Regenerative Growth of Axons of Hamster Optic Tract: Effects of Age, Substrate, and Growth-Promoting Factors

by

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Submitted to the
Department of Brain and Cognitive Sciences
in Partial Fulfillment of the Requirements
of the Degree of Doctor of Philosophy

at the

Massachusetts Institute of Technology
September 1989

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ABSTRACT

These experiments examined in vivo regenerative growth of neonatal hamster retinal ganglion cell axons after transection of the optic tract between the ages of postnatal day 3 (p3) and p25. In the first set of experiments, a neonatal neocortex-conditioned implant (containing primarily astrocytes) of gelfoam or collagen-glycosaminoglycan was used to bridge the gap between the proximal ends of the transected optic tract axons and their target, the superior colliculus.

In the second set of experiments, implants were conditioned in a potent cell mitogen, basic FGF. Basic FGF is primarily involved in promoting cell proliferation during early stages of neural and non-neural development, but it has also been found to promote survival and outgrowth of many CNS neurons. Study of axonal outgrowth revealed that both astrocyte-covered and bFGF-conditioned implants significantly enhanced the extent of retinal axon growth into implants. Although immunocytochemical staining of the astrocytic implants confirmed the presence of both laminin and bFGF, it is also possible that the growth-promoting effects of both types of implants result from indirect rather than direct influence on injured retinal axons. Thus, in addition to direct outgrowth-enhancing effects of these growth-promoting molecules, regenerative growth may result from (i) formation of an astrocytic substrate that is conducive to axonal growth, and (ii) reduction of the host glial scar that normally prevents injured axons from growing past the site of transection. Particularly dramatic retinal outgrowth was seen in the bFGF-conditioned implants, even in cases in which the optic tract was transected on postnatal days 18 and 25. The enhanced outgrowth in these latter cases may result from presence of an astrocytic substrate that forms as a consequence of the mitotic effects of bFGF on astrocytes.

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For Larry

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Chapter 1

Introduction

Following central and peripheral nervous system injury in non-mammalian species, damaged axons can regrow, reinnervate the appropriate target, and mediate normal behavioral function (Sperry, 1944, 1945). Similar regrowth and reinnervation occurs in the mammalian peripheral nervous system (PNS), but injured axons of the adult mammalian central nervous system (CNS) do not regenerate, showing at most a small amount of neuritic sprouting. It is not entirely clear why axons of the mammalian CNS do not regenerate, and through years of research on this topic, two primary explanations have been suggested: (i) that CNS neurons have an intrinsic inability to grow, or (ii) that mammalian CNS lacks a suitable extracellular substrate for supporting axonal growth. Pioneering work by Aguayo and co-workers showed that CNS axons can regrow if they are provided with a suitable environment--in this case, a piece of peripheral nerve (David and Aguayo, 1981; Vidal-Sanz et al., 1987). This finding provoked experimenters in the field of regeneration to refocus their attention on the extra-

axonal environment as a primary cause in the failure of CNS regeneration.

An understanding of what environmental factors are conducive to axonal growth can be gained from the study of axonal growth during development. Presumably, if CNS neurons retain the potential for growth throughout adulthood, as Aguayo's results indicate, then regenerative growth might be promoted by providing injured axons with an extracellular environment similar to that present during developmental outgrowth. This hypothesis requires a number of assumptions, and in particular, the assumption that adult CNS axons retain the ability (i.e., they possess the appropriate receptors) to respond to and interact with an environment similar to that encountered during normal development. (This assumption is discussed in greater detail later in this Chapter.) Thus, study of normal CNS development--of axon outgrowth as well as of the extracellular environment--can provide many insights into mechanisms of axonal growth, which can then be applied in the effort to induce regeneration in adult mammalian CNS. This Introduction reviews evidence for environmental influences on CNS neuritic growth in three conditions: (i) *in vivo* development, (ii) *in vitro* neuritic growth, and (iii) *in vivo* regeneration.

Neuronal outgrowth during development

Neuronal outgrowth can be divided into two distinct periods: elongation or pathfinding, in which growth cone-tipped axons navigate to their target, and target innervation or arborization, in which axons begin to branch and form terminal arbors within their target. Although there are other important stages of neural growth (e.g., competition, arbor refinement, synaptogenesis), the elongation and arborization periods have the most current relevance for nerve regeneration. Later stages of axonal arborization, including arbor refinement, competition, and synaptogenesis, will become more relevant to study of regeneration when the potential for functional recovery is closer to realization.

Development of the retinotectal projection

Axons of the developing retinotectal projection of hamster (Jhaveri et al., 1988a; Schneider et al., 1989a,b), chick (Thanos and Bonhoeffer, 1987), and Xenopus (Harris et al., 1985, 1987) show several distinct stages of growth. Elongation-type growth is very rapid (2-4 mm/day in rat and hamster corticospinal tract--Reh and Kalil, 1981; Shatz and Rakic, 1981; and 2 mm/day in developing and regenerating hamster retinofugal axons--Jhaveri et al., 1988; Carman et al., 1987; Cho and So, 1987) and is characterized by an unbranched and

fasciculated morphology (hamster: Jhaveri et al., 1988; Xenopus: Harris et al., 1985, 1987; chick: Thanos and Bonhoeffer, 1987). After reaching their target (and often after a "waiting period"), optic tract axons commence arborization (Harris et al., 1985, 1987; Jhaveri et al., 1988). Arborization-type growth in the hamster is much slower (8-10 um/hr) than elongation, is non-fasciculated, and axons grow with a branched morphology (Jhaveri et al., 1988). Equivalent growth stages have been described in development of Xenopus (Harris et al., 1985, 1987) and chick (Thanos and Bonhoeffer, 1987) retinotectal projections, and may be a general phenomenon in the development of many CNS tracts.

Influence of substrate on neural outgrowth in vivo

What mechanisms permit developing axons to achieve these growth stages so reliably--to navigate to the appropriate target, recognize it, and form terminal arbors? Much experimental evidence indicates that mechanical and molecular properties of the neural substrate play important, if not determining, roles in nerve outgrowth and target innervation (see Davies, 1987; Dodd and Jessell, 1988; Rutishauser and Jessell, 1988 for reviews). For example, during development of the chick limb buds, the formation of structural pathways within the limb mesenchyma influences not only the direction of

growth (Tosney and Landmesser, 1985; Swanson, 1985; Swanson and Lewis, 1982), but also the timing of outgrowth and the extent of axonal fasciculation (Swanson, 1985; Swanson and Lewis, 1982). The role of structural substrate guides in directing axonal outgrowth has also been indicated for CNS pathways such as the newt spinal cord (Singer et al., 1979), and the chick and mouse optic nerve (Kraynek and Goldberg, 1981; Silver et al., 1987; Silver and Sidman, 1980; Silver, 1984) and olfactory projection (Silver et al., 1987).

Tissue substrate can also influence the route and characteristics of developing axons by molecular means (Harris, 1989; see also Davies, 1987; Dodd and Jessell, 1988; Lander, 1989; Rutishauser and Jessell, 1988 for reviews). The expression of particular cell surface, extracellular matrix, or diffusible molecules at different stages of development is now known to be an important determinant in CNS axonal pathfinding (Harris, 1989; Letourneau, 1975; Tessier-Lavigne et al., 1988), target recognition (Bonhoeffer and Huf, 1980), and even axonal morphology (Harris et al., 1987) and axonal fasciculation (Rutishauser, 1985). The influence of molecular (as opposed to mechanical) mechanisms appears to be particularly relevant for studies of developing (and, by extension, regenerating) retinal projections.

During normal development of the mouse, Xenopus, and chick optic tract, single pioneer axons grow along a substrate formed by the endfeet of neuroepithelial processes and an adjacent basal lamina (Bork et al., 1987; Cohen et al., 1987; Lockerbie, 1987; Silver, 1984; Silver and Rutishauser, 1984; Silver and Sidman, 1980). Non-pioneers extend either along the same neuroepithelial surface in close apposition to the pioneer axons, or along the pioneers themselves (Lockerbie, 1987; see also Noble et al., 1984). A number of different cell surface molecules that are expressed in neuroepithelial processes may be involved in stimulating or guiding retinal axons during this early elongation stage of growth. A primary example is the neurite-promoting glycoprotein laminin, which is expressed in neuroepithelial processes during early retinal outgrowth in chick (Cohen et al., 1986, 1987), rat (McLoon et al., 1988) and mouse (Liesi and Silver, 1989). Other cell surface molecules that may influence retinal axon outgrowth include nerve cell adhesion molecule (NCAM; Rutishauser, 1985; Silver and Rutishauser, 1984) and N-cadherin (Hatta et al., 1987; Matsunaga et al., 1988); these will be discussed further in Chapter 3.

Influence of Substrate on Axonal Arborization

While laminin, NCAM, and N-cadherin guide axonal growth during elongation and pathfinding, other molecules influence target recognition and arborization (Bonhoeffer and Gierer, 1984; Harris et al., 1985, 1987; Gierer, 1987; Gottlieb and Glaser, 1980; Tessier-Lavigne et al., 1988). Sperry's (1963) chemoaffinity theory, that target cells have specific chemical markers that are recognized by their appropriate afferents, is widely accepted, although it has proven difficult to identify the particular molecules that mediate recognition (Lusmden and Davies, 1987; see Gottlieb and Glaser, 1980 and Landmesser, 1980 for reviews of CNS and neuromuscular specificity, respectively). Axons from a variety of CNS populations preferentially arborize in their appropriate targets (retinal ganglion cells: Bonhoeffer and Huf, 1980; Hankin and Lund, 1987a,b; Harvey et al., 1987; Lund and Harvey, 1981; Sefton et al., 1987; motoneurons: Keynes et al., 1987; Stirling and Summerbell, 1985; hippocampal afferents: Nilsson et al., 1988).

Certain morphological characteristics of arborizing axons also appear to be specified by their target; for example, hamster retinal ganglion cells that are made to terminate in aberrant targets will adopt ultrastructural characteristics that are consistent with the aberrant

target rather than with their normal morphology (Campbell and Frost, 1987; Crain and Hall, 1986; Kalil and Schneider, 1975). Further, patterns of retinal ganglion cell arborization vary with the age of target (i.e., tectal) tissue in mouse explants (Friedlander and Crain, 1985) and in vivo in rats with tectal transplants of various ages (Harvey and Lund, 1984). However, it is not known what properties of the target tissue effect these characteristics in the afferents; possibilities include target adhesivity (Letourneau, 1975) and cell surface markers (Bonhoeffer and Huf, 1980; Sperry, 1963). It has also been suggested that the timing of chick retinotectal arborization is determined by the cytoarchitectural maturation of the tectum (Rager and von Oeyenhauser, 1979; Thanos and Bonhoeffer, 1987).

Influence of substrate on neural outgrowth in vitro

The precise role of neuroepithelial and target substrates in determining various characteristics of elongating and arborizing axons in vivo is often difficult to determine. In vitro evidence can provide more definitive answers for how substrates influence neuritic growth. The morphology and direction of neuritic outgrowth in vitro can be determined by structural channels, by substrate adhesivity (Letourneau, 1975; Hammarback et al., 1988; Harris, 1989; Holley,

1987), and by the presence of particular soluble or cell surface molecules (that may or may not affect the substrate adhesivity) (Adler et al., 1985; Denis-Donini and Estenoz, 1988; Gunderson, 1987; Hammarback et al., 1988; Hopkins et al., 1985; Lander, 1989; Rogers et al., 1983). Thus, this technique can be used to more closely examine what cell surface molecules or properties of the neuroepithelial substrate are responsible for guiding and promoting retinal axon outgrowth. Because neuroepithelial cells are glial precursors, studies of retinal axon growth on monolayers of immature astrocytes may give insight into the mechanisms relevant during in vivo retinal outgrowth.

Influence of glia on neural growth

While immature astrocytes have been shown to support or promote neurite outgrowth in vitro (Denis-Donini and Estenoz, 1988; Fallon, 1985; Hatten et al., 1988; McCafferey et al., 1984; Noble et al., 1984; Tomaselli et al., 1988; Wigley and Berry, 1988), mature astrocytes fail to support, and may even inhibit, neuritic growth (McCafferey et al., 1984; Kalderon, 1988a,b; Smith et al., 1986). The precise properties or molecules responsible for inducing neuritic growth on immature astrocytes or glial precursors have not been entirely determined. There is evidence that immature astrocytes

express both soluble and cell-surface neurite-promoting molecules, such as fibroblast growth factor (FGF) (Ferrara et al., 1988; Hatten et al., 1988), laminin (Bernstein et al., 1985; Liesi et al, 1983, 1984; Tomaselli et al., 1988), fibronectin (Price and Hynes, 1985), N-cadherin (Hatta et al., 1987; Matsunaga et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988), NCAM (Keilhauer et al., 1985; Neugebauer et al., 1988; Noble et al., 1985), and other uncharacterized factors (Assouline et al., 1987; Banker, 1980; Lindsay, 1979; McCafferey et al., 1984; Muller and Seifert, 1982; Raju and Bennett, 1986; Wujek and Akeson, 1987). These factors have all been shown to enhance CNS neuritic outgrowth or neuronal survival in vitro (FGF: Hatten et al, 1988; Rydel and Greene, 1987; Unsicker et al., 1987; Walicke et al, 1986; laminin: Cohen et al., 1987; Ford-Holevinski et al., 1986; Lander et al., 1985a,b; Manthorpe et al., 1983; Smalheiser et al., 1984; fibronectin: Lander, 1983; Carri et al., 1988; Hall et al., 1987; Carbonetto et al., 1983; N-cadherin: Tomaselli et al., 1988; Matsunaga et al., 1988), and in vivo (FGF: Anderson et al., 1988; Sievers et al., 1987; laminin: Madison et al., 1987; Yoshii et al., 1987; Zak et al., 1987).

Growth-promoting effects of immature astrocytes have also been reported for in vivo experiments, although

these provide less evidence for the nature of the effective agent. Transplantation of purified astrocytes into a wound cavity enhances behavioral recovery after cortical injury (Emmett et al., 1988; Kesslak et al., 1986), and this effect is attributed to uncharacterized trophic factors that are released by transplanted astrocytes (Kesslak et al., 1986; Nieto-Sampedro et al., 1983; Rudge et al., 1985). Silver and co-workers have shown that immature (pre-critical stage) astrocytes can promote growth of developing or regrowing axons of the corpus callosum and spinal cord dorsal root (Smith et al., 1986; Silver and Ogawa, 1983; Kliot et al., 1988). This effect is thought to result from the favorable substrate or growth-promoting factors (e.g., laminin) expressed by the transplanted immature astrocytes (Smith et al., 1986; Liesi and Silver, 1989), or from a reduction in glial scar formation in host tissue (Kalderon, 1988a,b; Rudge et al., 1989; Kliot et al., 1988; Smith et al., 1986). Mature astrocytes may inhibit nerve regeneration by forming an impenetrable scar of astrocytic processes and a glial limitans (Kalderon, 1988a).

Characterization of Astrocytes

However, there are several different types of astrocytes, each of which may have different or even

opposing effects on neuritic growth. During normal development in rat optic nerve, type 1 astrocytes appear at E16 (Miller et al., 1985), and are located along the perimeter of the nerve (Miller and Raff, 1984). Type 1 astrocytes in the optic nerve develop form neuroepithelial cells of the optic stalk (Miller et al., 1989; Small et al., 1987), while type 2 astrocytes develop from precursors that migrate into the optic nerve (Small et al., 1987). Type 2 astrocytes develop after p7 and are located within the optic nerve (Miller and Raff, 1984). Only one type of astrocyte appears to be involved in promoting neuritic growth (Smith et al., 1986; Silver and Ogawa, 1983; McCafferey et al., 1984), although it is referred to by a variety of names. Type 1 astrocytes probably correspond to the immature (pre-critical stage) astrocytes described by Silver and co-workers (Silver and Ogawa, 1983; Smith et al., 1986) and to the non-process bearing (flat) immature astrocytes described by McCafferey et al., (1984). However, extracellular conditions greatly influence the morphology (Skaper et al., 1989), protein expression (Bologa et al., 1988; Ciesielski-Treska et al., 1988; Morrison et al., 1985), and thus the entire identity of various glial cells (Raff, 1989; Ingraham and McCarthy, 1989). Although indirect evidence indicates that reactive astrocytes may be similar or identical to immature or type 1 astrocytes,

there is as yet no direct proof that reactive astrocytes from adult CNS have molecular properties comparable to those of immature astrocytes.

The membrane protein A2B5 is commonly used to distinguish between type 1 and type 2 astrocytes; it is expressed by neurons, type 2 astrocytes, and by the common precursor of oligodendrocytes and type 2 astrocytes--the O-2A precursor, but not by type 1 astrocytes (Miller and Raff, 1984). [However, Miller et al. (1989) have recently noted that expression of A2B5 may not be reliable for distinguishing type 1 and type 2 astrocytes in tissue sections, where it stains intracellularly.] The ability of type 1 or immature astrocytes to support outgrowth may be related to their secretion of laminin (Liesi, 1985; McLoon et al., 1988; Smith et al., 1986); only immature (Liesi et al., 1983; McLoon et al., 1988) and reactive (Bernstein et al., 1985; Liesi et al., 1984; Liesi, 1985) astrocytes of the mammalian CNS express laminin <u>in vivo</u>. [Miller et al., 1986 have shown that reactive astrocytes are nearly all $A2B5^-$, and thus type 1 (see also Raff et al., 1987)]. It has also been suggested that the presence of laminin-secreting astrocytes may be a general property of nervous systems that regenerate (Hopkins et al., 1985; Liesi, 1985; McLoon, 1986).

Influence of humoral growth factors on neural growth

The existence of soluble growth factors that enhance survival and/or outgrowth of CNS and PNS neurons was first demonstrated with the discovery of Nerve Growth Factor (NGF). NGF has both tropic (Gunderson and Barrett, 1980; Letourneau, 1978) and trophic (Levi-Montalcini, 1982; Kromer, 1987) effects on sympathetic and sensory neurons, although its effects on other neuronal populations is more limited. In addition, NGF enhances survival of injured rat retinal ganglion cells when it is injected into the eye following optic nerve transection (Carmignoto et al., 1989; Maffei et al., 1989).

The discovery of the neurotrophic and neurotropic effects of NGF prompted a search for soluble factors that had similar effects on other neuronal populations. One such factor, fibroblast growth factor (FGF) appears to have trophic and mitotic effects on a wide variety of neural and non-neural cells (see Gospodarowicz, 1989 for review), although there have been no reports that has tropic effects.

Although FGF appears to be primarily involved in neural and non-neural cell proliferation during development (in chick: Munaim et al., 1988; in mouse: Wilkinson et al., 1989; in Xenopus: Slack and Isaacs,

1989), it also has trophic effects on developing and injured CNS neurons (Walicke, 1988a; Gospodarowicz, 1989). The influence of bFGF in CNS is discussed further in Chapter 4.

Influence of substrate on nerve regeneration in vivo

Understanding the influence of substrate or extracellular environment on normal neural development has been particularly important in furthering the study of CNS regeneration. One method for distinguishing between intrinsic and extrinsic causes of regeneration failure involves placing non-regenerating CNS tracts into environments in which regeneration normally occurs, e.g., PNS, embryonic CNS, or non-mammalian CNS. Through these efforts it has been shown that in vivo regenerative growth can be induced by altering the external environment of the injured CNS axons. Thus far, the greatest success has come from the use of uncharacterized biological materials: peripheral nerve implants (So and Aguayo, 1985; Vidal-Sanz et al., 1987; Cossu et al., 1987), human amnionic membrane (Davis et al., 1987; Blaker et al., 1988), transplanted embryonic neural tissue (Harvey et al., 1987; Kromer et al., 1981a,b), cultured PNS neural and glial constituents (Smith and Stevenson, 1988; Kromer and Cornbrooks, 1985), and transplanted astrocytic bridges (Smith et al., 1986;

Schreyer and Jones, 1987) all support in vivo growth or regeneration of CNS axons.

Inhibitory influences on CNS regeneration

Thus there is abundant evidence that glia alone or heterogeneous extractions of neurons and glia, can significantly enhance CNS regeneration. However, there is also a large body of work demonstrating inhibitory influences of CNS glia on axonal growth and regeneration. Recently, two proteins secreted by oligodendrocytes have been shown to be potent inhibitors of retinal ganglion cell outgrowth in vitro (Caroni and Schwab, 1988a,b; Caroni et al., 1988; Schwab and Caroni, 1988), and it has been suggested that expression of these proteins may be responsible for the lack of regenerative growth in the mammalian CNS (Schwab and Theonen, 1985; Caroni et al., 1988).

