phase high-performance liquid chromatography to apparent homogeneity as two closely related molecules of molecular size 16,600 and 16,800 that are highly potent mitogens for 3T3 cells (*38*). The amino acid composition of the basic molecule from the pituitary (*39*) strongly suggests that it differs from myelin basic protein. Partially purified acidic fibroblast and endothelial mitogens from retina (*40*), hypothalamus (*41*), pituitary (*42*), and human brain (*43*) could also be related to the acidic activity in brain FGF.

Neuropeptides. While most investigations of growth factors have concentrated on proteins, recent studies have shown that some neuropeptides have mitogenic activity (*43*). The activity of the *Hydra* head activator peptide has been referred to above. The neurohypophyseal nonapeptide vasopressin is a potent mitogen for 3T3 cells and acts synergistically with insulin and other growth-promoting agents. The tetradecapeptide bombesin also acts on 3T3 cells at subnanomolar concentrations to stimulate DNA synthesis and cell division (*44*). Although there is little information about their mitogenic effect on other cultured cells, there is a possibility that certain peptides may stimulate division in development and regeneration, and in proliferative disorders.

Astrocyte growth factors. Recent advances in culturing purified populations of cells from the astrocytic lineage has led to studies of factors affecting their proliferation and maturation. Glial maturation factor is an acidic protein with both mitogenic and maturation activities on astroblasts and has been partially purified from bovine brain (*45*). There is some evidence that the spectrum of these activities may change with developmental stage (*46*), but no definitive characterization of the molecules responsible has yet been reported.

Growth factors for Schwann cells. Cultured rat Schwann cells do not respond to some of the mitogens that act on other cell types (*18, 47, 48*), and they divide very slowly in conventional tissue culture media containing 10 percent serum. They are stimulated by two mitogens not detectable outside the nervous system; one is neurite surface mitogen, a surface component of nerve cells, and the other is a glial growth factor, which is soluble.

In explant cultures of rat sensory ganglia, the division of Schwann cells is enhanced by contact with neurites (*49*). It is feasible to obtain purified populations of neurons and of Schwann cells from peripheral ganglia by use of antimitotic agents (*50*) or by selective adhesion (*51*). When the purified populations are mixed, it is possible to demonstrate, either by autoradiography (*49*) or by thymidine incorporation (*51*), stimulation of proliferation in the nonneuronal population. Several criteria indicate that this stimulation is dependent on cell contact. For example, if rat sensory neurites and rat Schwann cells are separated by a 6-micrometer-thick collagen diaphragm, Schwann cell proliferation is prevented (*48*).

This interpretation has been further supported by studies in which cultured Schwann cells were stimulated to divide by plasma-membrane fragments prepared from cultured neurites and from central nervous system or peripheral nervous system axons (*52*). Membrane fragments from various other sources do

Table 2. Summary of purification of GGF from the pituitary. The activity was purified from 20,000 bovine anterior lobes as described (*56*). Details of the estimates are given in (*56*).

Step	Total protein (mg)	Recovery of activity (%)	Purification factor
Crude extract	400,000		
$(NH_4)_2SO_4$ fraction	202,000	100	2
CM-cellulose 1	1,200	30	100
CM-cellulose 2	210	15	250
AcA 44 gel filtration	19	6.8	1,250
Phosphocellulose	1.1	3.1	10,000
SDS–gel electrophoresis	0.08	0.45	~100,000

not stimulate Schwann cells, and rat fibroblasts are apparently not stimulated by axolemmal fragments (*53*). The activity of the fragments is sensitive to trypsin treatment or heating, but the molecules mediating adhesion to the Schwann cell and subsequent mitogenesis have not yet been identified.

In dissociated cultures of the neonatal rat sciatic nerve, all of the cells can be identified as Schwann cells or fibroblasts by using antibodies to cell surface antigens (*54*). The fibroblasts can be removed by complement-dependent lysis with antibody to the Thy 1 antigen (*54*). The resulting purified populations of Schwann cells are strongly stimulated by a basic protein present in extracts of bovine brain and pituitary (Fig. 3A). This activity is not detectable in extracts of nonneural tissue, and the rat Schwann cells do not respond to a number of purified hormones and mitogens, including those present in brain and pituitary FGF preparations (*47*). In view of the importance of the Schwann cell in identifying and purifying this activity, the active substance has been termed glial growth factor (GGF).