Another potential source of growth inhibition by glial cells comes from their tendency to form dense glial scars around areas of CNS injury (Reier et al., 1983). CNS glial scars are made up of dense accumulations of type 1 astrocytes (Miller et al., 1986) and invading fibroblasts and macrophages (Reier et al., 1983; Reier, 1986; Kruger et al., 1986), that effectively block CNS axons from growing out of host tissue (Reier et al., 1983; Tessler et al., 1988).

Regeneration of Neonatal Hamster Optic Tract Axons

Neonatal mammals have a greater capacity for regenerative growth than adult mammals (Kalil and Reh, 1979; Schneider, 1970, 1973, 1979; So et al., 1981), and in those mammals with particularly short gestation periods, functional regeneration can be studied postnatally. In the Syrian hamster, which has a gestation period of just 16 days, transected retinotectal axons will show functional regeneration only if they are transected on or before postnatal day 3 (p3) (So et al., 1981). Transection after p3 results in neuritic sprouting with no reinnervation of the superior colliculus (Harvey et al., 1986; Dyson et al, 1988; So et al., 1981). Similar results have been reported for the hamster lateral olfactory tract (Grafe, 1983; Devor, 1975). It is not known why retinal axons do not regenerate after p3; the work of Aguayo and others (So and Aguayo, 1988; Harvey et al., 1986; Dyson et al., 1988; Smith and Stevenson, 1988) on regeneration of the rodent optic nerve has effectively proven that these neurons have the intrinsic capacity to grow, so the cause of failure must lie in their environment. There are several possible explanations. First, formation of an astrocytic scar at the lesioned edge of host tissue may become increasingly dense and impenetrable after p3. Second, host tissue may cease expression of growth-

promoting molecules, or alternatively, may initiate expression of growth-inhibiting molecules. These possibilities were examined in the following studies.

The experiments presented in Chapters 3 and 4 examined the effects of several different growth-promoting cells and factors on regenerative growth of the neonatal hamster optic tract after transection between the ages of p3 and p25. For the experiments in Chapter 3, an implant of gelfoam or collagen-glycosaminoglycan harvested from neonatal neocortex (and known to contain an abundance of astrocytes), was used to bridge the gap between the proximal ends of the transected optic tract and the superior colliculus. Immature astrocytes have been shown to promote neuritic outgrowth of CNS neurons both in vitro (Fallon, 1985; Noble et al., 1984; McCafferey et al., 1984; Tomaselli et al., 1988) and in vivo (Smith et al, 1986). For the experiments in Chapter 4, gelfoam or collagen implants were conditioned in a growth factor, fibroblast growth factor, that is expressed by immature astrocytes (Ferrara et al., 1988; Hatten et al., 1988), and that has been shown to promote outgrowth of CNS neurons both in vitro (Bahr et al., 1989; Walicke, 1988a) and in vivo (Anderson et al., 1988; Sievers et al., 1987).

Chapter 2

Methods

The usual procedure for each experiment consisted of the following steps, which are described in greater detail below: neonatal hamsters were anaesthetized, the brachium of the right superior colliculus was transected, and a control or experimental implant was placed in the gap produced by the transection. Following a 1-2 week survival, the eye contralateral to the transected tract was injected with the neural tracer horseradish peroxidase (HRP) (Figure 1). Animals survived an additional 18-24 hours to permit transport of the HRP, then were deeply anaesthetized and perfused with an appropriate fixative. Brains and eyes were removed, then cut into 40 um sections and subjected to various histological and/or immunohistochemical reactions. Finally, reacted or stained tissue sections were photographed and quantified for cell survival and axonal growth.

Surgeries

For surgery on hamsters under the age of 7 days, hypothermia was used as anaesthetic. At older ages a combination of chloropent or nembutal and valium was used.

Transection of Brachium of the Superior Colliculus.

The brachium of the superior colliculus was transected at various postnatal ages, always under direct visual guidance after aspiration of cortical tissue overlying the pretectal/SC border. The brachium was then transected with a fine tungsten wire hook, which was inserted and pulled up through the superficial tissue to ensure complete transection. A fine glass suction tip was then used to create a ~.5-mm wide gap in the brachium. The various transplants were then placed in this gap, the midbrain was (in some cases) covered with a piece of collagen-glycosaminoglycan (C-GAG) film (made by Professor I.V. Yannas, M.I.T.), and saline-soaked gelfoam was placed dorsal to the midbrain, in the cortical gap. The skull flap was replaced, skin sutured, and animals less than 14 days old were returned to their mother.

Brachial Transection with Ablation of Superior Colliculus.

The brachium of the superior colliculus was transected as described above, then a fine glass suction tip was used

to aspirate superior colliculus just caudal to the transection site.

Implant Preparation: Harvested Implants.

A small suction lesion was made in the neocortex of the anaesthetized donor animal on p0, p1, or p10 and a saline-soaked piece of C-GAG polymer (Yannas et al., 1986), or gelfoam (Upjohn Co.) was placed in the resulting gap. To remove the implant for transplantation, the donor animal was deeply anesthetized and the implant was removed and transplanted directly to the host midbrain. A detailed study of the cell types harvested with this technique has recently been published (Rudge et al., 1989) and is discussed in Chapter 3.

Implant Preparation: Harvested and Rinsed Implants.

These were prepared exactly as described above for harvested implants, but implants were placed either in sterile saline for 30 minutes at room temperature or in an oxygenated tissue slice medium (pH 7.3, 37^{o}C), for 1 to 4 hours prior to transplantation. This procedure was originally intended to aid in surgery, but may have killed the harvested astrocytes or washed away growth-promoting molecules (see results, Chapter 3).

Implant Preparation: Harvested Implants with Freeze-Thaw.

Harvested implants were placed onto aluminum foil covering a piece of dry ice (without prior rinsing).

After 5 minutes of freezing, the foil was moved to a 37^0 hot plate for 5 minutes. This freeze thaw procedure was repeated 3 times, and after the final thaw, implants were transplanted into host animals.

Implant Preparation: Non-neural Implants.

A small cut was made through the meninges and the uppermost layers of the occipital muscle. A small piece of gelfoam or C-GAG polymer was placed into this cut in donor animals on p6 and harvested 3 days later.

Implant Preparation: FGF-soaked Implants.

Gelfoam or C-GAG implants were cut to the proper size for implantation, then placed in 4^oC solution of bFGF (15 ng/ml; FGF company, La Jolla, CA, or 50 ng/ml of a recombinant bFGF produced by Dr. Mike Klagsbrun) in 0.2% gelatin in phosphate-buffered saline (PBS) for 24 hours (with frequent agitation). Implants were kept at room temperature during surgery, approximately 1-5 hours prior to implantation. Recent reports on the binding of bFGF to gelfoam and collagen (Thompson et al., 1988), as well as to the particular GAG used in the C-GAG implants (Walicke, 1988b), are discussed in Chapter 4.

Eye Injections.

A solution of 50% HRP in 2% dimethylsulfoxide (DMSO) was injected into the eye through a glass micropipette at various post-lesion survival times, and the animal was

perfused 20-28 hours (depending on the age of the animal--older animals were perfused at 28 hours and the youngest were perfused at 20 hours) after the eye injection.

Perfusions.

Perfusions were done on deeply anaesthetized animals, with 0.9% NaCl-0.25% $NaNO_3$ sodium nitrite followed by 1% paraformaldehyde/ 1.25% gluteraldehyde, or, for tissue to be processed for immunohistochemistry, with 4%-paraformaldehyde. Brains were then cryoprotected in 30% sucrose-PO_4 and cut at 40 um on a freezing microtome (except for hosts receiving ^{3}H-thymidine labeled implants; these brains were cut at 30 um).

Preparation of Retinas.

Eyes were removed following perfusions, and a thread was tied around the superior rectus to indicate proper orientation. Eyes were further fixed in 5% formalin, then the lenses and vitreous were removed, and the eye was embedded at a known orientation in albumin gelatin with gluteraldehyde. Eyes were cut into 30 um sections on a freezing microtome. Sections were mounted and stained for Nissl substance with cresylecht violet.

^{3}H-Thymidine labeling of Donor Implants.

Donor animals received gelfoam implants on p1 as described above, and were injected intraperitoneally with

5 uCi/g body weight of ^{3}H-thymidine (New England Nuclear; specific activity = 86 Ci/mmol) 1 hour later. They received a supplementary dose of 2.5 uCi/g body weight an additional 18 hours after implantation. Implants were harvested on p3 and transplanted to the midbrain of host animals that had received brachial transection on p6. After a 7 day survival, the left eye of host animals was injected with HRP, and hosts were perfused 24 hours later. Brains were cut at 30 um into three series; one series was reacted for visualization of HRP, and a second series was mounted and processed for autoradiography.

Histology

Tract tracing methods.

Tissue was reacted for visualization of HRP, generally with a modified AHM-TMB method (Olucha et al., 1985). This method is less sensitive than the SNF-TMB method of Mesulam (1978), but the latter produces excessive artifact (Jhaveri et al., 1988b). Both methods were used, often on alternate sections to enable optimal interpretation of the sections. Tissue was counterstained with neutral red or hematoxylin and eosin; the latter permits good discrimination of the C-GAG polymer.

Thymidine-labeling and Autoradiography.

Host tissue containing implants from ^{3}H-thymidine labeled donors was cut at 30 um, mounted, coated with Kodak NTB-2

photographic emulsion, and exposed in the dark for 6 weeks at 4°C. The slides were developed in Kodak D-19 developer, fixed, and counterstained with cresyl violet.

Immunohistochemistry.

Harvested, non-transplanted implants were rinsed for 10 minutes in 4% paraformaldehyde in PO_4. These fixed implants and fixed tissue sections were stained by the avidin-biotin and immunofluorescent methods with antibodies against the neurofilament proteins GFAP and vimentin[1], and with antibodies against the growth-associated molecules laminin and bFGF (generous gifts from Dr. Seth Finkelstein and Dr. Mike Klagsbrun, respectively). In addition, the harvested, non-transplanted implants were stained with antibodies to the membrane protein A2B5 (gift from Dr. Ron McKay), which requires staining of live cells and thus could not be done on fixed tissue sections.

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1. Glial and many other neural and non-neural cells also express the intermediate filament protein vimentin (Chiu et al., 1981), although the relationship between vimentin expression and glial type has not yet been clearly elucidated. Vimentin is expressed by radial glia during the earliest stages of development (Pixley and DeVellis, 1984; McDermot and Lantos, 1989), and appears in reactive astrocytes following CNS injury in rodent (Pixley and DeVellis, 1984). Although some have used it as a marker of astrocyte immaturity (Manthorpe et al., 1986), the fact that vimentin can be rexepressed in many cells in response to injury or to various culture conditions, suggests that it cannot be used to identify cell type or maturational state.

Double-labeling with immunofluorescent markers was used to colocalize GFAP/vimentin, GFAP/FGF, GFAP/A2B5, and GFAP/laminin. The specific protocols used in each of these procedures can be found in Appendix A.

Assay of Rinse Solution

In order to determine what factors were lost from harvested implants when they were rinsed prior to transplantation, two types of assays were done on samples of of PBS in which harvested implants had been rinsed for 4 hours. A Lowrey phenol-biuret assay for protein was performed by Dr. Judi Schaechter and an assay for mitogenic aactivity (assessed by incorporation of ^{3}H-thymidine in Balb C 3T3 cells) was done by Jonathon Foster and Dr. Seth Finkelstein.

Implant Materials

Collagen-glycosaminoglycan polymer matrices

C-GAG polymers were generously provided by Professor I.V. Yannas (Department of Polymer Science, M.I.T.). This polymer is composed of type IV collagen from rat tail tendon and chondroitin 6-sulfate glycosaminoglycan from shark cartilage, which are crosslinked to form a porous matrix (Yannas et al., 1980 a,b). The pore size, orientation, and extent of crosslinking could be varied to produce a matrix structure compatible with use in the CNS or other tissues (Yannas et al., 1986; 1989). For the

experiments reported here, we used a collagen-glycosaminoglycan (C-GAG) polymer with random pore orientation and pore size of 20 to 30 um.

Heparin-sepharose (HS) beads

HS-bFGF beads were generously provided by Professor Robert Langer and Dr. Elazer Edelman (Whitaker College, M.I.T.). Basic FGF was bound to the H-S beads, and the entire complex was encapsulated by mixing with sodium alginate to form small (~0.8 mm diameter) microspheres. These enabled controlled release of bFGF at the site of the optic tract lesion. In addition to acting as a carrier for bFGF, the use of heparin stabilizes the molecule (Edelman et al., 1989; Gospodarowicz, 1989), and enzymatic manipulation of the heparin-bFGF bonds enables regulation of the rate of FGF release (Edelman et al., 1989). Approximately 5-10 ng of bFGF (FGF Company, La Jolla) was bound to each HS bead and a single microsphere was implanted dorsal to the lesion site in each animal. The mitogenic activity of over 90% of bFGF released by H-S beads has been demonstrated *in vitro*, although not *in vivo* (Edelman et al., 1989). Based on *in vitro* evidence, it is expected that *in vivo* release will continue for more than two weeks (Edelman et al., 1989).

Methods of Analysis

Axonal Growth

Regenerative growth of HRP-labeled optic tract axons was quantified by superimposing a grid (0.0016 mm^2 at 25x magnification) over the implant in every brain section in which the implant could be seen. This method of restricting growth quantification to labeled axons within the implant ensured that only regenerating neurites were included in the analysis, and eliminated any error due to misinterpretation of retinal axons spared by the transection. In addition, this excluded axons growing on thin membraneous strips that commonly form after neonatal brain injury (Harvey et al., 1986; Dyson et al., 1988). The total number of grid squares in which labeled (i.e., retinal) axons could be identified within or on the implant was counted for every other section in each case. (Each brain was cut in 40 um sections into two series; one series was always reacted with the AHM-TMB method and the other series was reacted either with AHM-TMB or SNF-TMB. For each case, only the series showing the most sensitive reaction was counted.) Totals for all sections were summed, producing a single score for growth into the implant for each case.

Only cases meeting certain minimum criteria were included in these analyses. The criteria are (1) dense

HRP label of the optic axons, (2) complete or nearly complete tract transection, (3) implant located immediately adjacent and normal to the transected axons. The total number of cases meeting these criteria in each condition (and thus the number of cases included in the analyses) is given in Table 2. Mean growth for each condition is given in Table 2 and raw scores are given in Table 3.

Survival of Retinal Ganglion Cells

All of the cell-survival comparisons were done with animals that received tract transection on p6 and were perfused on p14. Eye injections often caused retinal distortion in the corresponding eye, so it was difficult to choose a truely representative segment of the retina from which to make a sample count of the number of surviving retinal ganglion cells (RGCs). Therefore, following the method of Sengelaub et al. (1986), one section from each retina in the plane of the optic disk was selected and two sectors (encompassing 30% of a single retinal section) were chosen for counting RGCs.

All RGCs were counted in one sector from temporal retina and one sector from nasal retina (Figure 10). The sector locations were chosen according to the following criteria (in order of importance): (1) no folds or distortion of the retina within that sector, (2) sector

extended from a point 1/3 the distance between optic disk and and outer edge of retina, to 1/2 that same distance (see Figure 10), and (3) relatively little curvature within the sector. Using the Neurotrace computer-aided microscopy system, an outline of the section was drawn at 10x magnification. The sectors were chosen at 20x and marked by drawing a line at the edges of the microscope field. Finally, using a 100x oil-immersion lens, all RGCs within each sector were digitized and marked with a circle that approximated the size of the cell.

Actual cell counts and estimated totals for each retina are shown in Table 4. Following the method of Frost (1975), the estimated total number of RGCs in each eye was computed as follows:

* Eye diameter, d, was taken from the circle that best approximated the curvature of the embedded retina.

* Retinal arc, a, was measured with a map reading tool from a projected image of the retina at a known magnification.

* Using a formula derived by Frost (1975), the total area of the retina, A, is given by:

$$A \approx \pi/2(ad - 0.57d^2)$$

* Percent of retina represented by each sector, p, is given by:

$$p = \frac{\text{sector arc} * \text{thickness of section after shrinkage}}{A}$$

* Finally, the estimated total, T_e is given by:

$$T_e = \frac{\text{number of cells}}{p}$$

Immmunohistochemical Characterization of Experimental and Control Implants

Brain sections and implants were reacted with antibodies against the intermediate neurofilament proteins GFAP and vimentin, and against the growth-associated molecules laminin and bFGF. In addition, harvested implants were reacted with antibodies against the membrane protein A2B5. Double-labeling with immunofluorescent secondary antibodies was used to co-localize GFAP and FGF, GFAP and laminin, GFAP and vimentin, and GFAP and A2B5. The immunohistochemical procedures are described in greater detail in Appendix A.

Chapter 3

Influence of Implants harvested from Neonatal Neocortex on Regenerative Growth of Retinal Axons

Astrocytes and their derivatives play an important role in guiding neurons and axons in a wide range of instances in the developing CNS (Hatten and Mason, 1986). As radial glia, they serve as neuronal guides during development of the cerebral cortex (Rakic 1971, 1972; Rakic et al., 1974), cerebellar cortex (Caviness and Rakic, 1978; Hatten and Liem, 1981), and hippocampus (Nowakowski and Rakic, 1979). More recently, astrocytes have been shown to act as neuronal guides or scaffolds during development of the barrel fields in mouse (Steindler and Cooper, 1987) and olfactory glomeruli in moth (Tolbert and Oland, 1989). Via their formation of neuroepithelial sheets (i.e., surfaces formed by the collective endfeet of neuroepithelial processes), astrocytic processes also guide, or at least provide appropriate substrates for, the developing optic tract in

a variety of species (Bork et al., 1987; Cohen et al., 1987; Lockerbie, 1987; Singer et al., 1979; Silver, 1984; Silver and Rutishauser, 1984; Silver and Sidman, 1980). What are the mechanisms or molecules by which immature astrocytes or their precursors promote neuritic growth? In vivo work can provide possible candidates by correlating stages of axonal growth with presence of adhesive cell-surface molecules or their receptors. So, for example, because neuroepithelial endfeet transiently express laminin at times corresponding to the initial stages of optic axon outgrowth in chick (Cohen et al., 1986; 1987), rat (McLoon et al., 1988), and mouse (Liesi and Silver, 1989), it has been suggested that neuroepithelial cells enhance or guide retinal axon outgrowth via their production of laminin (Liesi and Silver, 1989; McLoon and McLoon, 1988; see also Letourneau et al., 1988a).

Astrocytes are also preferred substrates for growth of CNS neurons in vitro (Denis-Donini and Estenoz, 1988; Fallon, 1985; Hatten et al., 1988; McCafferey et al., 1984; Noble et al., 1984; Tomaselli et al., 1988; Wigley and Berry, 1988; Wujek and Akeson, 1987), where their growth-promoting effects can be more effectively linked to specific mechanisms or molecules. Many of these studies have suggested that astrocytes promote neuritic outgrowth via their expression of laminin (Leisi et al.,

1983; Wujek and Akeson, 1987), although several report that antibodies against laminin do not block neuritic growth on cultured astrocytes (Tomaselli et al., 1986; Denis-Donini and Estenoz, 1988; see also Ard and Bunge, 1988; David, 1988; Johnson et al., 1988; Wigley and Berry, 1988). Probably the best evidence for what mechanisms might underlie neuritic growth on astrocytes comes from studies involving inhibition of receptors for specific adhesion molecules.

Mechanisms of Neuritic Growth on Astrocytes

CNS neurite outgrowth on astrocytes is not inhibited by antibodies (e.g., JG22 and CSAT) that block integrin receptors for the growth-promoting extracellular matrix proteins laminin and fibronectin (in chick CNS: Cohen et al., 1986; Hall et al., 1987; Letourneau et al., 1988b; Neugebauer et al., 1988; Tomaselli et al., 1986), although it is inhibited following detergent extraction of the astrocytes or in the presence of antibodies against both N-cadherin and integrin receptors (Tomaselli et al., 1986, 1988). This suggests that neurite-astrocyte interactions involve at least two different types of receptors-- N-cadherins, as well as integrin receptors for ECM neurite-promoting molecules (Hatta et al., 1987; Matsunaga et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988). Effectiveness of these

receptors in mediating neuritic outgrowth is age related; integrin receptors appear to lose their effectiveness with increasing neuronal age (Tomaselli et al., 1988; Hall et al., 1987; Cohen et al., 1986, 1987; Schinstine and Cornbrooks, 1988), although N-cadherins remain effective (Neugebauer et al., 1988; Tomaselli et al., 1988). However, integrin responsivity to laminin appears to be regulated by contact with target tissue, and these ECM receptors may be up-regulated by target ablation (Tomaselli and Reichardt, 1988).

There is some debate about what other adhesion molecules or receptors are involved in neurite-glia adhesion, and there is evidence for involvement of J1-tenascin (Steindler and Cooper, 1987; Kruse et al., 1985; Grumet et al., 1985), AMOG (Antonicek et al., 1987), N-CAM (Keilhauer et al., 1985; Noble et al., 1984; Edmondson et al., 1988; Neugebauer et al., 1988), and Ng-CAM (Grumet and Edelman, 1984, 1988). Although N-CAM is involved in axonal fasciculation and neuron-astrocyte adhesion <u>in vivo</u> (Silver et al., 1987; Silver and Rutishauser, 1984; Halfter and Dreiss, 1986; Fraser et al., 1988; Miragall et al., 1988; Thanos et al., 1984), as well as <u>in vitro</u> (Keilhauer et al., 1985; Noble et al., 1985), other evidence indicates that N-CAM is not a necessary component for neuritic adhesion to astrocytes (Neugebauer et al., 1988; Tomaselli et al., 1988). Thus,

it appears that neuritic growth on astrocytes is mediated by at least three different types of receptors via their interactions with several different cell-surface adhesion molecules. More specific identification of receptors and antigens involved in this interaction probably depends upon the neuronal source and age, and the type and age of the astrocyte population.

In addition to promoting growth via their expression of cell surface adhesive proteins, immature astrocytes may promote neuritic outgrowth via their production of soluble growth factors such as bFGF (Ferrara et al., 1988; Finkelstein et al., 1988; Hatten et al., 1988), NGF (Furukawa et al., 1987), or other as-yet unidentified factors (Assouline et al., 1987; Banker, 1980; Muller et al., 1984; Muller and Siefert, 1982; Kesslak et al, 1986; Nieto-Sampedro et al, 1983; Politis and Miller, 1985; Rudge et al., 1985). Effect of bFGF on neuritic growth is discussed in greater detail in Chapter 4.

Influence of Astrocytes on CNS Regeneration

Studies of astrocyte influence on CNS regeneration in vivo actually preceeded much of the in vitro evidence for growth-promoting effects of astrocytes. Much of this work stemmed from studies on the role of glia in the development of the corpus callosum. During normal development of the mouse corpus callosum, callosal axons

cross the cortical midline on a glial bridge that connects the two hemispheres (Silver et al., 1982; Silver and Ogawa, 1983). If this glial bridge is severed prenatally, the callosal axons are unable to cross the midline and instead form large neuromas on both sides of the midline (Silver and Ogawa, 1983). However, if a bridge of nitrocellulose is placed between the two hemispheres during early outgrowth of the callosal axons, the axons can find their way across the midline (Silver and Ogawa, 1983). The callosal axons are preceeded on the nitrocellulose bridges by immature astrocytes that migrate onto the nitrocellulose and thus provide a substrate for the developing callosal axons (Silver and Ogawa, 1983). However, if the nitrocellulose bridge is implanted after p8, astrocytes fail to migrate onto the bridge and axons fail to cross the bare nitrocellulose (Smith et al., 1986). In later studies, it was found that if the nitrocellulose bridges are pre-coated with immature (younger than p8) astrocytes, callosal axons from much older mice (p17 and p34) will leave the neuromas and cross the midline (Smith et al., 1986). These studies indicate that immature, but not older (i.e., 'post-critical') astrocytes are capable of providing a substrate or factors that are conducive to axonal growth (Smith et al., 1986).

Evidence from Liesi and Silver (1989) suggests that astrocytic expression of laminin plays a crucial role in promoting growth of the mouse optic nerve, corpus callosum, and fornix, although, as noted above, there is considerable evidence from in vitro studies that astrocytic promotion of CNS outgrowth can be mediated by cell-surface receptors not involving laminin (Wigley and Berry, 1988; Johnson et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1986; Denis-Donini and Estenoz, 1988; Ard and Bunge, 1988). It has also been suggested that immature astrocytes may promote CNS regeneration by reducing the formation of glial scars in response to injury (Kalderon, 1988b; Rudge et al., 1989; Smith et al., 1986).

Gliosis after CNS Injury

Glial scar formation has long been thought to be a major cause of the lack of neural growth after CNS injury (Reier et al., 1983; Reier, 1986; Stensaas et al., 1979), although this hypothesis appears in conflict with many studies showing that CNS axons of non-mammalian species are able to grow through or around glial scars (Reier, 1979; Scott and Foote, 1981; Stensaas and Feringa, 1977). However, studies of glial scarring in mammalian and non-mammalian species indicate that species and maturational differences in composition and morphology of reactive

astrocytes may determine whether a CNS scar does or does not inhibit neural growth (Wujek and Reier, 1984). Using the freeze-fracture technique to examine intramembranous particles (IMPs) of reactive astrocytes, Wujek and Reier (1984) found that IMPs form dense orthogonal arrays in the plasma membrane of mammalian reactive astrocytes, but not in amphibian astrocytes. In addition, formation of orthogonal arrays occurs to a far lesser extent in immature than in mature astrocytes (Wujek and Reier, 1984; Anders and Brightman, 1979), which may help to explain the differences in glial scarring reported for immature and mature callosal implants (Silver and Ogawa, 1983; Smith et al., 1986).

Astrocytic scarring also affects the extent of CNS neuritic growth into transplants of neuronal tissue (Kruger et al., 1986) and into transplants of non-neuronal tissue (Rudge et al., 1989). A detailed study of CNS scar formation between lesioned adult host tissue and transplanted embryonic donor tissue shows that three distinct types of host-donor scarring or integration can be identified (Kruger et al., 1986). First, in cases where there is no transplanted donor or in regions where the donor tissue does not appose host tissue, a dense scar of astrocytes, macrophages, and fibroblasts forms along the lesioned edge of host tissue (Kruger et al., 1986; Reier et al., 1983). In regions where host and

donor tissue are directly apposed, either a dense astrocytic scar forms between host and donor tissue, or the donor tissue becomes integrated with little or no noticeable scar (Kruger et al., 1986; Houle and Reier, 1988). Similar variations in host-transplant integration have been reported for non-neuronal (i.e., astrocytic) implants (Rudge et al., 1989).

There are several explanations for why donor tissue is integrated in some regions or separated by a glial scar in others, often even within the same animal. One possibility is that fetal tissues (or immature astrocytes) that are closely apposed to the host prevent invasion of fibroblasts into the lesion site (Kruger et al., 1986; Rudge et al., 1989), second, that immature donor tissue releases inhibitory factors (e.g., plasminogen activators) that interfere with host scar formation (Kalderon, 1988b; Kruger et al., 1986), or third, that the morphology of astrocytic processes aid in integration of host and donor tissue (Gage et al., 1988; Rudge et al., 1989). Whatever the underlying cause of host-implant integration, this tight fusion of host and donor neuropil appears to be a primary necessity in order for host axons to grow into donor implants (Houle and Reier, 1988).

Oligodendrocyte proliferation at the site of the lesion is likely to inhibit neural growth not only by forming a mechanical barrier, but also via oligodendrocyte expression of specific proteins that are inhibitory to adhesion and neurite extension (Schwab and Theonen, 1985; Caroni and Schwab, 1988a,b). Two proteins, of 35 and 250 kDa, have been identified as the primary inhibitors of neuritic growth on cultures of adult oligodendrocytes (Caroni and Schwab, 1988a,b). Expression of these proteins is related to the age of the oligodendrocytes; immature oligodendrocytes, like immature astrocytes, (from 7 to 12-day old rats) are not inhibitory for neuritic growth (Caroni et al, 1988).

The experiments reported in this chapter examined the effects of transplanted astrocytes on transected retinal axons of neonatal hamsters. If immature astrocytes are capable of creating a substrate that is conducive (or inductive) for CNS neuritic outgrowth, then a 'bridge' of immature astrocytes linking the proximal ends of transected axons to their tectal target, might permit regenerative growth and target reinnervation. In order to determine what properties of the astrocyte-covered bridges were involved in supporting or promoting growth of retinal axons, several different types of harvested bridges were implanted. Axonal outgrowth into

the implant was assessed by labeling the regenerating axons with HRP; harvested implants were characterized by immunohistochemical staining against various cell specific antigens and growth-promoting molecules.

Results

Regenerative Growth

Table 2 shows the number of successful cases in each condition and the mean extent of growth into the implant. [These n's represent perhaps 1/3 of all cases operated, since many cases did not meet the criteria outlined in Chapter 2.] For host animals receiving optic tract transection on p6, gelfoam implants harvested from neonatal neocortex significantly enhanced extent of neural growth into the implant when compared to implants soaked in buffer [$t(7)=2.676$, $p=.032$, collapsing over donor age at harvest]. In addition, harvested C-GAG implants that were transplanted directly into the host were possibly (see Table 2), although not significantly, more effective in promoting retinal outgrowth than harvested C-GAG implants that were soaked in buffer or oxygenated tissue slice medium prior to transplantation [$t(19)=1.946$, $p=.067$]. No differences were seen in extent of growth into gelfoam implants harvested on p3 vs. p13 [$t(4)=1.912$, $p=.129$], or into gelfoam implants

harvested from donor neocortex vs. non-neural tissue [t(9)=0.512, p=.616, collapsing over age of harvest]. Repeated freezing and thawing of harvested implants prior to transplantation did not reduce the ability of implants to support growth; growth into these implants was significantly greater than growth into saline-soaked implants [t(4)=3.123, p=.035]. Type of implant material (gelfoam vs. C-GAG) had no effect on extent of axonal growth [t(19)=0.333, p=.743, collapsing over donor age at harvest (see Figure 11)].

For host animals receiving optic tract transection on p9 or later, there was little or no growth into implants harvested on p3. Statistical comparisons were not done at these ages because of the small number of cases in each condition, although the means are given in Table 2.

Target Reinnervation

Reinnervation of the superior colliculus was observed in 6 cases receiving tract transection on p6, but only in those surviving for at least 2 weeks after surgery. In all cases, the patch of reinnervation was small and was located in rostrolateral SC, nearest the implant.

Thus, retinal axons transected on postnatal day 6 show enhanced outgrowth onto C-GAG and gelfoam implants harvested from neonatal neocortex and non-neural tissue

(Figure 2a-d), but only if these implants are transplanted directly, without an intervening rinse. Harvested implants that were repeatedly frozen and thawed prior to transplantation also enhanced retinal axon outgrowth. The fact that rinsing, but not freezing, harvested implants prior to transplantation causes a loss of growth-promoting effect has several possible explanations. First, neurotrophic factors from donor neocortex may be rinsed away or inactivated. Assays of saline in which harvested astrocytes were rinsed for 4 hours, showed that the rinse fluid contained high levels of protein and was mitotic for Balb C 3T3 cells. Thus, some unidentified mitogenic proteins <u>are</u> being lost from the implant during the rinse procedure. Another possible explanation is that rinsing kills a significant proportion of the donor cells. Loss of growth-promoting effect occured when harvested implants were rinsed for approximately 30 minutes in unoxygenated sterile saline (at room temperature) or when harvested implants were placed in an oxygenated tissue slice medium (pH=7.3 for 1 to 4 hours). Labeling of donor animals with ^{3}H-thymidine prior to harvest and transplantation of the implant, showed that donor cells do survive <u>direct</u> transplantation (Figure 5a,b). However, the fact that substantial regenerative growth did occur in the presence of implants repeatedly frozed and thawed (but not rinsed) prior to

transplantation, suggests that cell survival is not necessary in order for harvested implants to support regenerating neurites. All of this evidence seems to indicate that the positive effect of harvested implants on regenerative growth is mediated by a soluble factor produced by donor neocortex or by transplanted astrocytes.

Retinal Ganglion Cell Survival

Comparison of estimated total number of RGCs in cases receiving PBS/gelfoam with those receiving harvested gelfoam implants showed no effect of harvested implant on RGC survival [$t(4)=0.334$, $p=.755$]. Comparison of estimated total number of RGCs for eyes contralateral and ipsilateral to the transected optic tract showed no difference in number of surviving cells. Because others have observed loss of 70-90% of RGCs after ablation of the SC at this age (Perry and Cowey, 1982), it is possible that the intact SC provides trophic factors that sustain RGCs after tract transection. This possibility was examined by comparing RGC survival in animals undergoing ablation of the right superior colliculus (SCabl) in addition to the brachial transection. Estimated total number of RGCs was not reduced in SCabl animals in comparison to animals receiving tract

transection alone [t(4)=1.305, p=.262], but, unlike animals not receiving tectal ablation, was significantly reduced in comparison to normals [t(4)=3.281, p=.030]. Mean estimated total number of cells is shown in Figure 14 and Table 4.

Size of Surviving Retinal Ganglion Cells

A histogram showing the distribution of sizes of surviving retinal ganglion cells is given in Figure 15, and the mean size for each animal is given in Table 3. Only cases receiving SC ablation in addition to the tract transection had a significantly smaller mean RGC size [t(4)=6.155, p=.004].

Immunohistochemistry

Antibody specificity was confirmed by comparing patterns of antibody staining in host tissue with known staining patterns. Staining with antibodies to vimentin (Figure 13c) was specific to radial glia and to ependyma surrounding the midbrain and lining the ventricles. Staining with anti-GFAP (Figure 13d) mostly overlapped with much of the anti-vimenting staining. Anti-laminin stained blood vessels in host tissue (Figure 6e) and discrete, punctate staining could be seen in host tissue at the lesion site (Figure 6d) and within harvested implants (Figure 6a-c). Anti-bFGF stained cell bodies in host tissue surrounding the lesion site (Figure 7a,b),

within harvested implants (Figure 7c,d) and in ependymal cells lining the third ventricle (Figure 7e), but nowhere else.

Results of immunohistochemical staining of harvested implants are summarized in Table 1.

Implants harvested p3--first implanted on p0 or p1

These implants contained cells that stained positively with antibodies against GFAP and vimentin. Nearly all $GFAP^+$ cells or processes were also $vimentin^+$ (Figure 5c-e), although a few $vimentin^+/GFAP^-$ cells were seen. Only a single $GFAP^+/A2B5^+$ cell was seen, although very light punctate A2B5 staining was observed in a few other $GFAP^+$ cells. Thus, the vast majority of cells in the harvested implants appeared to be $GFAP^+/A2B5^-$, type 1 astrocytes (Miller and Raff, 1984). No $GFAP^-/A2B5^+$ cells (i.e., neurons) were seen in the implants.

Many cells in implants harvested on p3 and in the host tissue surrounding the implant were $GFAP^+/laminin^+$. Anti-laminin staining was mostly punctate (Figure 6a-d), and was not wide-spread. Antibodies against bFGF stained cells in the implant and in host tissue immediately adjacent to the implant (Figure 7a-d). $bFGF^+$ cells tended to form small clusters in host tissue, but appeared clustered or singly within the implant (Figure 7). [Most of the FGF^+ cells were associated with $GFAP^+$

cells and processes, although the reverse was not necessarily true.]

Implants harvested on p13--first implanted on p10 or p11

These implants contained cells that stained positively with antibodies against GFAP and vimentin. Just as for implants harvested on p3, the vast majority of $GFAP^+$ cells were also $vimentin^+$. No $GFAP^+/A2B5^+$ cells were seen in implants harvested on p13.

Many cells in implants harvested on p13 and in the host tissue surrounding the implant were $GFAP^+/laminin^+$. Anti-laminin staining was punctate and was closely associated with regions of $GFAP^+$ processes. Anti-bFGF staining was diffuse (i.e., cytoplasmic) rather than punctate and was always closely associated with regions of $GFAP^+$ cells and processes. No $FGF^+/GFAP^-$ regions were observed. Thus, for the antigens examined here, there were no observable differences between implants harvested on p3 and those harvested on p13.

Implants harvested from non-neural tissue

A scalpel blade was used to make an incision in the meninges and upper layers of the occipital muscle in p6 hamsters, and gelfoam and C-GAG implants were placed in this incision. These non-neural implants were harvested after 3 days (on p9) and transplanted into the host midbrain in the gap left by the transected brachium.

These implants contained no GFAP$^+$ cells or processes, although they stained quite heavily with antibodies against vimentin.

Implants harvested on p13 and rinsed prior to fixation

These implants contained some GFAP$^+$ cells, although there appeared to be fewer GFAP$^+$ cells in rinsed than non-rinsed implants. However, the most noticeable difference was in the number and length of GFAP$^+$ processes; cells in rinsed implants generally had fewer and shorter processes.

^{3}H-thymidine labeling of harvested implants

Donor animals were injected with ^{3}H-thymidine 1 hour and 18 hours after gelfoam was implanted, and implants were harvested for transplantation 30 hours after the second injection. Thus, labeled cells must have undergone DNA synthesis within 48 hours after the cortical lesion, and any postmitotic cells that migrated into the implant remained unlabeled. This limited labeling of donor cells most likely leaves the majority of donor cells unlabeled, and can be used only to confirm the presence and survival of donor cells that divide in response to cortical injury and subsequently migrated into the polymer implant. Therefore, the scarcity of ^{3}H-labeled cells in host tissue does not necesssarily reflect the size of the

donor cell population. Figure 5 (a,b) shows several heavily labeled cells, located within the transplanted polymer in the host midbrain. No labeled cells were seen beyond the borders of the transplanted polymer. An average of 18.2 cells were observed in the five animals that received ^{3}H-thymidine transplants (range = 4 to 56). Because only 1/3 of all brain sections were processed for autoradiography, it is estimated that, on average, each transplant contained 49 labeled cells.

Gliosis and Axonal growth

Intense glial proliferation around the edges of the lesion site was visualized with immunofluorescent staining for anti-GFAP (Figure 8c-e) and histochemical staining with Cajal's gold chloride method (Figure 8a,b). Both of these methods are specific for astrocytes. In regions where the implant was closely apposed to host tissue, astrocytes often appeared to extend between host tissue and implant (Figure 8b,d). But in other regions, especially at those sites where the implant was displaced away from the lesion edge, a dense glial accumulation formed a distinct border at the edge of host tissue (Figure 8c,e and Figure 13c,d). Thus, astrocytic process extension into the implant appears to integrate the substrates, while astrocytic process accumulation along the host/implant border that merely parallels, but not

joins, the two substrates, creates a barrier that effectively segregates host from implant. Formation of the dense glial border or of the more integrated host-implant astrocyte reaction appeared to be dependent on several factors. An integrated morphology was seen only in cases in which the implant was closely apposed to the lesioned edge of host tissue. The extent of integration was also greatly influenced by the type of implant material used (probably even more than the medium in which the implant was conditioned); because gelfoam swelled more in vivo than the C-GAG polymer, regions of host-implant apposition, and therefore integration, were far more common in cases receiving gelfoam implants. Indeed, a far greater number of CG implants than gelfoam implants were completely displaced from the midbrain or the lesion site. Thus, these mechanical effects may be responsible for the observed differences in extent of axonal growth on gelfoam or C-GAG implants (see Table 1 and Chapter 4). Finally, the age of the host animal also affected the glial response; dense gliosis along the lesion edge occured far more often in older than younger hosts.

Regenerating optic tract axons entered the implant only in regions of host-implant integration, and failed to cross dense astrocytic accumulations (Figure 2e). However, the prefered site for axonal growth appeared to

be along glial 'bridges' that commonly formed along the dorsal surface of the implant (Figure 9e). Such bridges may be similar, either in formation or in cellular content, to glial bridges that form in cases receiving midbrain suction lesions without implants (Dyson et al., 1988). These bridges stained very heavily with antibodies against GFAP (Figure 9a-c) and vimentin (not shown). Although they appeared to consist mainly of $GFAP^+$ processes, occasional $GFAP^+$ cells were also visible within the bridges (Figure 9b). The glial bridges often contained $bFGF^+$ and/or $laminin^+$ cells, although these antigens were not consistently present on glial bridges. Due to methodological incompatibilities, only a few cases were reacted for both HRP visualization and immunohistochemistry (Figure 9). Thus, any relation between growth on tissue bridges and presence of these growth-promoting antigens cannot be precisely determined, and is based on observations from separate cases. In other words, labeled axons are commonly seen growing on glial bridges in sections reacted for visualization of HRP, and $laminin^+$ and $bFGF^+$ staining are often seen in glial bridges in sections processed for immunofluorescence, but the direct correspondence between regenerating axons and presence of these growth-promoting antigens can not be determined.