The activity in the pituitary resides in a basic protein of molecular size 31,000 as shown by two lines of evidence. (i) After 5000-fold purification of the activity, the resulting preparation (*55*) was used to derive four monoclonal antibodies that form specific complexes with it (*56*). All four antibodies react with a 31,000-dalton protein in partially purified preparations (Fig. 3B). (ii) After analysis by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis in the absence of disulfide-reducing agents, the activity is recovered from the gel at 31,000 daltons (*56*). A minor peak of activity is sometimes detected at 56,000 daltons (*56*).

The 31,000-dalton species has been purified to apparent homogeneity by combining ion exchange and gel filtration steps with preparative electrophoresis (*56*). Table 2 is a summary of a purification from 20,000 bovine anterior lobes, in which a purification factor of approximately 100,000 was achieved. The estimated dissociation constant for the putative high-affinity receptor on Schwann cells is approximately $10^{-10}M$, a value comparable to that obtained with epidermal growth factor (EGF) and PDGF. A detectable effect on DNA synthesis of cultured rat Schwann cells is observed at a concentration of $5 \times 10^{-12}M$. A rigorous comparison with other growth factors must await sequencing studies; however, the localization of GGF in the nervous system and its action on Schwann cells appear to make it distinct. Although there are some striking similarities to PDGF, GGF does not give rise to fragments when treated with reducing agents, and PDGF is not active on cultured rat Schwann cells (*56*). The two molecules may, however, belong to the same family of growth factors.

Glial growth factor activity is detectable in all areas of the bovine brain but is highest in the caudate nucleus (*57*). The activity in partially purified caudate preparations is indistinguishable from that in the pituitary by several criteria, including recovery of activity and reaction with monoclonal antibodies at