Discussion

In summary, axonal growth after optic tract transection is enhanced in the presence of implants harvested from neural or non-neural tissue (even if they are frozen prior to transplantation), but rinsing the harvested implants prior to transplantation causes a loss of the growth-promoting effect. No differences in growth-promoting effect were detected for implants harvested from neocortex on p3, neocortex on p13, or muscle/meninges on p9. Results of immunohistochemical staining of these harvested implants for anti-GFAP, anti-vimentin, anti-laminin, anti-bFGF, and anti-A2B5 are summarized in Table 1. With the exception of GFAP, which was not present in the implants harvested from muscle/meninges, no dramatic differences were detected in the presence of these antigens in these three implants. The fact that the growth-promoting effect was lost when implants were rinsed prior to transplantation suggests that either the astrocytes were killed by this procedure, or that the effective growth-promoting factor(s) was denatured or washed away. Assays of saline in which harvested implants had been rinsed confirmed that protein and mitotic agents were present in the rinse saline. In addition, the fact that implants frozen prior to transplantation supported growth, provides further

support for the conclusion that the effective agent is a soluble factor.

There are several possibilities for what properties of the implants might promote retinal axon outgrowth: (i) the presence of laminin in transplanted astrocytes might stimulate growth by enhancing substrate adhesivity, (ii) growth factors released by transplanted astrocytes may directly stimulate retinal axon growth, or might indirectly enhance outgrowth by inducing astrocytic proliferation, or (iii) astrocytes may enhance growth indirectly by increasing integration of the implant with host tissue. The first possibility, that laminin is the crucial element, seems unlikely for several reasons. First, laminin$^+$ cells were present throughout the implant, but axonal growth was relatively restricted to strips of GFAP$^+$ tissue and to the dorsal surface of the implant (Figure 2c,d); axonal growth into the interior of the implant was observed (Figure 2a,b), although this was relatively rare. Laminin$^+$ cells were often present on the dorsal surface of the implant, as well as on strips of GFAP$^+$ tissue, so while laminin may be involved in promoting neuritic outgrowth, it was probably not the primary determinant for neuritic growth.

Although <u>in vivo</u> evidence indicates that developing rodent and chick retinal axons may be guided by laminin

(McLoon et al, 1988; Cohen et al, 1986; Liesi and Silver, 1989), in vitro studies of neuritic response to laminin in chick RGCs and ciliary ganglion neurons show that integrin receptors for laminin lose their affinity with increasing neonatal age (Cohen et al., 1986; Hall et al., 1987; Neugebauer et al., 1988; Tomaselli et al., 1986), probably due to age-related receptor modifications (Hall et al., 1987). However, other in vitro evidence indicates that astrocytic substrates can promote CNS neuritic growth by receptor mechanisms other than laminin-integrins (David, 1988; Neugebauer et al., 1988). Thus, laminin does not necessarily need to be expressed by astrocytes to achieve the neurite-promoting effects. Neurite-astrocyte interactions can be mediated by N-cadherins, which appear not to be down-regulated with increasing age in chick ciliary ganglia (Tomaselli et al., 1986, 1988; Hatta et al., 1987), as well as by NCAM (Neugebauer et al., 1988).

Growth factors released by transplanted astrocytes may enhance neuritic outgrowth either by directly stimulating neurons or by inducing astrocytic proliferation, which then increases the total surface area of growth-promoting substrate. The most likely candidates are NGF or FGF. Fibroblast growth factor, like NGF (Furukawa et al., 1987), is expressed in immature and reactive astrocytes (Ferrara et al., 1988;

Finkelstein et al., 1989; Nieto-Sampedro et al., 1988), and which is a potent mitogen for astrocytes and other cells (Eccleston et al., 1985; Gavaret et al., 1989; Giulian et al., 1986; Gospodarowicz et al., 1987; Pruss et al., 1982). Possible effects of bFGF are discussed further in Chapter 4, where the effects of bFGF-conditioned implants on retinal axon outgrowth are examined more directly.

The decrease in growth-promoting effect after rinsing of the harvested implant may result from denaturing of astrocytic factors, or astrocytic death or process retraction. However, the hypothesis that astrocytes are the effective agent in promoting outgrowth fails to explain the growth-enhancing effects of $GFAP^-$ implants harvested from meninges/muscle tissue (Table 2 and Figure 11). One possibility is that cells harvested from meninges/muscle secrete mitotic factors that induce astrocytic proliferation, thus causing formation of an astrocytic substrate. FGF and other astrocytic mitogens (e.g., EGF--Morrison et al, 1987) are secreted by many neural and non-neural tissues (reviewed in Gospodarowicz, 1989), but the implants harvested from muscle/meninges have not been characterized for cellular or molecular content (Table 1).

Another possible growth-promoting effect of the harvested implants stems from the influence of transplanted astrocytes on scar formation and host-implant integration. Silver and co-workers, as well as others, report that transplants of immature containing mostly astrocytes reduce scar formation in host tissue surrounding the implant (Kalderon, 1988a,b; Kliot et al., 1988; Rudge et al., 1989; Smith et al., 1986), and that extent of scar formation is related to age of astrocytes: transplants of immature astrocytes are more effective than older astrocytes in reducing scarring. Although in the experiments reported here, no differences in growth-promoting effects were observed for implants harvested on p3 or p13, host age did appear to influence extent of host-implant integration, which may be influenced or determined by astrocytic response. Good host-implant integration can be characterized by a lack of dense gliosis at the host/implant border; astrocytes that are present extend processes <u>into</u> the implant--perpendicular to the border, rather than along the border. This integrative type of astrocytic response occurs far more often in younger hosts, and in implants that have not been rinsed prior to transplantation. Thus, immature astrocytes appear to enhance outgrowth onto implants by two primary means: first, they increase host-implant integration and reduce glial barrier formation by

extending processes that link, rather than segregate, the two substrates (Figure 8b,d), thus effectively knitting host and implant together. Second, they increase growth into the implant by providing a cell-surface substrate that is suitable for neuritic growth, or by secreting mitotic factors that induce further astrocytic proliferation and substrate formation.

Chapter 4

Influence of Fibroblast Growth Factor on Regenerative Growth of Optic Tract Axons

Growth factors play an important role in promoting survival and directing outgrowth of developing CNS neurons (Berg, 1984; Dodd and Jessell, 1988; Theonen and Edgar, 1985). The prototype neuronotrophic factor, NGF, has both trophic (Levi-Montalcini, 1982; Kromer, 1987) and tropic (Gunderson and Barrett, 1980; Letourneau, 1978) effects on sympathetic and sensory neurons, as well as on retinal ganglion cells (Carmignoto et al., 1989; Maffei et al., 1989). The discovery of NGF's potent neurotrophic and neurotropic effects prompted a search for other soluble factors that might be similarly effective in other CNS cell populations. One of the more recently discovered factors is a potent mitogenic protein, fibroblast growth factor (FGF), that exists in two closely-related forms; basic FGF is a 146 amino acid polypeptide and acidic FGF is a 140 amino acid polypeptide. These two forms of FGF have very different

distributions in neural and non-neural tissues, and may have different target populations (Gospodarowicz et al., 1987) (although, because they bind the same receptors, this is not entirely certain--see Neufeld and Gospodarowicz, 1986).

Although FGF was initially identified because of its potent effects on endothelial cell proliferation (Gospodarowicz, 1974), it has since been discovered that FGF, and in particular bFGF, is effective in promoting proliferation, survival, and outgrowth of certain neuronal populations. The survival- or outgrowth-promoting effects of bFGF have been shown in vitro for neurons from mammalian cerebellum (Morrison et al., 1988), cerebral cortex (Morrison et al., 1986), hippocampus (Walicke et al., 1986), mesencephalon (Ferrari et al., 1989), PC12 cells (Rydel and Greene, 1987; Claude et al., 1988), retinal ganglion cells (Bahr et al., 1989; Lipton et al., 1988), as well as other CNS cell populations (Morrison et al., 1987; Walicke, 1988a). In addition, bFGF supports survival of injured septal and retinal neurons in rat in vivo (Anderson et al., 1988; Sievers et al., 1987). The effects of aFGF are similar to those of bFGF for some neuronal populations (Rydel and Greene, 1987; Walicke, 1988a), although they have differing effects in other neuronal populations (Lipton et al., 1988; Walicke, 1988a), which may be related to

differences in receptor affinities, rather than to differences in the type of receptor for the two proteins (Neufeld and Gospodarowicz, 1986).

There has been some debate over whether bFGF directly influences neuronal survival and/or outgrowth, or whether its effects are mediated indirectly, via non-neuronal cells such as astrocytes. The effects of astrocytes or factors secreted by astrocytes on neuronal proliferation, survival, and outgrowth are well-documented; this evidence was reviewed in Chapter 3. The fact that bFGF is a potent mitogen for type 1 astrocytes (Eccleston et al., 1985; Ferrara et al., 1988; Giulian et al., 1986, 1988; Gavaret et al., 1989; Miller et al., 1989; Morrison and DeVellis, 1981; Pettmann et al., 1982; Pruss et al., 1982) suggests the possibility that bFGF causes glial proliferation, and that these glia in turn release some other factor (which, in an interesting recursive twist, may even be bFGF --Hatten et al., 1988) that acts directly to promote neuronal survival or outgrowth. Convincing evidence against the possibility that bFGF has only indirect (i.e., glial-mediated) effects on neuronal survival <u>in vitro</u> has been presented (Walicke and Baird, 1988), although it is still possible that bFGF effects are mediated by <u>both</u> direct and indirect mechanisms.

The role of bFGF in development is not certain, although its presence in various neural and non-neural tissues during early stages of development suggests that it is primarily involved in regulation of embryonic cell proliferation (in chick limb buds: Munaim et al., 1988; in various neural and non-neural mouse tissues: Wilkinson et al., 1989; in Xenopus embryonic tissues: Slack and Isaacs, 1989), and in angiogenesis (Baird et al., 1985; Thompson et al., 1988). Similarly, the primary functions of bFGF in CNS are not known, although it is present in many CNS regions (see Gospodarowicz, 1989 for review) and it binds to both neurons and astrocytes (Walicke and Baird, 1988). Basic FGF is expressed in astrocytes (Ferrara et al., 1988; Hatten et al, 1988), and there is also evidence that it is expressed in CNS neurons (Janet et al., 1988; Pettmann et al., 1986). The mechanisms for its release are not known (Ferrara et al., 1988; Gospodarowicz, 1989), although several studies indicate that astrocytes release bFGF in response to tissue injury (Finkelstein et al., 1988; Nieto-Sampedro et al., 1988; Rogister et al., 1988).

The purpose of this series of experiments was to determine whether implants conditioned in bFGF could enhance the survival and/or outgrowth of transected axons of the optic tract <u>in vivo</u>. This is of interest not only for what it might show about the effect of bFGF on

injured retinal ganglion cells *in vivo*, but it might also help in determining what factors were responsible for the growth-enhancing effects seen in the previous experiments in which harvested astrocytes were transplanted into the transected optic tract.

Results

The same method for quantifying growth was used as that described in Chapter 2; means for axonal outgrowth onto the implant are given in Table 2 and Figure 12. In comparison to cases receiving gelfoam implants soaked in buffer, FGF-conditioned implants enhance the extent of regenerative growth into the implants (Figures 3 and 12). This effect was significant for transection at postnatal day 6 [$t(10)=3.148$, $p=.010$], p10 [$t(6)=3.11$, $p=.021$], p18 [$t(6)=2.901$, $p=.027$], and p25 [$t(7)=2.673$, $p=.032$]. Unfortunately, no older animals were tested in this condition. Animals receiving bFGF bound to HS beads on p10 had greater mean growth into gelfoam implants in comparison to those receiving PBS/gelfoam (Table 2), although this was not significant [$t(5)=2.31$, $p=.069$]. Seven control animals receiving HS beads with no bFGF all died following surgery. Comparison of growth into FGF/gelfoam or FGF/C-GAG implants for animals operated on p6 showed an apparent effect of type of implant: mean

growth onto FGF/gelfoam implants was enhanced [although not significantly, $\underline{t}(12)=2.916$, $p=0.130$] relative to that on FGF/C-GAG implants]. This effect of implant was not observed in any other conditions (see Chapter 3 results), and may result from a 'floor effect'--i.e., with most other conditions growth was not extensive enough to permit detection of small growth differences due to implant material. Possible explanations for this difference in extent of growth onto the two implants are discussed below. Comparison of retinal axon growth into FGF/gelfoam implants with growth into harvested gelfoam for animals transected on p6 showed a significant enhancement of growth in the presence of bFGF [collapsing over the two donor ages, $\underline{t}(14)=3.211$, $p=0.006$]. Although the means (see Table 2) hint that a similar effect might exist at p10, the use of different types of implant at these two ages confound the interpretation. [I.e., A comparison of bFGF/gelfoam and harvested/cg implants in p10 hosts contains two independent variables.]

Approximate number of axons regenerating into PBS and FGF conditioned implants was calculated by measuring the diameter of the regenerating bundle, and dividing this by the approximate axonal diameter. Because of differences in extent of myelination and axonal fasciculation, it is impossible to make accurate comparisons between different ages. However, if we assume

that regenerating retinofugal axons have a diameter of 0.4 um, and are maximally packed, then the number of regenerating axons ranges up to 5,000 axons.

While most regenerating axons grew along the dorsal surface of the implant (Figure 3), there were also small fasciculated bundles of labeled axons that extended into the implant (Figure 4, a-d). These axons often appeared to follow the contours of the porous implants. Reinnervation of the superior colliculus occured in several cases that were transected on p6, but only one case transected on p10, and in none of the cases transected at older ages. In each of these, there was a small patch of HRP indicating reinnervation only in the region of tectum closest to the implant. Thus, those regenerating axons that reentered host tissue did not extend very far within host neuropil, and arbors remained fairly small. This apparent refractory property of host superior colliculus was clearly age related: although large numbers of regenerating retinal axons grew into FGF-conditioned implants after p18 and p25 transection (Figure 3b,d,f), no target reinnervation occured. Lack of neuritic arborization in mature CNS tissue is an increasingly well-documented problem in studies of CNS regeneration (Vidal-Sanz et al., 1987; Crutcher, 1989; Harvey et al., 1986; Caroni and Schwab, 1988b; reviewed in Reier et al., 1988). These current results indicate

that the apparent growth-inhibiting properties of maturing hamster superior colliculus become effective at postnatal days 12 to 16. This time course roughly corresponds to the myelination of hamster optic tract; Gallyas staining shows myelination of the brachium of superior colliculus beginning at about p14 (L. Rava, unpublished data).

The majority of the cases reported here were examined 8-10 days after tract transection, but three cases that received bFGF/gelfoam implants on p10 survived for 10 weeks after surgery. Because such extensive retinal outgrowth occured within 8 days after transection in such cases (Figure 3 a,c,e), I wanted to determine whether there was enough of a retinotectal projection to warrant behavioral testing. None of these cases showed any innervation of the superior colliculus, and there were very few or no labeled axons in the gelfoam implant (which appeared to have partly disintegrated, but was still identifiable--Figure 13a,b). In addition, the implant appeared to be less well integrated with host tissue (as compared to similar cases with short survival), and a dense glial accumulation had formed along the lesioned edge of host tissue.

Retinal Ganglion Cell Survival

Comparison of retinal ganglion cell survival (in the left eye, contralateral to the transected tract) in animals receiving tract transection on p6 showed no difference between FGF/gelfoam and PBS/gelfoam implants [$t(4)=1.006$, $p=.371$], just as no difference was found for PBS/gelfoam and harvested gelfoam implants (see Chapter 3). In addition, counts of RGCs in the right (ipsilateral) eye of animals receiving optic tract transection revealed no significant difference in RGC survival between eyes contralateral and ipsilateral to the lesion. However, there was significant RGC death in cases receiving ablation of the superior colliculus in addition to the brachial transection in comparison to normals [$t(4)=3.281$, $p=.030$], but not in comparison to cases receiving tract transection alone [$t(4)=1.305$, $p=.262$]. The mean estimated number of retinal ganglion cells for these conditions are given in Table 3 and Figure 14.

Size of Surviving Retinal Ganglion Cells

A histogram showing the distribution of sizes of surviving retinal ganglion cells is given in Figure 15. Mean size of RGCs in animals with tract transection alone (with any type of implant), was significantly different

from normals [t(4)=4.551, p=.010], and animals receiving tectal ablation in addition to tract transection were also different from normals [t(4)=6.155, p=.004]. However, animals undergoing tectal ablation in addition to the tract transection did not show a significant reduction in mean cell size in comparison to animals receiving only tract transection [t(4)=1.589, p=.187]. Mean RGC sizes are given in Table 3 and Figure 15.

Discussion

There are several possibilities for what mechanisms might underlie the enhanced outgrowth of transected optic tract axons in the presence of implants conditioned in bFGF. First, bFGF may directly stimulate axonal and/or dendritic outgrowth. Second, bFGF may enhance outgrowth indirectly, via its mitotic effects on host cells, and particularly astrocytes. The first possibility, that bFGF acts directly upon retinal ganglion cells to enhance outgrowth, requires that bFGF be taken up at the transected ends of the retinal axons, where it either acts locally or is transported retrogradely to the soma. Little is known about the mechanisms of bFGF activity or about the neuronal localization of receptors, but there is a great deal of evidence showing that other trophic factors are taken up at nerve terminals and at the

proximal end of severed axons (Dahm and Landmesser, 1988; Korsching and Theonen, 1983), and are transported to the soma where they regulate gene transcription.

Stimulation of neuronal outgrowth by bFGF has been shown for a variety of mammalian CNS neurons, both in vivo (Anderson et al., 1988; Sievers et al., 1987) and in vitro (Morrison et al, 1986, 1987, 1988; Walicke et al., 1986; Bahr et al, 1989; Ferrari et al., 1989; Lipton et al., 1988), although the mechanisms of its effects are not known (Gospodarowicz, 1989). However, one additional possibility is that bFGF affects neuronal outgrowth into gelfoam and C-GAG implants indirectly, via its mitotic effects on host astrocytes (Ferrara et al., 1988; Giulian et al., 1986, 1988; Gavaret et al., 1989; Pruss et al., 1982). Although mature astrocytes can inhibit regenerative growth by forming a dense scar at the lesioned edge of host tissue (Kalderon, 1988a; Miller et al., 1986), paradoxically, immature astrocytes can also aid in promoting regenerative growth either by directly stimulating neuritic growth (Gage et al., 1988; Hatten et al, 1988; Liesi and Silver, 1989; Lindsay, 1979), by inhibiting formation of the dense glial-fibroblastic scar (Rudge et al., 1989; Kliot et al, 1988; Kalderon 1988b), or by increasing the astrocytic surface area available as a suitable substrate (Denis-Donini and Estenoz, 1988;

Fallon, 1985; McCaffery et al., 1984; Noble et al., 1984; Silver and Ogawa, 1983; Wigley and Berry, 1988).

Axons growing into the gelfoam implants tend to grow mostly, although not exclusively, on the dorsal surface of the gelfoam implants (Figure 3a-f, Figure 4a-d), rather than uniformly invading the implant. This may result because the dorsal surface provides the most direct, unimpeded route to their tectal target, which exerts a neurotropic influence on growing retinotectal axons in rodent (Lund et al., 1988; Smalheiser et al., 1981a,b). Alternatively, host astrocytes proliferating in response to presence of bFGF are likely to migrate along the exposed borders of the implant, thus creating a substratum of astrocytes along the dorsal-most face of the implant. Silver and Ogawa (1983) reported a similar phenomenon when they implanted bare, non-harvested nitrocellulose filters between the hemispheres of acallosal mice; for mice younger than p8, host astrocytes migrated onto the filter and created an astrocytic substrate that supported outgrowth of callosal axons. However, if non-harvested filters were implanted in older animals, no such astrocytic migration occured, and callosal axons did not grow onto the bare filters (Smith et al., 1986). In many control cases receiving buffer-soaked gelfoam implants on p6, p10, p18, and p25, a small amount of axonal growth into the implant was observed.

Thus, it is possible that bFGF-conditioned implants enhance growth of retinofugal axons by causing astrocytic proliferation, which results in formation of an astrocytic substratum across the dorsal face of the implant.

Examination of anti-GFAP staining in animals receiving FGF-conditioned implants at 2, 4, 6, and 8 days after transection showed a progressive invasion of the implant by $GFAP^+$ astrocytes. Although many of these astrocytes appeared to be migrating from host neuropil, an even greater number appeared to migrate from host glial limiting membrane at the lesion site. These current studies did not include examination of astrocytic proliferation and migration for hosts of different ages, but others have reported a reduction in rate and extent of astrocytic migration at older ages (Rudge et al., 1989; Silver and Ogawa, 1983). Studies are currently underway to compare astrocytic proliferation and migration in the presence or absence of bFGF.

Astrocytic proliferation may also aid in inhibition of glial scar formation by enhancing integration of the implant with host tissue. In regions of close integration, host and/or donor astrocytes appeared to extend processes between implant and host tissue (Figure 8b,d), in effect knitting these two substrates together.

Integration of implant with the lesioned edge of host tissue was a significant determiner of whether retinal axons grew into the implant (see also Kruger et al, 1986; Tessler et al., 1988; Rudge et al., 1988). Thus, part of the apparent growth-promoting effect may result from bFGF-induced astrocytic proliferation, which then aids in host-implant integration.

Recent studies by Harvey et al. (1986) and Dyson et al. (1988) show that neonatal optic tract transection in the brachial/pretectal region of rats, at ages comparable to those used here, results in formation of bridges of glial and connective tissue at the lesion site. These bridges are composed mainly of astrocytes, but also include fibroblasts, macrophages, endothelial cells, pericytes, and collagen (Dyson et al., 1988), and they are favored substrates for regenerating retinal axons. Such bridges, as well as considerable axon growth onto them, were also observed in the cases reported here (although these were not included in any analyses). Formation of such bridges in response to suction lesions may be a unique property of astrocytes in the pretectal/brachial region, since they have not been reported after lesion of other heavily myelinated regions such as the spinal cord and optic nerve.

It is possible that growth into PBS or FGF conditioned implants is related to formation of these bridges; if formation of astrocytic bridges is due to an exceptional migratory or protein synthesizing capacity of pretectal astrocytes, then that same exceptional astrocytic behavior might also contribute to retinal axon growth into gelfoam implants. Thus, the enhanced regenerative growth observed in this model may be quite specific to this region, and the effect of astrocyte-covered of FGF-conditioned implants on promoting growth of injured CNS axons may be different for lesion of other CNS tracts.

One unexpected finding was the difference in extent of neurite outgrowth on bFGF/gelfoam and bFGF/C-GAG. [Differences in axonal growth on gelfoam or C-GAG implants were not noted until they were used with bFGF; the lack of obvious differences for other conditions (e.g., when implants contained harvested astrocytes--see Chapter 3) may reflect a 'floor effect': extent of outgrowth was relatively small, so differences in extent were not apparent.] There are several possible explanations for this effect; first, that bFGF binds less readily to the C-GAG polymer so less bFGF is actually delivered to the site of injury; second, that neurites grow less readily on collagen substrates; and third, that structural differences between gelfoam and the C-GAG

polymer affect the extent of implant-host integration and thus restrict the number of axons that actually enter the implant. Binding of bFGF to gelfoam and collagen has been demonstrated in vitro (Thompson et al., 1988), and gelfoam-based introduction of bFGF has been shown to stimulate angiogenesis in vivo even at very low levels of growth factor (Thompson et al., 1988). Although Thompson et al. (1988) demonstrated that bFGF also binds effectively to type IV collagen, Walicke (1988b) has demonstrated that, relative to other GAGs, chondroitin sulfate (which was used in the C-GAG implants--Yannas et al., 1980a,b) is less effective in binding bFGF. However, studies comparing the bFGF binding affinity for gelfoam and C-GAG have not been reported.

Although there is evidence from in vitro studies of CNS neuritic growth that neurites grow less readily on collagen substrates (Carbonetto et al., 1983), the fact that a couple of FGF/C-GAG cases showed growth comparable to that seen in FGF/gelfoam implants suggests that something other than, or at least in addition to, implant material may influence extent of outgrowth. If retinal axon outgrowth is relatively inhibited on collagen or C-GAG, then there should be greater disparity in extent of growth for all cases, not just in the means. Another possibility is that the observed difference in extent of neuritic outgrowth on gelfoam or C-GAG implants is due to

structural differences between the two materials. Because of their extensive crosslinking, C-GAG implants tend not to swell when hydrated, while gelfoam implants swell several fold. As a result, gelfoam implants generally filled the entire lesion cavity while C-GAG implants were less well integrated with host tissue and often fell out of the lesion site. [Cases in which implants were not directly apposed to host tissue were not used in any analyses.] The lack of host-implant integration may contribute to formation of a glial border (Figure 8a,c,e), while close apposition of host and implant permits integration as host astrocytes extend processes into the implant (Figure 8b,d). In addition, if the lesioned edge of host tissue is not closely apposed to to the implant, fibroblasts may be more likely to invade the lesion site and thus contribute to formation of a glial-fibroblastic scar that inhibits growth into the implant (Reier, 1986; Reier et al., 1983).

Target Reinnervation

The potential for using bFGF implants to promote recovery of behavioral function was assessed by examining the optic tract projection in three animals that received bFGF/gelfoam on p10, and were perfused ten weeks later. Although labeled axons could be seen at the border of the

lesion site, few labeled axons remained within the implant, and none were seen in host tectum (Figure 13a,b). In addition, a dense glial accumulation similar to that seen in animals lesioned in adulthood, had formed at the border between implant and host (Figure 13a). Because large bundles of regenerating retinal axons were observed in implants of similar cases at shorter survival times (Figure 3a,c,e), the lack of a projection in long-surviving cases indicates that regenerating axons retracted between 2 and 10 weeks after surgery. Such retraction probably indicates that retinal axons never reached or arborized in the superior colliculus (although small patches of tectal arborization were noted in several of the short-survival cases). Similar lack of host neuropil invasion has been reported by others (Vidal-Sanz et al., 1987; Harvey et al, 1986; Crutcher, 1989), and it is rapidly becoming apparent that the problem of CNS target innervation (as opposed to growth in general) will be the next major hurdle in study of CNS regeneration.

Lack of arborization in the superior colliculus might be caused by inhibitory effects of oligodendrocytes (Caroni and Schwab, 1988a,b; Schwab and Caroni, 1988); myelination of the hamster optic tract appears in the brachium at around p15 (L. Rava, unpublished data based on Gallyas staining). Although small patches of tectal

arborization were observed in the SC in harvested gelfoam and FGF/gelfoam cases transected on p6 (and in one transected on p10), no tectal reinnervation was observed in cases transected on p18 or p25, in spite of substantial axonal growth into the conditioned implants. Thus, the time course of myelination roughly corresponds to the time at which retinal innervation of superior colliculus is no longer observed.

Retinal ganglion cell survival

Survival of retinal ganglion cells was not affected by presence of bFGF-conditioned implants, probably because the continued presence of the superior colliculus sustained RGCs following tract transection. In the hamster, normal RGC death occurs between p5 and p10 (Sengelaub et al., 1986), so in these experiments, introduction of the bFGF-conditioned implant on p6 occurs at a time when RGCs should be maximally receptive to the influence of neurotropic factors. In addition, Perry and Cowey (1982) have shown that RGC death in response to lesions of the superior colliculus is greatest if the lesions are made before RGCs have established collateral projections. Therefore, ablating the superior colliculus on p6 should produce maximal cell death and, consequently, introduction of neurotrophic factors at this age should have the greatest survival-enhancing

effect. However, in transecting the optic tract, the tectal target remained mostly intact, so transected axons may still have had access to diffusible trophic factors from the SC.

Significant RGC death occured only when the superior colliculus was ablated in addition to the tract transection. The survival-enhancing effect of explanted target tissue has been shown in vitro for chick and rat RGCs (Cunningham et al., 1988; Korshing and Theonen, 1983a,b; McCafferey et al., 1982, 1984; Armson and Bennett, 1983; Nurcombe and Bennett, 1981), and at least one of the responsible factors has been identified (Johnson et al., 1986). Thus, these findings provide in vivo confirmation for the survival-enhancing effect of the rodent superior colliculus. Evidence that cell death results from loss of target factors rather than from the trauma of transection has been presented for other CNS neuronal populations as well (Cunningham et al, 1987). The continued presence of the SC target after optic tract transection probably provides trophic factors that sustain RGCs even after axonal transection, and any additional survival-enhancing effects of bFGF may be negligible by comparison.

One interesting phenomenon observed in nearly all of the cases receiving bFGF-conditioned implants was that

the regenerative growth of the transected retinal axons was almost always directed towards the tectal target (Figure 3). This was probably true in other cases as well, but the greatly enhanced growth in the presence of bFGF made this phenomenon particularly apparent. Because the shape and pore orientation of both C-GAG and gelfoam implants is random, this target-oriented growth must result either from a chemotropic response of transected axons to their tectal target, or from a purely mechanical tendency of axons to continue growing in the established orientation of the proximal stump. Chemotropic guidance of developing and regenerating axons is well established for many different CNS axonal populations (Tessier-Lavigne et al., 1988), including the rodent retinotectal projection (Lund et al., 1988; Smalheiser et al., 1981a,b). In the two representative cases shown in Figure 3, the regenerating axons grow mostly along the dorsal surface of the conditioned implant, which is closely apposed dorsally to an unconditioned piece of gelfoam that was used to fill the gap left by aspiration of cortical tissue. (Thus, regenerating axons appear to be growing directly through a single implant towards the target.)

Conclusions

"While it is often a great advantage to be able to study the complex behavior of cells in the strictly defined conditions of a culture dish, the observations must sooner or later be checked against the behavior of cells in their natural environment *in vivo*." (Alberts et al., 1983. Molecular Biology of the Cell, p.161)

This series of experiments was intended to apply findings from *in vitro* studies of CNS neuronal growth to *in vivo* studies of injured retinofugal axons in the neonatal hamster. An increasingly large number of molecules and substrates have been shown to be effective in supporting or promoting CNS neuritic outgrowth *in vitro*, and enough evidence has accumulated that these findings can now be applied to living animals.

Study of axonal outgrowth in this model revealed that astrocyte-covered implants significantly enhanced the extent of retinal axon growth into these implants. This effect was reduced if implants were rinsed prior to transplantation, but was not reduced when implants were repeatedly frozen and thawed prior to transplantation. These results, as well as assays showing that astrocyte-covered implants released protein and mitogenic molecules

into the rinse solution, indicated that a soluble factor might be involved in promoting retinal axon outgrowth. Because of several reports indicating that astrocytes secrete basic fibroblast growth factor, as well as *in vivo* and *in vitro* evidence that bFGF promotes growth and survival of rodent retinal ganglion cells, bFGF-conditioned implants were also implanted using the same model. Basic FGF-conditioned implants also enhanced outgrowth of transected retinal ganglion cell axons, although they did not affect cell survival.

The rodent retinotectal projection is an ideal system for study of nerve regeneration and for application of *in vitro* findings. First, the relatively easy isolation of pure populations of RGCs has contributed to their extensive use *in vitro*, thus providing extensive detailed information about effects of various factors on RGCs at specific developmental stages. Second, the relative isolation of RGCs from the rest of CNS also makes it easy to label these cells *in vivo* (e.g., via eye injection) without involving other neural populations. Finally, because behavioral functions of the retinotectal projection have been clearly documented for both normal and lesioned animals (Schneider, 1969), functional recovery can also be determined.

However, as the studies presented here have made apparent, the complexity of the CNS makes clear interpretation of results exceedingly difficult. Although implants soaked in bFGF or harvested from neonatal neocortex can act as a bridge for regenerating retinofugal axons, the actual mechanisms mediating the enhanced growth are still uncertain: does bFGF directly stimulate neuritic growth, or does it induce formation of an astrocytic substrate by stimulating glial proliferation?

In addition, several other factors can greatly influence whether outgrowth occurs, and these interfere with clear interpretation of results. For example, is the loss of growth-promoting effect of harvested implants at older host ages due to increasing host scar formation, or is there a loss of axonal response to growth-promoting factors (e.g., laminin or bFGF) that are expressed by astrocytes? The influence of scar formation on retinal axon outgrowth in vivo is one important factor that prevents direct translation of in vitro findings to in vivo experiments.

Although these experiments represent a first step in determining what factors influence regeneration of retinal axons in neonatal hamster, future studies should make more use of specific antibodies to block antigens or

receptors that might be involved in promoting regenerative growth and they should include more of the techniques that are commonly used *in vitro*, including membrane extraction of receptors and assays to determine presence of specific growth-promoting molecules.

Appendix A:
Immunohistochemical Methods

All controls for immunohistochemical reactions were incubated in 1% normal serum in PBS with no primary antibody. Except for those reactions against the membrane protein A2B5, all incubations in primary and secondary antisera and in PBS also included Triton X-100 for permeabilization of cell membranes. Tissue and implants were mounted on glass slides and coverslipped with Immumount.

Anti-GFAP staining.

Free-floating brain sections and fixed non-transplanted, harvested implants were stained by both immunofluorescent and the avidin-biotin method. For the latter, sections were incubated in methanol with 0.3% hydrogen peroxide, normal goat serum (NGS, Sigma; 1% in PBS), primary antisera (rabbit monoclonal anti-GFAP IgG, Boehringer-Mannheim, 1:50 in 1% NGS), secondary antisera (biotinylated goat anti-rabbit IgG, Vector, 1:100 in 1% NGS), avidin-biotin complex (Vector) and DAB (0.05% in 0.01% hydrogen peroxide).

Immunofluorescent staining for GFAP was combined with staining for vimentin, FGF, and laminin. For

GFAP/FGF and GFAP/laminin double-labeling, mouse monoclonal anti-GFAP IgG (1:50, Boehringer-Mannheim) was combined with, respectively, rabbit monoclonal anti-bFGF IgG (1:1000, gift from Dr. Mike Klagsbrun) or rabbit monoclonal anti-laminin IgG (1:2000, Gibco, gift from Dr. Seth Finkelstein). Primary antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG (1:200, ICN Immunobiologicals) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:200, Boehringer-Mannheim) For GFAP/vimentin double-labeling, rabbit monoclonal anti-GFAP IgG (1:50) was combined with mouse monoclonal anti-vimentin IgG (1:50, Boehringer-Mannheim). These primary antibodies were visualized with FITC-conjugated goat anti-mouse IgG (1:200) and rhodamine-conjugated goat anti-rabbit IgG (1:200).

Anti-vimentin staining.

Immunofluorescent staining used the same protocol as that for GFAP, but mouse monoclonal anti-vimentin IgG (1:50, Boehringer-Mannheim), normal horse serum, and rhodamine-conjugated horse anti-mouse IgG (1:200) were used.

Anti-FGF staining.

Immunofluorescent staining was done with rabbit monoclonal anti-bFGF IgG (1:500, gift of Dr. Mike Klagsbrun), NGS, and rhodamine-conjugated goat anti-rabbit IgG (ICN Immunobiologicals, 1:200).

Anti-laminin staining.

Immunofluorescent staining was done with rabbit monoclonal anti-laminin IgG (1:400, Gibco), NGS, and rhodamine-conjugated goat anti-rabbit IgG (1:200).

Anti-A2B5 staining.

Staining for this cell-surface antigen requires live cells, so it could be done only on harvested, non-transplanted implants. Implants were harvested from p3 or p13 donors, rinsed briefly in PBS, then incubated for 30 minutes in undiluted primary antisera. Anti-A2B5 and anti-GFAP were stained by double immunofluorescence using mouse anti-A2B5 IgG (gift from Dr. Ron McKay) and rabbit monoclonal anti-GFAP IgG (1:50). Primary antibodies were visualized with FITC-conjugated goat anti-mouse IgG (1:200) and rhodamine-conjugated goat anti-rabbit IgG (1:200). Labeled implants were then fixed in methanol (95% with 5% acetic acid) and wet-mounted with Immumount.

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TABLE 1

Immunohistochemical Characterization of Harvested and Transplanted Polymer Bridges

PRIMARY ANTIGEN	IMPLANT CONDITION			
	p3 harvest	p13 harvest	harvest & rinse	non-neural harvest
GFAP	+++	++	+	-
vimentin	+++	++	+	++
A2B5	-	-		
FGF	+	+		+
laminin	+	+		+
GFAP/vimentin	+/+	+/+		-/+
GFAP/A2B5	+/-	+/-		
GFAP/FGF	+/+	+/+		
GFAP/laminin	+/+ +/-	+/+ +/-		

Table 2

Effect of Implant Condition
on Axonal Growth
(mean growth into implants)

IMPLANT CONDITION	AGE OF HOST AT TIME OF TRACT TRANSECTION				
	p3	p6	p10	p18	p25/26
gelfoam buffer	n=4 259	n=3 27.7	n=4 51.1	n=4 22.75	n=4 26.0
gelfoam bFGF		n=9 380.4	n=4 329.2	n=4 110.0	n=5 110.0
gelfoam p3 harv.		n=4 116.5			
gelfoam p13 harv.		n=2 219.5			
gelfoam non-neural		n=5 126.6			
C-GAG buffer	n=4 117.7				
C-GAG bFGF		n=5 115.8			
C-GAG p3 harv.	n=1 176	n=15 134.7	n=2 6.5		
gelfoam harv+rinse		n=6 46.7	n=2 11		
gelfoam freeze+thaw		n=3 373.3			
HS bead FGF		n=3 124.7	n=3 185.9		

TABLE 3

Effect of Implant Condition on Survival of Retinal Ganglion Cells

	IMPLANT CONDITION				
	gel/buffer	gel/FGF	p3 harvest	Normals	SC ablation
	n=2	n=3	n=3	n=3	n=3
$total_1$	416	612	356	426	322
$nasal_1$	241	308	209	240	147
$temp_1$	175	304	147	186	175
$total_2$		561	494	638	281
$nasal_2$	312	298	293	373	147
$temp_2$		263	201	262	134
$total_3$		369	455	467	275
$nasal_3$		218	271	260	124
$temp_3$	195	151	184	207	151
means	461.5	514	444.6	532	292.7
Estimated total number of retinal ganglion cells (in 1000s, includes only cells larger than 5.8 micrometers)					
$total_1$	259.4	301.6	193.5	244.8	172.5
$total_2$	158.7	332.3	171.9	301.9	146.6
$total_3$	185.9	156.8	284.5	210.0	163.4
means	201.3	263.6	216.6	252.2	160.8
Retinal ganglion cell size (in micrometers): (includes only cells larger than 5.8 micrometers)					
$mean_1$	7.34	7.51	8.79	8.18	7.27
$mean_2$	7.08	9.00	7.66	8.53	6.99
$mean_3$	7.68	8.01	7.65	8.80	6.68
means	7.37	8.20	8.03	8.50	6.98

Figure Captions

Figure 1. Coronal sections from a normal hamster brain and one with an optic tract transection. The projections of the left eye (right optic tract) are shown. In the experiments described here, the right optic tract of the host animal is transected with a wire hook just rostral to the superior colliculus (small arrow=site of transection), and a small amount of midbrain is aspirated to make room for the implant (i). Various implants (harvested from neonatal neocortex, conditioned in FGF, or controls) are placed in the midbrain gap. After a survival period of 1-2 weeks, the left eye is injected with HRP to label the regrowing retinal axons. These coronal sections indicate the plane in which brains were cut during histology, and can be used in understanding the orientation of the implant and host tissue in the photographs.

Figure 2. Dark-field photographs of labeled optic tract axons extending from the host midbrain into implants harvested on p3 and transplanted into the host on p6 (A-D). Axons tend to grow along the dorsal edge of the implant (C, D), although in many cases small fasciculated bundles can be seen extending into the implant (B and D, arrows). Control cases in which

harvested gelfoam implants were rinsed prior to transplantation show less retinal axon growth into the implant, even in cases where the gelfoam is closely apposed to the host tissue at the site of tract transection (E). All photos taken at 10x.

[For all figures, i=implant, h=host tissue, solid arrows indicate regenerating axons within the implant, and open arrows indicate approximate border between host tissue and implant]

Figure 3. Large bundles of labeled retinal axons extend into gelfoam implants that have been conditioned in bFGF. Unlike harvested implants, FGF-conditioned implants promote consistent axonal regeneration at ages above p6; labeled axons are shown here growing into the implant in hosts with optic tract transection on p10 (A, C, E, same tissue section), and p18 (B, D, F, same tissue section). In most cases, axons appeared to grow on the dorsal edge of the gelfoam implant, which is closely apposed to a second, unconditioned, piece of gelfoam. The similarity of appearance of the unconditioned and conditioned pieces of gelfoam produces the impression that the regrowing axons are growing directly through a single piece of gelfoam (A and B). [Open arrows indicate the approximate border between host tissue and implant.]

A and B taken at 4x, C and D at 10x, E and F at 20x.

Figure 4. Axons extending into harvested and FGF-conditioned implants (solid arrows) tend to grow in small fasciculated bundles (A-D). Note that axons typically enter the implant only in a few discrete regions (A, B, D), rather than as a mass of extending axons. A and D taken at 10x, B at 20x, and C at 40x. Growth into the implant does not occur in all cases in which the implant is closely apposed to the lesioned edge of host tissue (E and F are 10x and 20x views of a single case that received a rinsed harvested implant on p6, respectively).