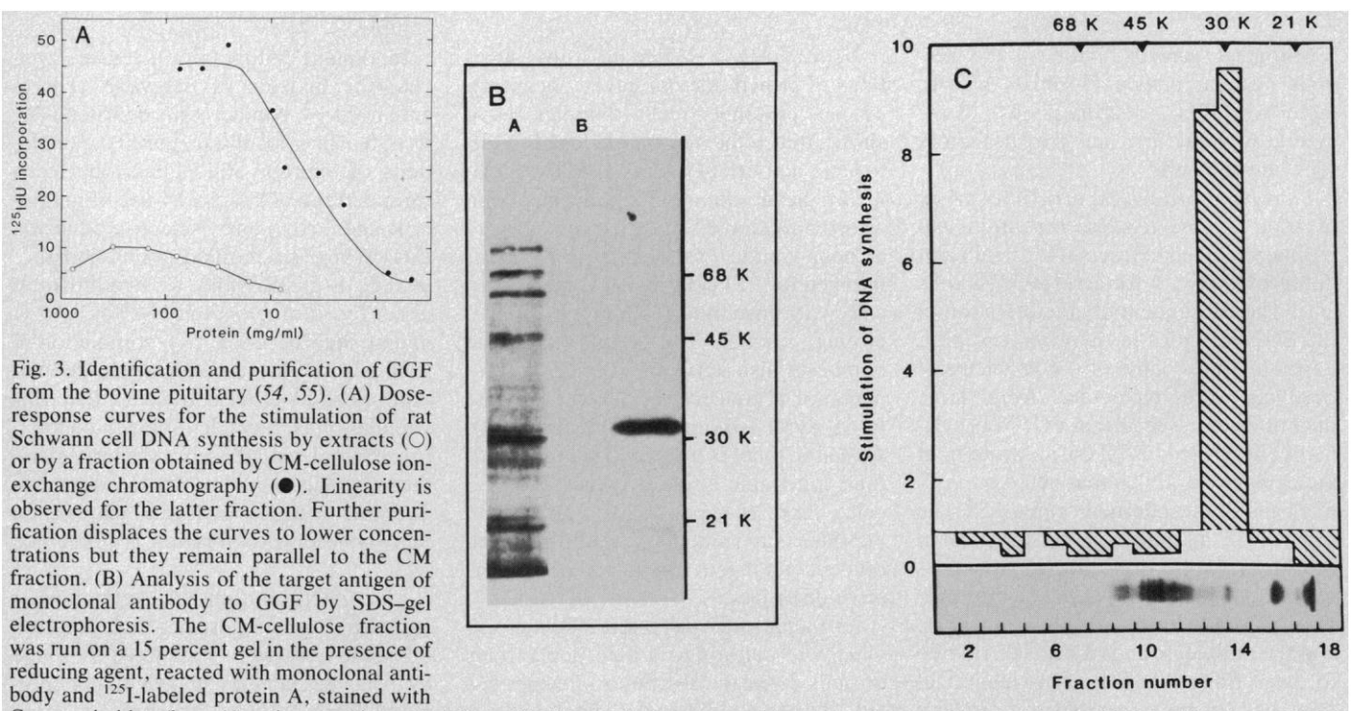

Fig. 3. Identification and purification of GGF from the bovine pituitary (*54*, *55*). (A) Dose-response curves for the stimulation of rat Schwann cell DNA synthesis by extracts (○) or by a fraction obtained by CM-cellulose ion-exchange chromatography (●). Linearity is observed for the latter fraction. Further purification displaces the curves to lower concentrations but they remain parallel to the CM fraction. (B) Analysis of the target antigen of monoclonal antibody to GGF by SDS–gel electrophoresis. The CM-cellulose fraction was run on a 15 percent gel in the presence of reducing agent, reacted with monoclonal antibody and ^{125}I-labeled protein A, stained with Coomassie blue (lane A) and autoradiographed (lane B). The antibody reacts with a minor band at 31,000 daltons. (C) Identification of GGF activity after SDS–gel electrophoresis. The phosphocellulose fraction (see Table 2) was run on a 12.5 percent gel in the absence of reducing agent. The gel was sliced and eluted, and the eluants were assayed for their stimulation of rat Schwann cell DNA synthesis. Activity was recovered at 31,000 daltons. [(B) and (C) are reprinted with permission of the *Journal of Neuroscience*]

31,000 daltons (*18, 56, 57*). The highest activities have thus far been found in extracts of human acoustic neuromas [Schwann cell tumors arising around the auditory nerve (*58*)]. These cells may be a suitable source for attempting to clone the gene for GGF.

The action of the purified molecule is not confined to Schwann cells. It acts over a comparable concentration range on cultured astrocytes (*55, 59*) and fibroblasts (*57*), and the activities against all three cell types migrate together on electrophoresis of purified preparations. As with other purified growth factors, the cell specificity is quite broad. The distinguishing feature is that the rat Schwann cell responds to GGF but not to other soluble growth factors.

Criteria for Identifying the Neural Factor

A number of different molecules may be acting as mitogenic growth factors for the blastema, possibly in a synergistic fashion on overlapping populations of cells. As in the case of neurotransmitter identification, a number of criteria need to be satisfied. First, the molecule in question must be present in the blastema; second, its level should decrease after denervation; third, it should stimulate division of a blastemal population normally dependent on the nerve; and fourth, an antiserum specific for the molecule should abrogate the normal effect of the nerve when introduced into the blastema. This demonstration was achieved in the case of nerve growth factor (*60*), but it has not so far been reported for any mitogenic growth factor.

In the case of brain FGF, some effort has been made to satisfy the first and the third criteria (*61*), but the interpretation of these experiments is now complicated by uncertainty about the identities of the active species. Although crude extracts of regenerating axolotl limbs were reported to stimulate 3T3 cells, and extracts of normal limbs not to, the investigators acknowledge that neither the identity of the agent nor its neural derivation was established. A drawback with 3T3 cells in this context is that they respond to several growth factors. Injections of microgram quantities of brain FGF preparations into denervated blastemas raised the mitotic index, and concentrations of 10 to 100 nanograms per milliliter had a twofold effect on thymidine incorporation into cultured blastemas (*61*).

A second proposal has been that neuropeptides, and particularly the undecapeptide substance P, may be important (*62*). In one sense it is a surprising candidate in view of the difference in level between motor and sensory axons, but there may be a related peptide in motor axons. There is some evidence that the third criterion was satisfied in that substance P had a small effect on the mitotic index of explanted blastemas. Further investigations of these effects will be of interest.

In the case of GGF, recent work has provided evidence to satisfy the first and second criteria. Although it has been characterized in detail only in bovine pituitary and brain, it is possible to detect an activity that stimulates rat Schwann cells in rat pituitary (*47*), and in human (*58*), chicken (*63*), frog, newt, and axolotl brain. The major peak of activity in newt brain elutes from the cation exchange resins phosphocellulose and mono S at an ionic strength close to that for the bovine pituitary and brain activity (Fig. 4A). In addition, the newt brain activity has been recovered after SDS–gel electrophoresis at 31,000 daltons. Together with the fastidious requirements of rat Schwann cell proliferation discussed earlier, it seems likely that a molecule closely related to bovine GGF is present in the Urodele nervous system.

The data of Figs. 4B and 5 show that it is possible to assay this activity in small groups of blastemas by fractionating extracts on a column and assaying the residues in the rat Schwann cell proliferation assay. The level in the blastema rises about sevenfold after amputation, as compared to that in the normal limb (Fig. 4B). In the experiment of Fig. 5, extracts of innervated and denervated blastemas were assayed by the same procedure, and at least 90 percent of the activity in the blastema was lost after 6 days of denervation.

These data provide evidence that GGF activity is present in the newt nervous system, that its level increases in the blastema, and that it is lost on denervation. The effect of bovine GGF or the partially purified newt brain species (Fig. 4A) on the division of 22/18–positive