Figure 5. Implants were harvested on p3 from donors injected with ^{3}H-thymidine, then transplanted to host animals. ^{3}H-labeled cells (A and B, arrows) were observed in the host within the implant, thus confirming that donor cells survive the transplant procedure. Implants harvested on postnatal day 3 (but not transplanted) contain numerous GFAP^{+} (C) and vimentin^{+} cells (D), and these two intermediate neurofilament proteins are co-localized in the vast majority of harvested cells (E, double exposure of same fields shown in C and D). All photographs taken at 40x magnification.

Figure 6. Implants harvested from neonatal neocortex contain laminin^{+} cells (A-C), which is usually quite

punctate (B-D), but occasionally appears to be cytoplasmic (A). Host tissue at the edge of the lesion site also shows punctate staining (D). Laminin staining was quite specific, and was seen only within the implant, in host tissue adjacent to the lesion site, and in blood vessels throughout the host tissue (E). Figures A and E taken at 40x magnification, all others taken at 20x.

Figure 7. Implants harvested on p3 and p13 (but not transplanted) contain numerous $bFGF^+$ cells (C,D), which are closely associated with $GFAP^+$ cells and processes. In addition, host tissue shows enhanced expression of bFGF at the edges of the lesion (A,B), and these $bFGF^+$ cells are generally closely associated or colocalized with $GFAP^+$ cells and processes. $bFGF^+$ cells were seen only within the implant, at the lesion site, and a few $bFGF^+$ cells were seen in the ventricular zone of the third ventricle (E). Figures A and D taken at 40x, B, C and E taken at 20x.

Figure 8. Intense glial proliferation at the border between implant and host tissue can be seen with Cajal's gold chloride stain (A and B) and with immunohistochemical staining for GFAP (C, D, and E). In some cases there is a dense scar formed at the lesion edge of host tissue (A, C, and E), especially in those regions where the donor implant has been displaced from

the edge of host tissue. However, for cases in which the donor implant is closely apposed to the lesion edge, there appears to be more integration (i.e., less dense scar formation) between host and donor glia (B and D). Figure F shows an area of transition between host and implant with little or no scar formation. Figure A taken at 10x magnification, B and C at 20x magnification, and D, E, and F at 40x magnification.

Figure 9. Glial bridges appear to be made up mostly of $GFAP^+$ processes (A and C), although occasional $GFAP^+$ cells are also seen (B). Glial bridges stain positively with antibodies against bFGF (D); the staining is diffuse and is closely associated with regions of heavy $GFAP^+$ staining (C and D, same field). Glial bridges are preferential sites for growth of regenerating optic tract axons (E and F, arrows). (The tissue section shown in E, which is adjacent to those in C and D, has been reacted for visualization of HRP and is photographed in polarized light.) Figure B taken at 40x magnification, all others taken at 20x.

Figure 10. Cross-sections of the left eye from 9 different animals from 3 different experimental conditions. Large arrow indicates representative sector in which all retinal ganglion cells were counted. Small

arrow indicates the optic disk, where retinal ganglion cell axons leave the eye.

Figure 11. Mean growth of retinal axons into various types of implants for animals receiving optic tract transection on p6. Growth was estimated by counting the number of grid squares in which labeled axons could be seen in the implant. See Table 2 for actual means.

Figure 12. Mean growth of retinal axons into bFGF- or buffer-conditioned implants, for animals receiving optic tract transection on p6, p10, p18, or p25. See Table 2 for actual means.

Figure 13. Animals receiving bFGF-conditioned implants on p10, with 10 week post-lesion survival, showed no evidence of tectal reinnervation and there were very few labeled axons on the implant (A, B, both at 4x magnification). C and D show low power (4x) views of host tissue stained with antibodies against vimentin (C) and GFAP (D).

Figure 14. Mean estimated total number of retinal ganglion cells for animals receiving tract transection with various implants, no transection, or transection with ablation of the superior colliculus.

Figure 15. A. Mean retinal ganglion cell size for each of the different lesion and implant groups, and the

unlesioned controls. n=3 in each of the different groups. B. Histogram showing retinal ganglion cell sizes for each of the different lesion and implant groups.