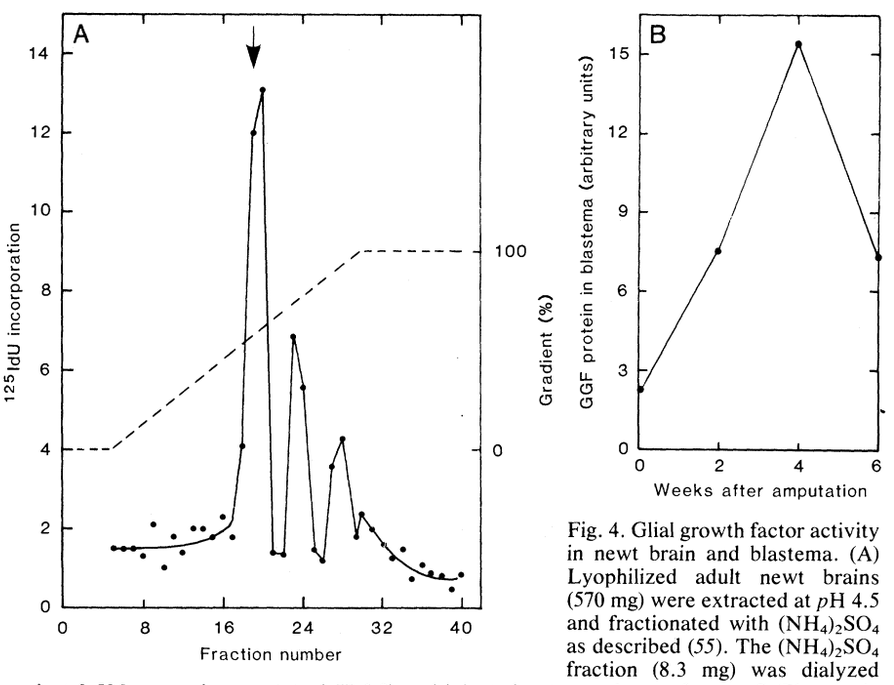

Fig. 4. Glial growth factor activity in newt brain and blastema. (A) Lyophilized adult newt brains (570 mg) were extracted at *p*H 4.5 and fractionated with $(NH_4)_2SO_4$ as described (*55*). The $(NH_4)_2SO_4$ fraction (8.3 mg) was dialyzed against 0.5*M* ammonium acetate (*p*H 6.0) and injected onto a column of mono S (Pharmacia FPLC). After being washed with 0.5*M* buffer the column was eluted with a programmed 20-ml 0.5 to 1.3*M* ammonium acetate gradient at *p*H 6.0. Portions of each fraction were evaporated to dryness in the presence of carrier protein and tested in the rat Schwann cell proliferation assay. The major peak is eluted close to the position (arrow) of bovine brain and pituitary GGF. The minor peaks are not reproducible and account for less than 15 percent of the activity when corrected for the logarithmic dosage relationship (*56*). The activity in fractions 20 and 21 migrated at 31,000 daltons when analyzed by SDS–gel electrophoresis as in Fig. 3C. The numbers on the ordinate represent the magnitude of stimulation as a multiple of the control value. (B) Time course of appearance of GGF activity in the blastema. A group of 50 adult newts were bilaterally amputated at time zero, and the amputated segments were stored at −80°C for the initial time point. Blastemas were removed from groups of newts at 2, 4, and 6 weeks and stored frozen; extracts were prepared at *p*H 4.5, dialyzed against 0.5*M* ammonium acetate (*p*H 6.0), and equivalent amounts of protein (0.7 mg) analyzed by FPLC on mono S as described for (A). The activity in the GGF peak was corrected to the quantity of GGF by using the logarithmic dosage relation (*56*).

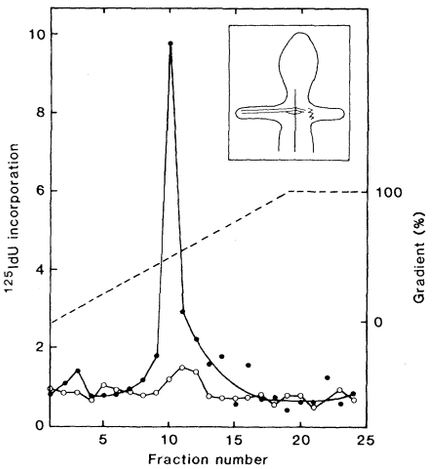

Fig. 5. Glial growth factor activity in the forelimb blastema decreases on denervation. A group of 20 newts had bilateral amputations and were denervated at the brachial plexus on the right side after 14 days. Six days after denervation, the left innervated (●) and right denervated (○) blastemas were removed and extracted; equivalent amounts of protein were analyzed by FPLC on mono S as described in Fig. 4A. The fractions were evaporated in the presence of carrier protein and tested in the rat Schwann cell DNA synthesis assay. The peak of activity from the innervated side elutes close to the position of bovine pituitary GGF. The inset is a schematic diagram of the newt with left innervated and right denervated blastemas.

cells is being studied in explanted blastemas. There is some appeal to the concept of involvement of GGF in view of the evidence that Schwann cells are an important contributor to the early blastema. It is tempting to speculate that the 22/18–positive early blastema cells retain for a period the characteristic mitogenic dependence of the Schwann cells that gave rise to them.

The Aneurogenic Limb

This view of the cellular basis of nerve-dependent proliferation may also be relevant to the phenomenon of the aneurogenic limb, which has yet to be explained at a cellular and a molecular level. The issue is an important test for understanding the controls exerted over proliferation. It is possible to remove most of the neural tube from an *Ambystoma* embryo and to sustain the embryo by parabiosis with a normal partner of the same stage. Under these circumstances the limb buds in the abnormal embryo develop in the virtual absence of a nerve supply, at least in favorable cases. Such aneurogenic limbs are capable of regenerating in the absence of the nerve (*64*). After being transplanted to a normal host larva, the limb is innervated by the brachial nerves and rather abruptly becomes nerve-dependent for regeneration (*65*).

One hypothesis is that the limb becomes "addicted" to the nerve as a source of mitogen (*21, 66*). The cellular implementation of this addiction could involve negative influences that the nerve mitogen might exert on the susceptibility of cells to other growth factors, or on the production of growth factors by other cell types. The present proposal is that the aneurogenic blastema is composed largely of the 22/18–negative population whose proliferation is independent of the nerve. This is based on the antibody evidence for heterogeneity in the normal blastema and the fact that Schwann cells cannot contribute to the aneurogenic blastema. After innervation of an aneurogenic limb, amputation would produce a normal blastema that was predominantly 22/18–positive in its early stages, but the aneurogenic blastema would not form after denervation and amputation. Certain aspects of this proposal can be readily tested by using the marker antibodies to investigate the cellular composition of the blastema before and after innervation of the aneurogenic limb.