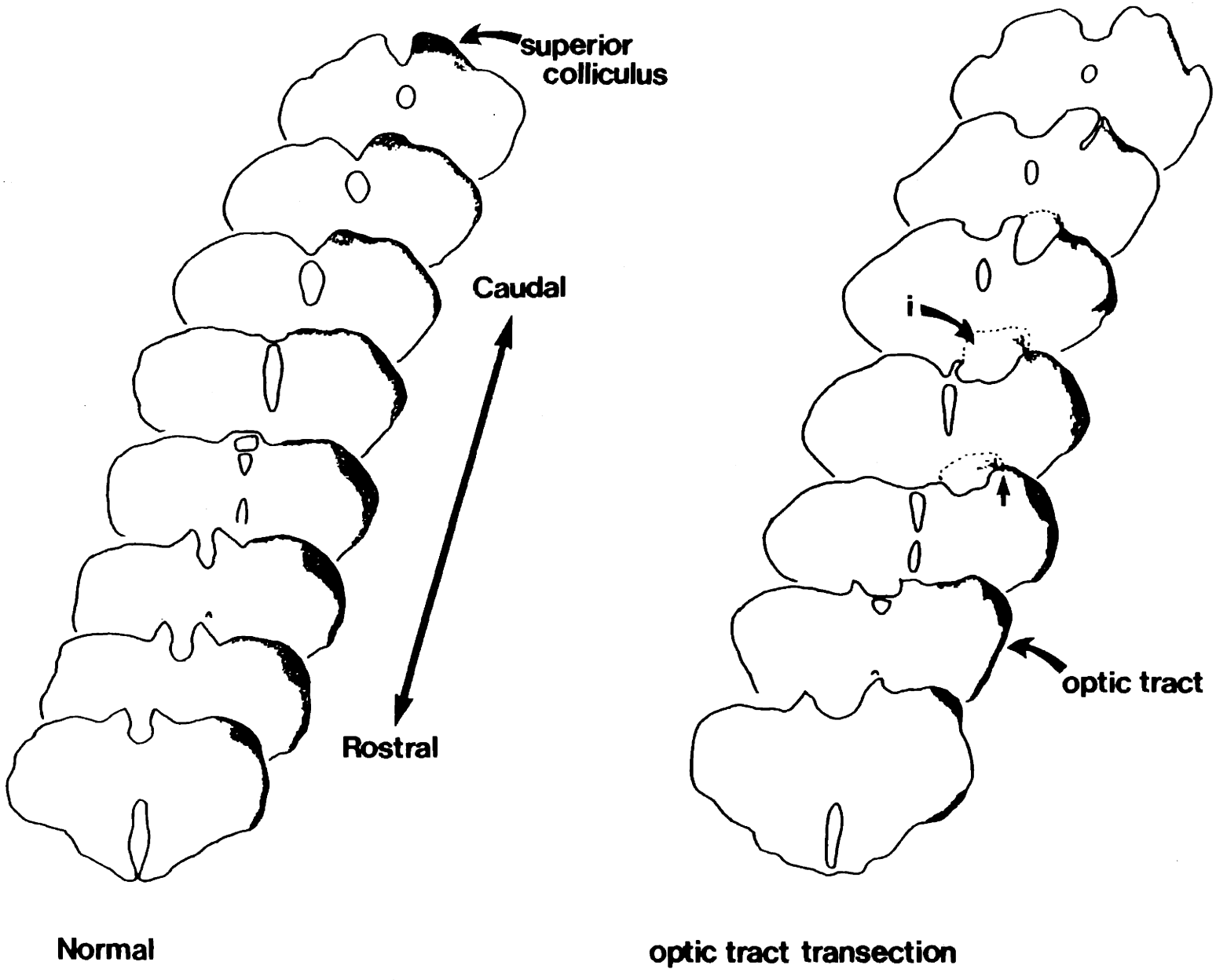

superior
colliculus
Caudal
Rostral
Normal
i
optic tract
optic tract transection

FIGURE 1

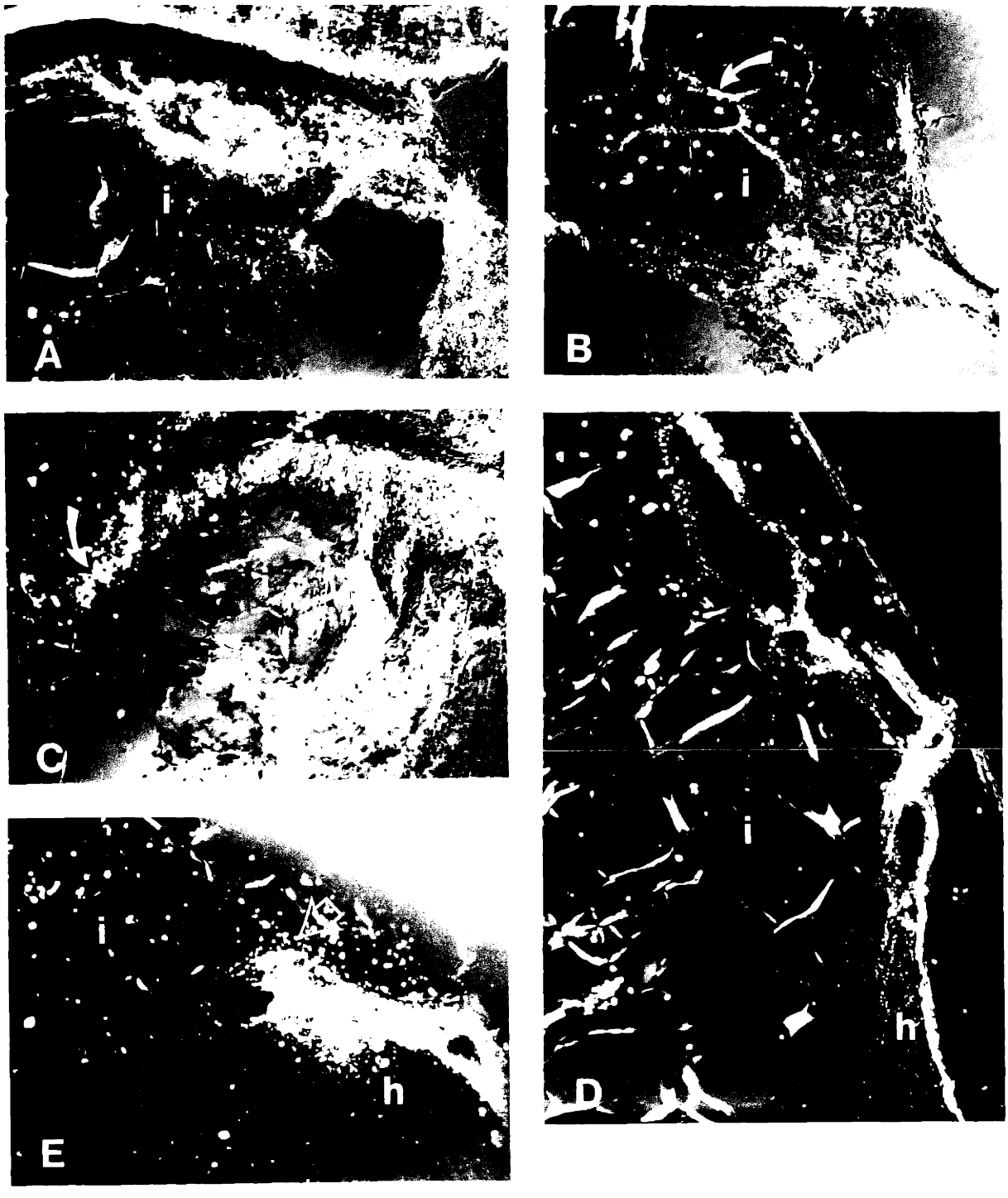


Figure 2

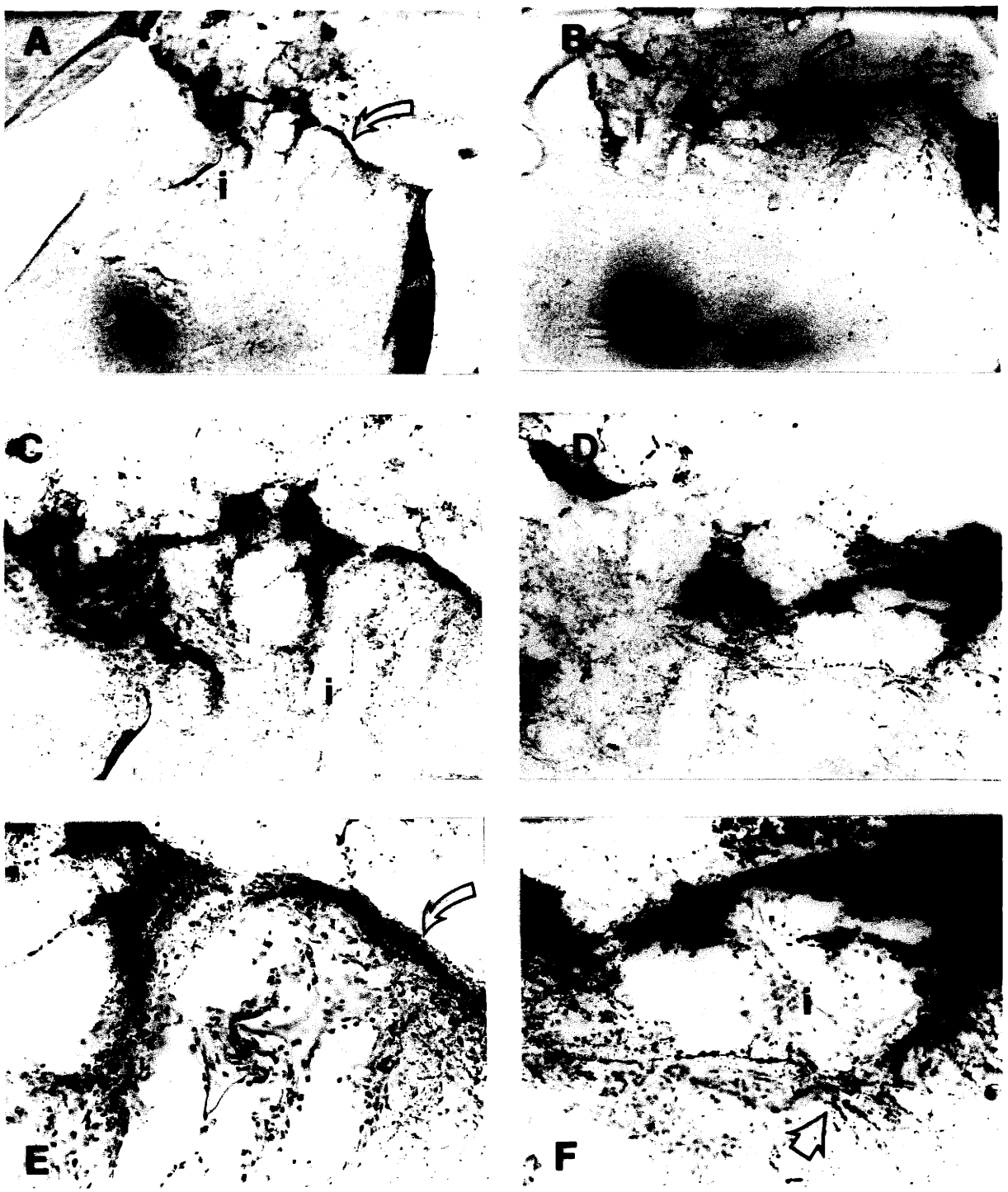


Figure 3

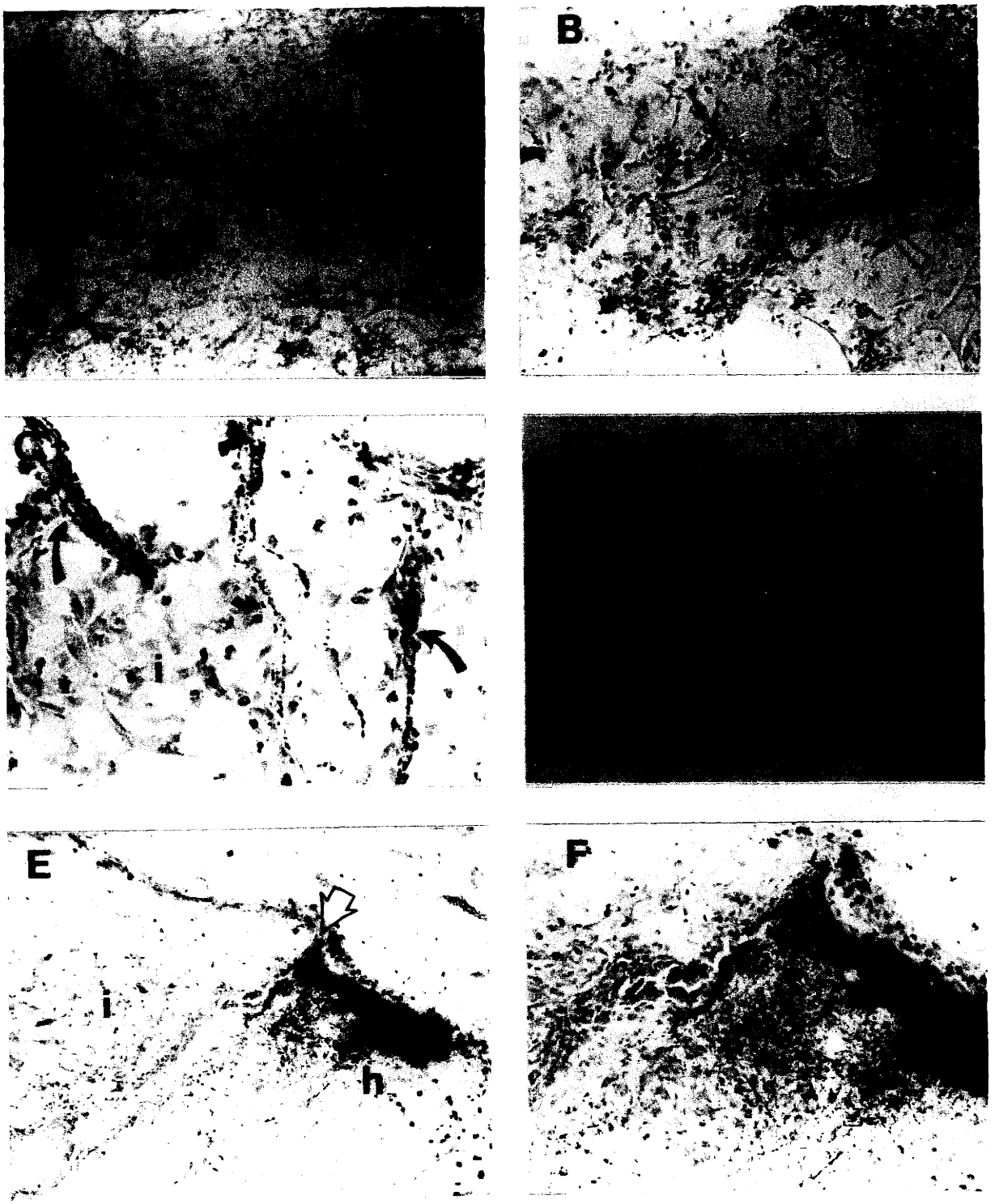


Figure 4

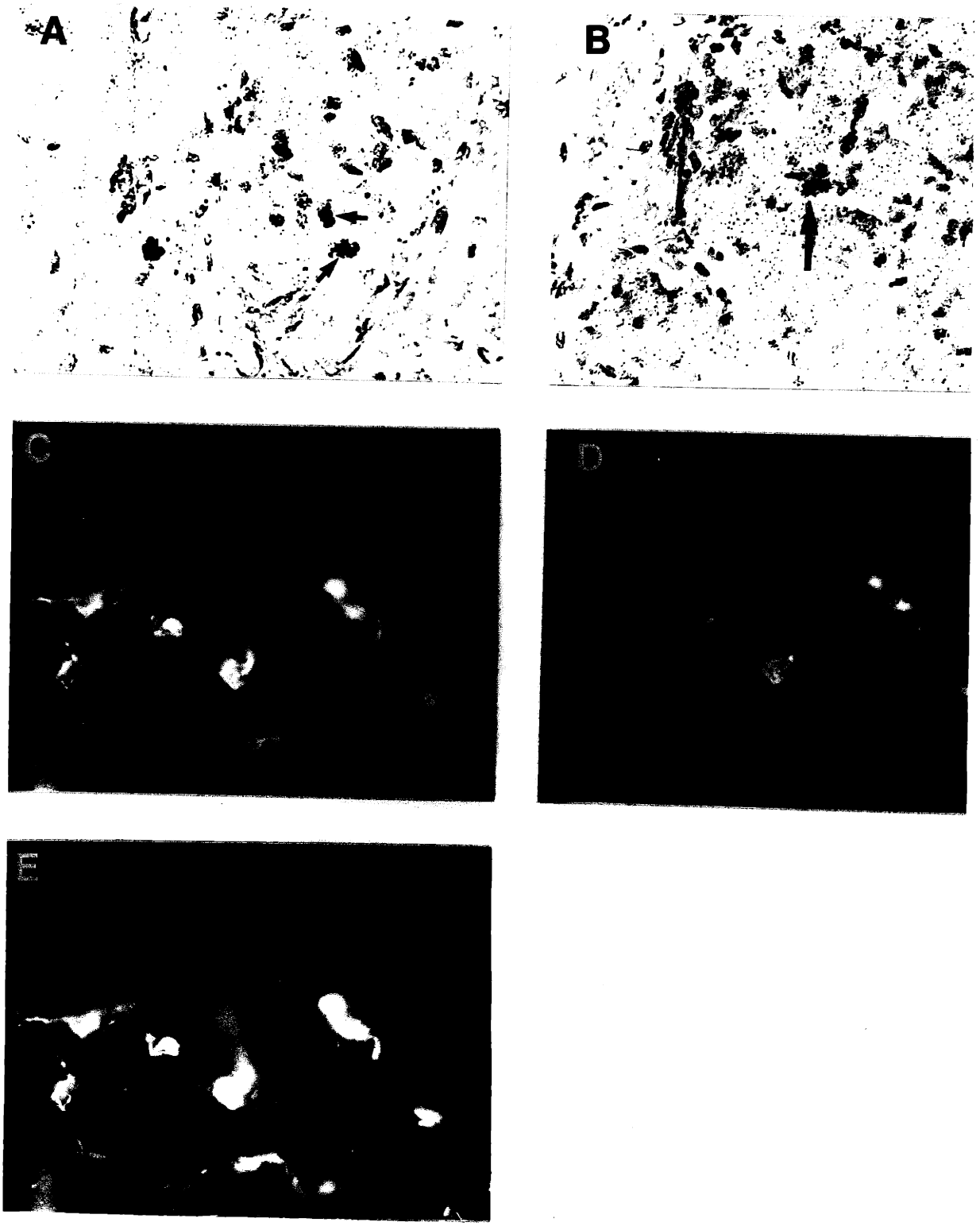


Figure 5

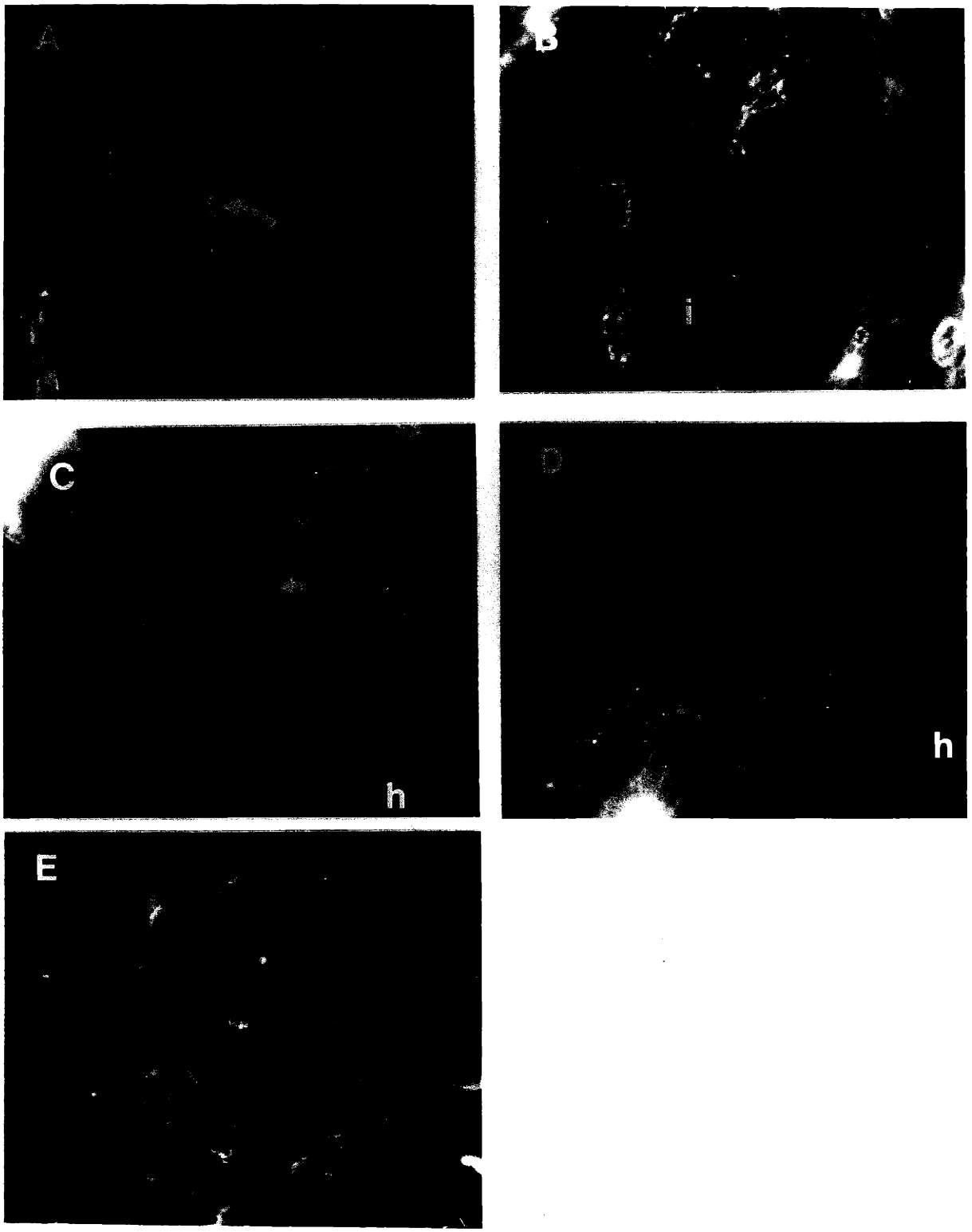


Figure 6

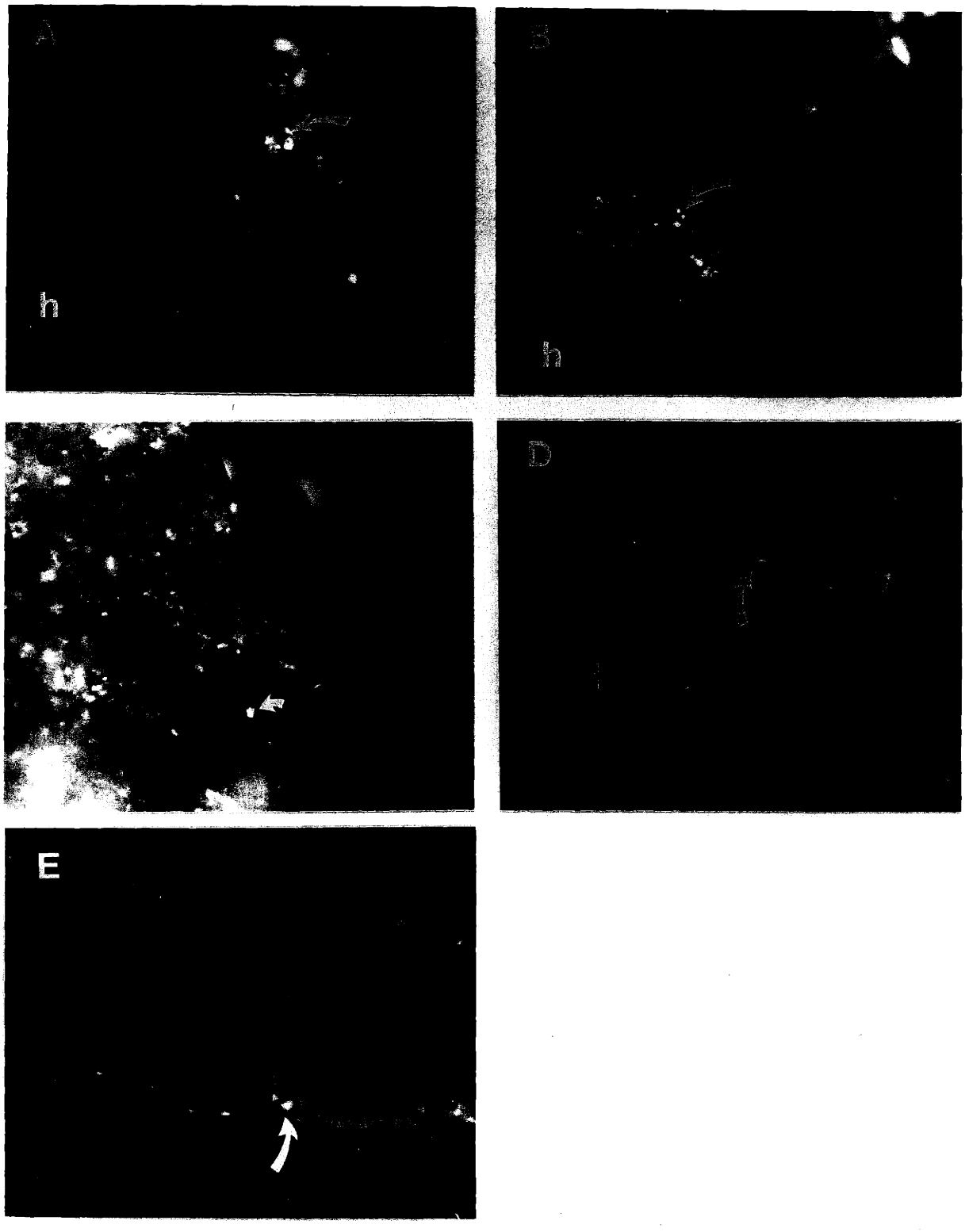


Figure 7

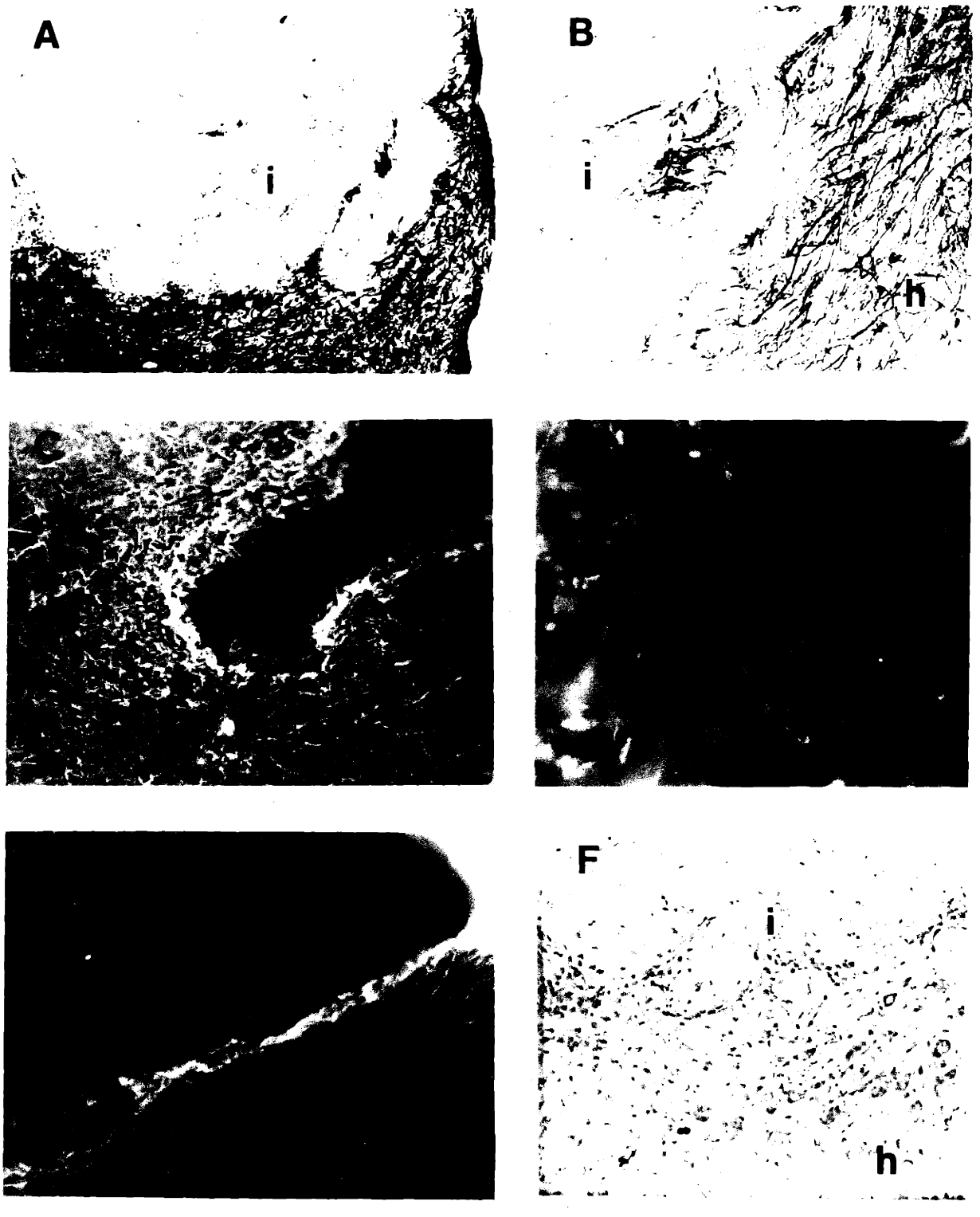


Figure 8

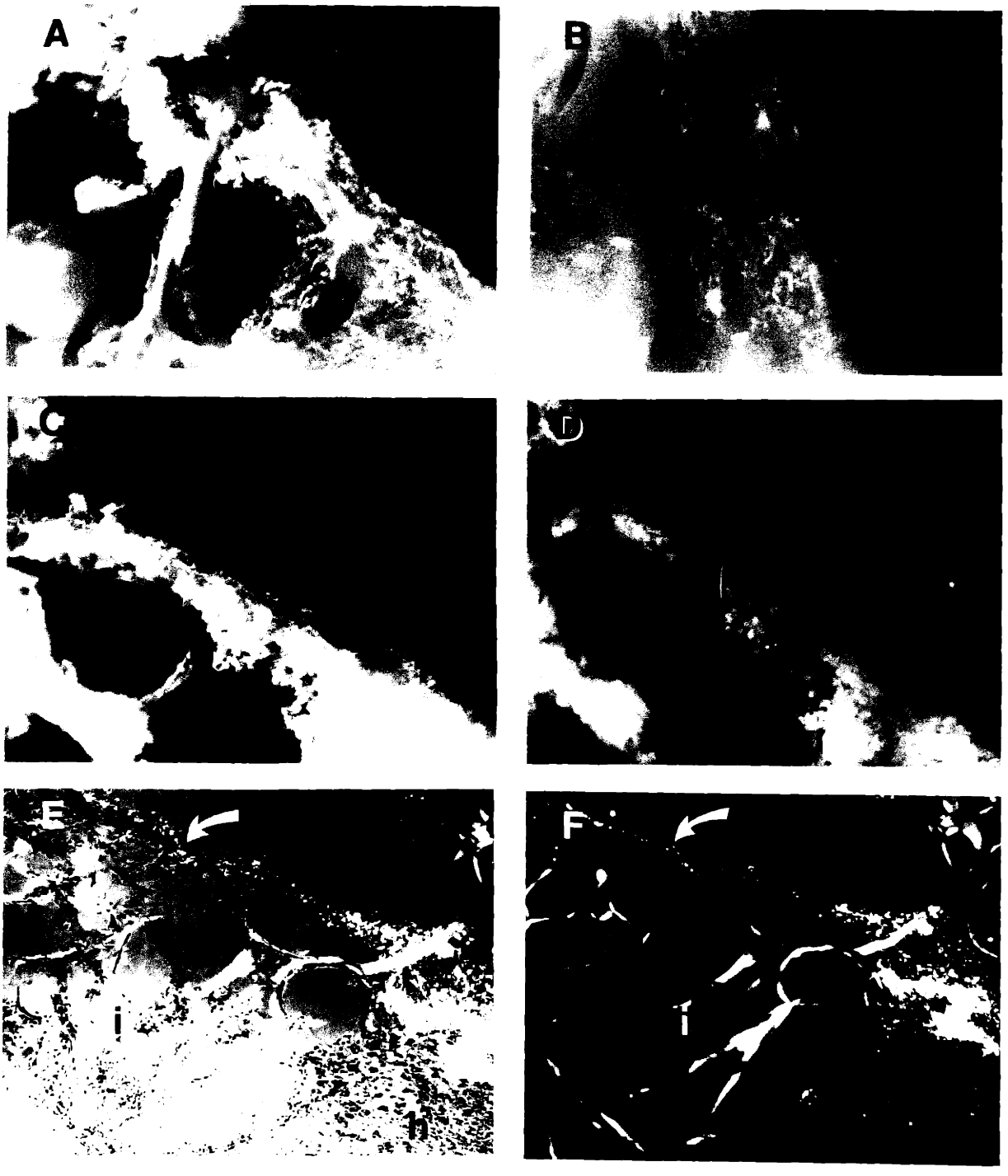
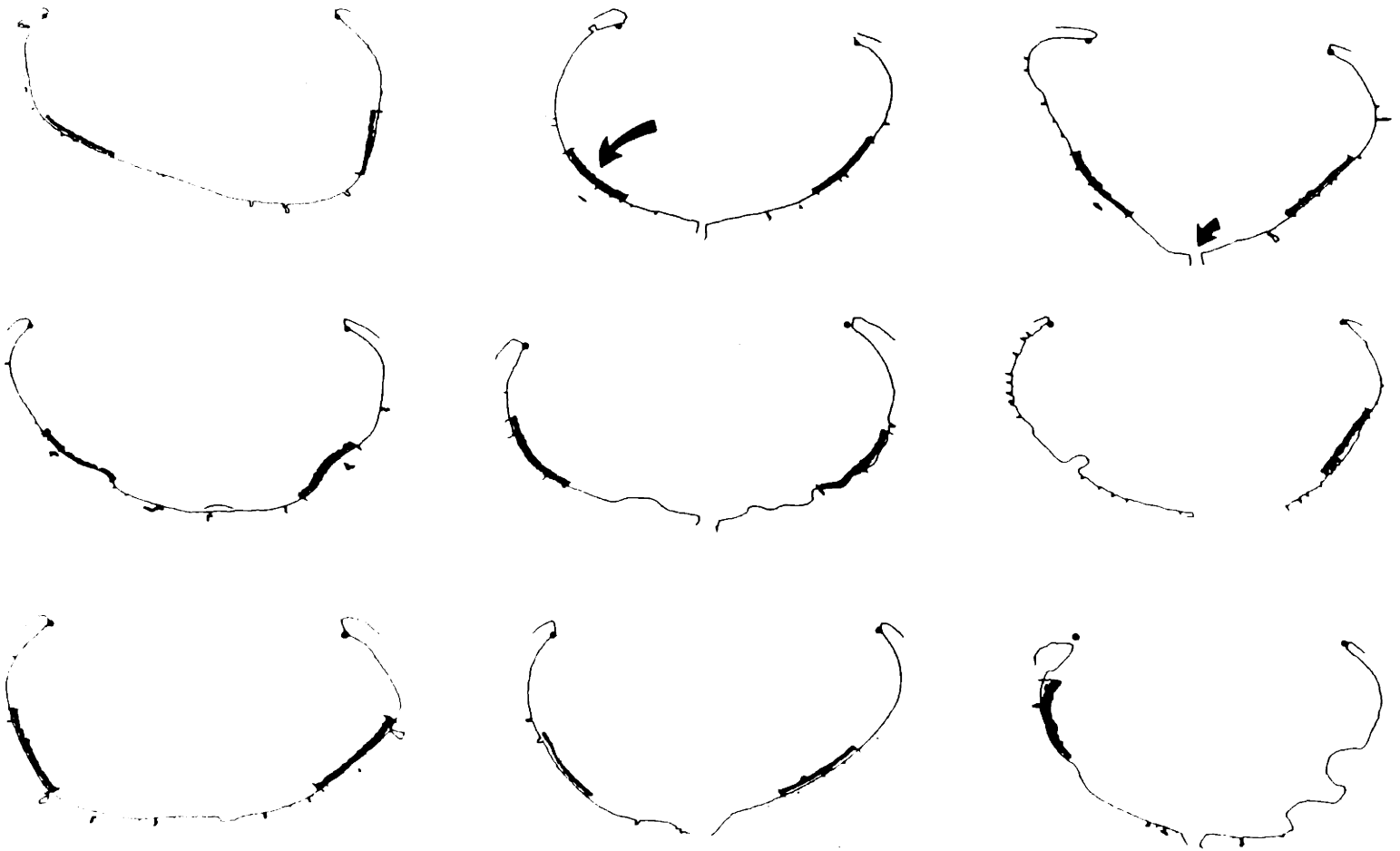