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Cell Biology of Synaptic Plasticity

Carl W. Cotman and Manuel Nieto-Sampedro

Summary. The nervous system of mammals retains throughout the animals' lifespan the ability to modify the number, nature, and level of activity of its synapses. Synaptic plasticity is most evident after injury to the nervous system, and the cellular and molecular mechanisms that make it possible are beginning to be understood. Transplantation of brain tissue provides a powerful approach for studying mechanisms of synaptic plasticity. In turn, understanding the response of the central nervous system to injury can be used to optimize transplant survival and integration with the host brain.

The nervous system is specialized to mediate the adaptive response of the organism to environmental changes or changes in the organism itself. To this end the nervous system is uniquely modifiable, or plastic. Neuronal plasticity is largely the capability of synapses to modify their function, to be replaced, and to increase or decrease in number when required (Fig. 1). Neuronal plasticity is maximal during development and is expressed after maturity in response to external or internal perturbations, such as changes in hormonal levels, environmental modification, or injury.

In this article we review the types of changes that occur in the mature central nervous system (CNS) of mammals, where they occur, what type of neurons are most plastic, and the stimuli that evoke synaptic growth. We will discuss lesion-induced synaptic plasticity in the adult together with recent work on the integration of transplanted embryonic CNS tissues into neonatal and adult hosts. Transplantation is a powerful means of eliciting the potential plasticity of the host CNS and studying selected aspects of its mechanism. Furthermore, transplants have therapeutic potential to alleviate the behavioral deficits that result from neuronal death caused by injury, degenerative disease, and aging. Most studies on synaptic plasticity have been carried out at the cellular and subcellular levels, but molecular mechanisms are beginning to emerge that are leading to new insights on the control of neuronal growth in the normal and injured CNS.

We have found it helpful to introduce the term "synapse turnover" to discuss synaptic plasticity and its mechanisms in the mature CNS (*1*). The turnover of a synapse is defined as its loss and replacement; the turnover time of a synapse population is the period required for the disconnection of old junctions and the initiation, establishment, and maturation of the new connections (Fig. 1). The term synapse turnover unifies in one concept the increasingly evident common features of synaptic plasticity in the mature CNS.

Synaptic Growth Evoked by Partial Denervation

The most extensive evidence of synaptic growth in the CNS comes from studies in which lesions serve as the stimulus. Liu and Chambers (*2*) first described axon sprouting in the cat spinal cord. After severing adjacent dorsal roots, the residual root sprouted new axons into the denervated zones. Raisman (*3*) showed that new synapses can form in the rodent septal nucleus in response to lesions. An extensive literature now exists on the extent of lesion-induced synaptic replacement in the CNS, its rate, and the type of growth evoked. The most complete studies have been carried out on the rodent hippocampal formation; accordingly we will make use of this system to illustrate key concepts.

The hippocampal formation consists of two major subdivisions, the dentate gyrus and the hippocampus proper with its subdivisions (CA1, CA2, CA3, and CA4). Granule cells are the major cell type of the dentate gyrus, and pyramidal cells are the major type in the hippocampal subfields (Fig. 2). The major extrinsic input to the hippocampal formation originates from a special subdivision of the cerebral cortex, the entorhinal cortex (*4*).

The response to unilateral ablation of the entorhinal cortex illustrates many of the general principles of lesion-induced synapse turnover. One day after the lesion is made, 90 percent of the synapses in the outer two-thirds of the dendritic tree of the granule cells on the side of the lesion are lost. A massive growth response by all of the residual fiber systems in the dentate gyrus except the raphe begins 3 days after the lesion. New synapses are formed by sprouting fibers originating in the contralateral entorhinal cortex (*5*), the septum (*6*) and in the CA4 area (commissural-associational system) (*7*). Synapse replacement is complete by about 60 days. The newly formed synap-

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