Figure 9

FIGURE 10



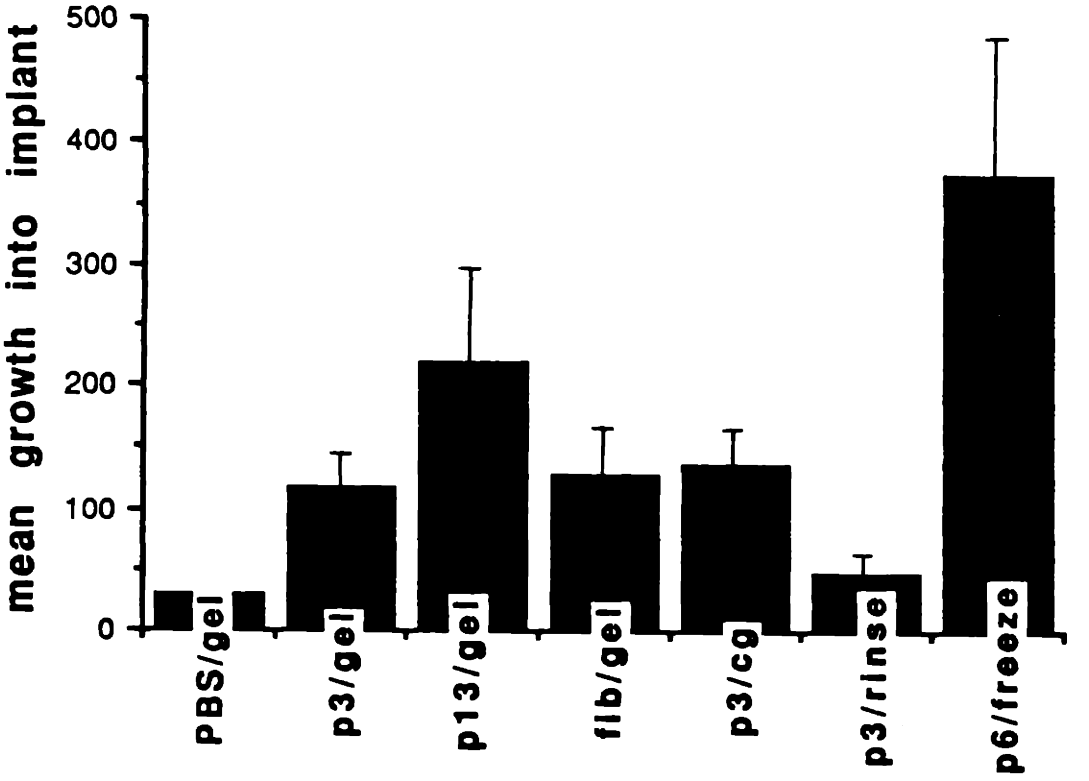

mean growth into implant
500
400
300
200
100
0
PBS/gel
p3/gel
p13/gel
flb/gel
p3/cg
p3/rinse
p6/freeze

FIGURE 11

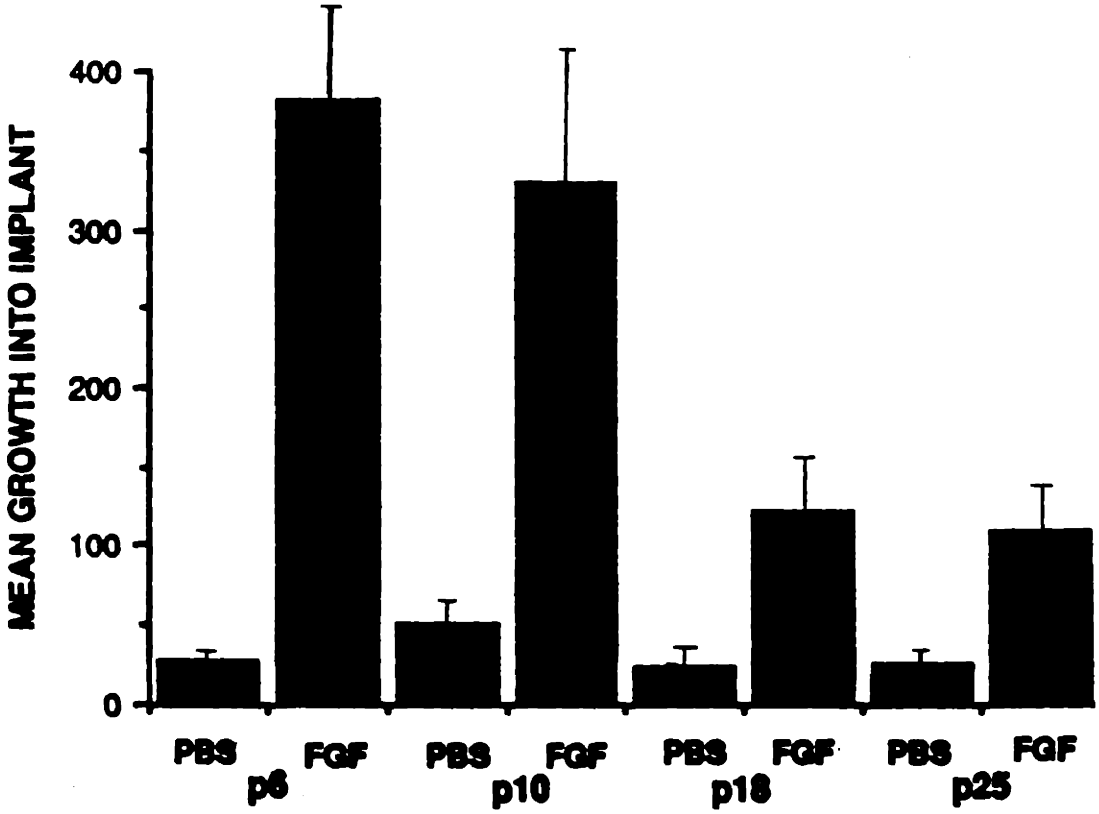

MEAN GROWTH INTO IMPLANT
400
300
200
100
0
PBS
FGF
PBS
FGF
PBS
FGF
PBS
FGF
p6
p10
p18
p25

FIGURE 12

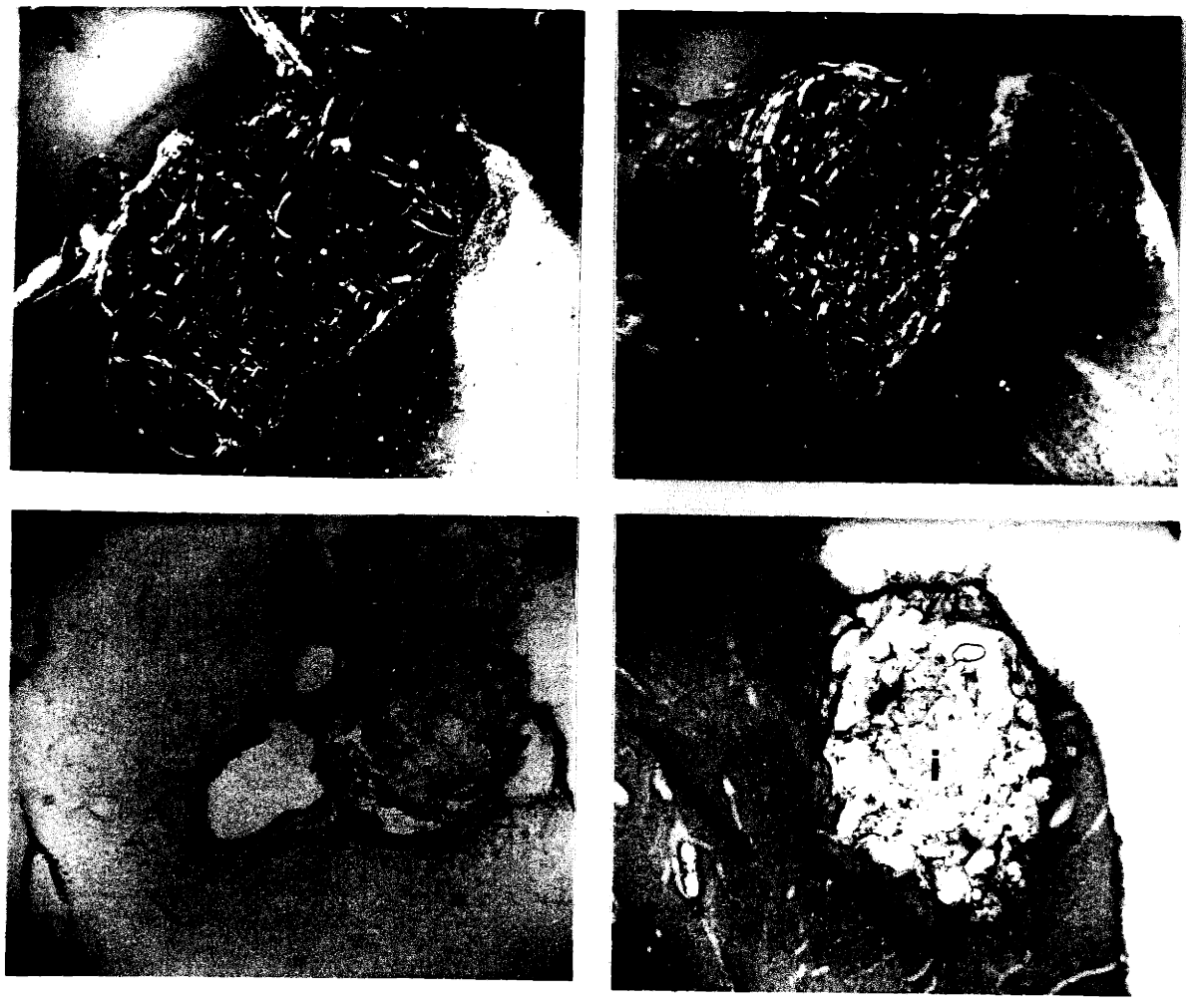


Figure 13

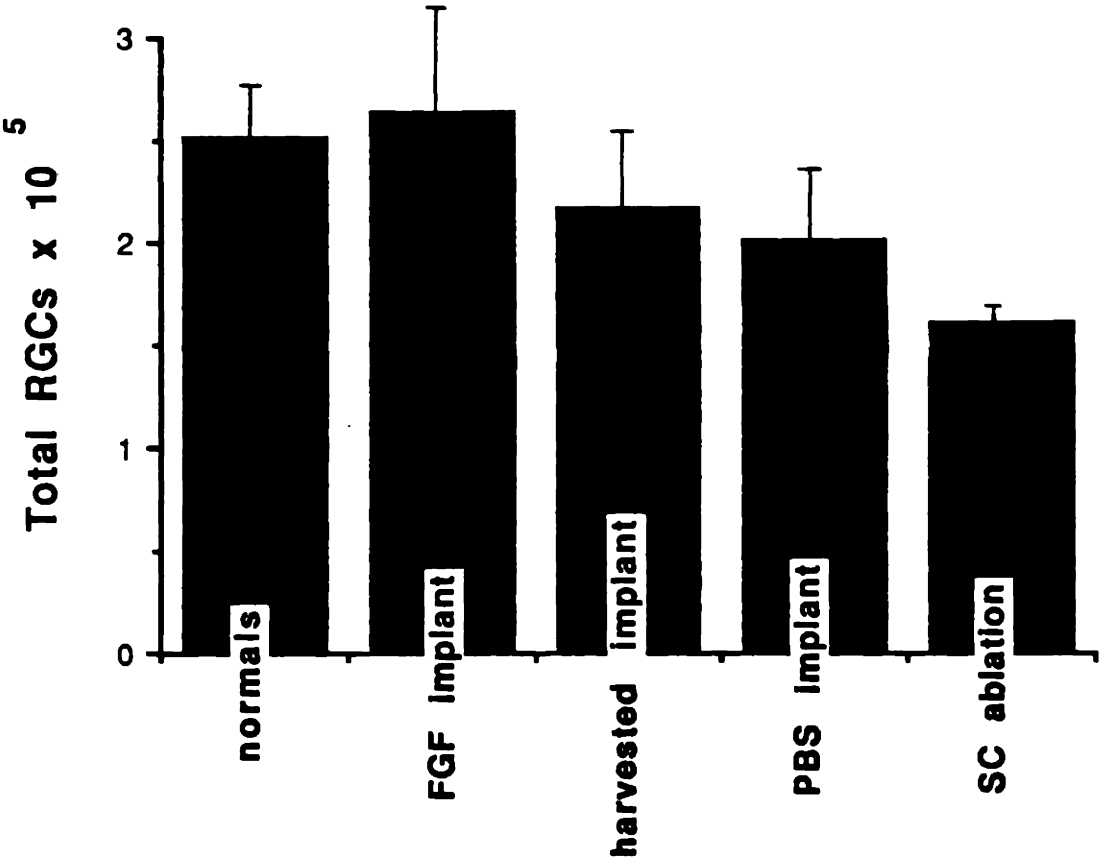

Total RGCs x 10 5
3
2
1
0
normals
FGF implant
harvested implant
PBS implant
SC ablation

FIGURE 14

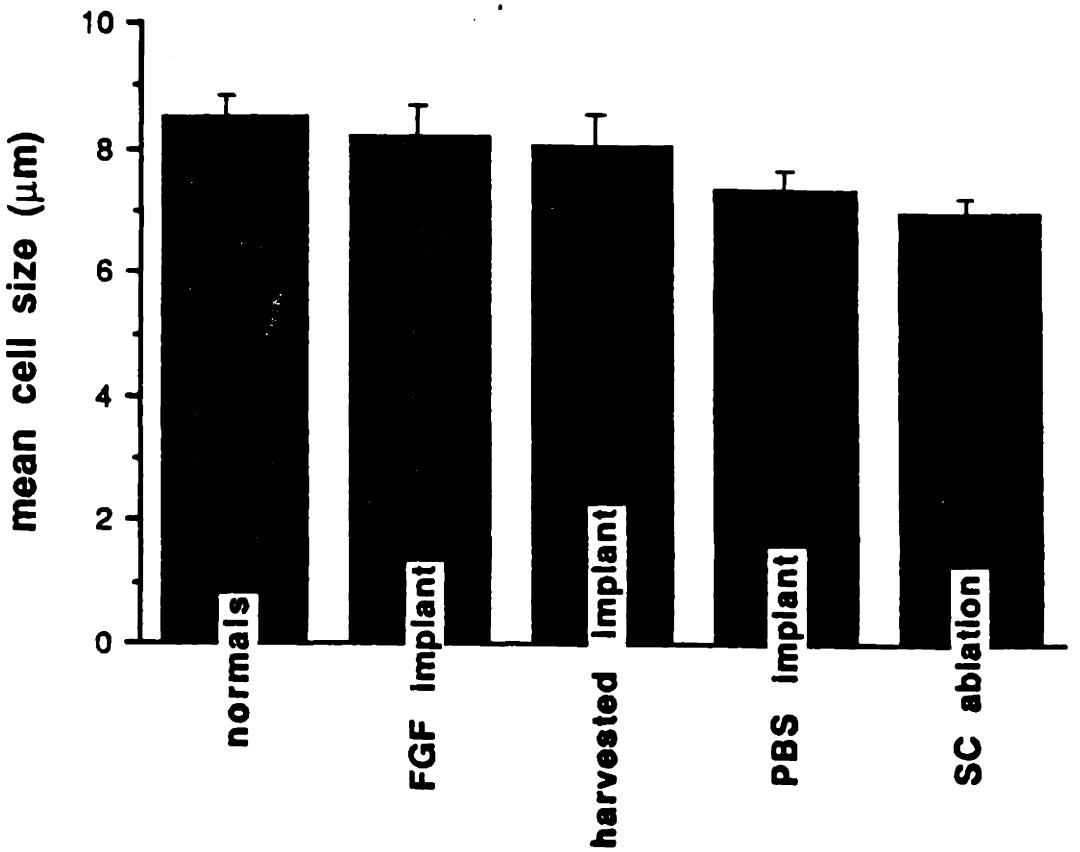


FIGURE 15A

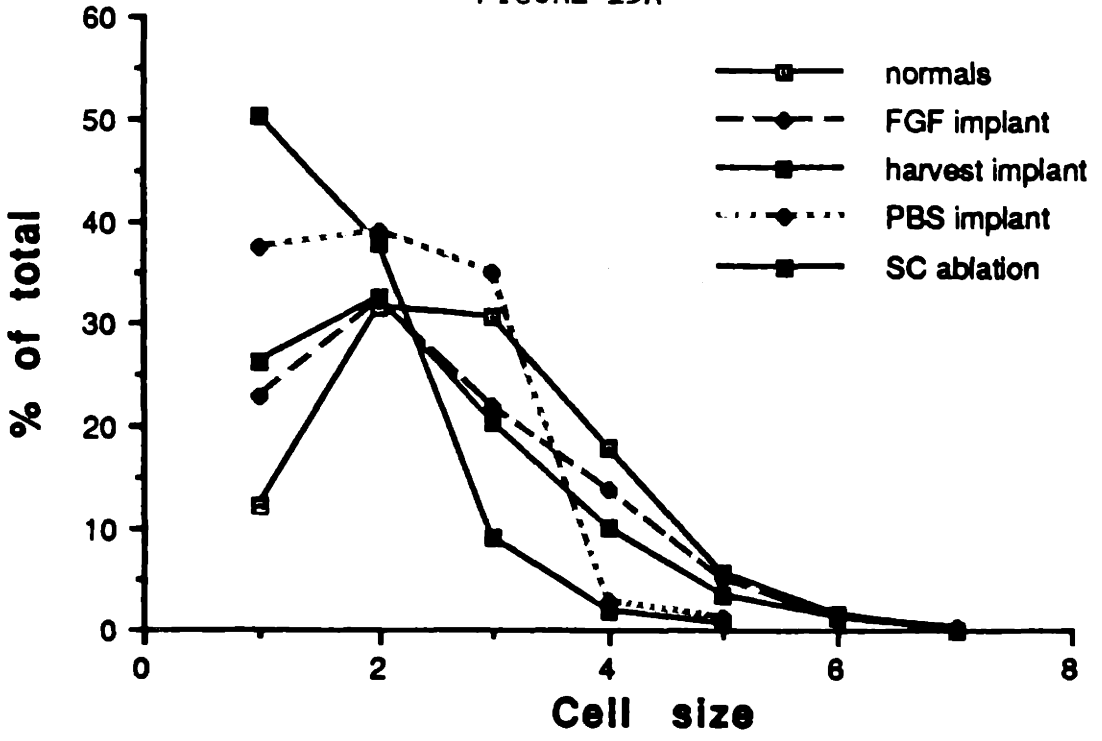


FIGURE 15B

Acknowledgements

I would like to thank my advisor, Professor Jerry Schneider, and my thesis committee members, Professors Arthur Lander, Jerry Silver, and Mriganka Sur, for detailed and extensive comments in the writing of the thesis. I am especially grateful to Arthur Lander for his suggestions for experiments that were critical for understanding what properties of the harvested implants might be responsible for enhancing axonal outgrowth.

I'd also like to thank my many collaborators who have generously provided important new technologies, as well as many suggestions and lessons. These include Dr. Elazer Edelman and Professor Robert Langer, who developed and generously encouraged use of their sepharose beads for slow release of fibroblast growth factor. Professor Ioannis Yannas provided the collagen-glycosaminoglycan polymer implants, and was very helpful throughout the thesis with suggestions. Dr. Seth Finkelstein provided several critical suggestions, and helped with the mitogenic assay.

Many people provided training, without which this thesis could not have been completed. In particular, Professor Jerry Schneider taught me the delicate surgical

skills necessary for this study, and encouraged me to pursue my ideas. Dr. Sonal Jhaveri helped me to learn the difficult techniques required for neuroanatomical study of the neonatal hamster.

Others have also contributed their time in teaching me new techniques, and I am grateful for their teaching and their patience. Dr. Judi Schaechter performed the protein assay, and made helpful suggestions for improving it; Da Yu Wu patiently taught me immunocytochemistry; Pat Harlan helped me with the autoradiography; and Tony Passera miraculously got my computer up and running in time for me to write the thesis.

Finally, I'd like to thank Larry Parsons, my family, and my many friends at MIT and beyond. Jan Ellertsen, Laurie Collier, and Jong-on Hahm deserve special mention for the countless times they provided emotional support during difficult times.