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A novel role of macrophages in peripheral nerve regeneration

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by

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Abstract

A novel role of macrophages in peripheral nerve regeneration
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We studied the regeneration of peripheral neurites by culturing adult mouse dorsal root ganglia (DRG) explants on top of unfixed, longitudinal cryosections of adult mouse sciatic nerve. DRG neurites are able to grow on cryosections of both uninjured and post-injury (distal segment) sciatic nerves. However, on uninjured nerves, DRG neurites begin to degenerate shortly after contact with the substrate while neurites growing on post-injury nerve substrate remain healthy for up to 9 days in culture, implying that the sciatic nerve environment undergoes a transformation during Wallerian degeneration such that it becomes supportive of stable neurite outgrowth. Using a semi-quantitative scoring system, we found that such capability to support stable outgrowth peaks at 8 days after injury, but gradually decreases at 10 days until it disappears by 20 days after injury, even when regenerating neurites are prevented from entering the distal nerve segment. Macrophages, which begin to accumulate in the distal segment by 4 days after injury, is a likely candidate cell type responsible for the transformation of the post-injury nerve environment since uninjured nerve cryosections can be rendered supportive of stable outgrowth by pre-incubation with macrophage-conditioned medium. The activity of the macrophage-conditioned medium has protein-like biochemical properties and is secreted in increased amount on activation of macrophages. Two hypotheses may account for the molecular mechanism of post-injury nerve transformation: (1) A presumptive "degeneration signal" molecule normally present in the adult nerve is removed by

macrophages after injury; (2) A presumptive "permissive signal" molecule is laid down, directly or indirectly, by infiltrating macrophages in the post-injury nerve. Several lines of evidence appear to favor the "degeneration signal" model but not the "permissive signal" model. First, as macrophages are known to secrete several matrix-degrading proteases during tissue remodeling, normal nerve cryosections could be transformed by brief treatment with trypsin, a non-specific protease. Second, a battery of growth factors secreted by macrophages failed to mimic the effect of macrophage-conditioned medium. Third, even in the presence of combinations of neurotrophic factors, regenerating neurites degenerated on normal nerve cryosections, implying that the support of stable outgrowth is not induced via neurotrophic factors.

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CHAPTER 1: BACKGROUND TO THE THESIS PROJECT

Introduction

The mammalian peripheral nervous system is remarkable in its ability to repair itself after injury. In some of the more extraordinary cases, such as the transplantation of completely severed adult human hand, meticulously repaired nerves can regenerate for a distance of 360 mm in 365 days, from the site of severance all the way to the fingertip (Owen et al., 2001). The regeneration rate was a steady and rapid 1 mm per day and the patient eventually regained both sensory and motor functions, which were demonstrated by the sensation of pinprick and "pinch" gripping with his index finger and thumb.

The complicated process of nerve regeneration has been the subject of intense investigation since the time of Ramon y Cajal in the late 19th century (Ramon y Cajal, 1913). As a result of a series of century-long studies, many of the complex cellular and molecular events that follow nerve injury have been documented in detail (Fawcett and Keynes, 1990; Hall S, 2001). However the exact purpose and contribution of each event towards subsequent regeneration are far from being fully understood. It is the goal of this thesis project to elucidate how some of these events contribute to successful repair.

Wallerian degeneration following nerve injury

As a starting point of discussion, we shall review the series of events occurring after the sciatic nerve of a mouse is surgically severed, a typical peripheral nerve injury under experimental conditions. Transection of the sciatic nerve divides the axons into proximal segments (attached to the cell bodies) and distal segments (attached to the target sensory organ). Despite the trauma of the injury, most of the neurons survive as the axonal membrane at the cut ends quickly reseal, preventing an excess loss of axoplasm (Vestergaard et al., 1997; Jessell, 1991). Within a day, the cell bodies swell up, the Nissl substance (rough endoplasmic reticulum) begins to break up (chromatolysis) (Grafstein, 1983) and injury-specific genes such as c-Jun are expressed (Broud et al., 1997; Gu et al., 1997; Wu, 1996).

By 2 to 4 days after injury, the distal segments of the injured axons begin to degenerate and a series of cellular activities occur concurrently within the non-neuronal environment of the nerve. The onset of these events, collectively known as Wallerian degeneration, is marked by the invasion of a large number of hematogenic macrophages (Perry et al., 1987), believed to be recruited by TNF-α secreted by denervated Schwann cells (Liefner et al., 2000). These macrophages migrate from the wound site into the Schwann cell basement membrane tubes of the entire distal segment, while the proximal segment remains free of invading macrophages except near the wound site.

The invading macrophages induce the degeneration of morphology in the distal segment, as longitudinal histological sections show axons fragmented into

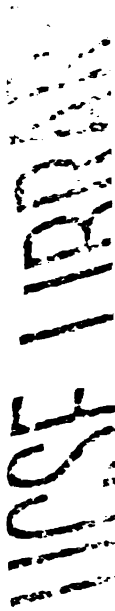
islands of debris instead of distinct, parallel axon bundles. Schwann cells, induced by IL-1 secreted by macrophages (Brown et al. 1991), dedifferentiate and shed their myelin sheaths that once wrapped around healthy axons and may even phagocytize the myelin material themselves (Beuche and Friede, 1984). The bulk of the axonal and myelin debris is later phagocytized by macrophages.

Following dedifferentiation, the Schwann cells undergo proliferation (Salzer and Bunge, 1980) and up-regulate the expression of several growth factors including nerve growth factor (NGF) (Matsuoka et al., 1991), brain-derived neurotrophic factor (BDNF) (Meyer et al., 1992), leukemia inhibitory factor (LIF), interleukin-6 (Kurek et al., 1996) and transforming growth factor β1 (TGF-β1) (Scherer et al., 1993). The low-affinity NGF receptor (NGFR or $p75^{NGFR}$) was also highly expressed (Heumann et al., 1987). Interestingly, the primary function of Schwann-cell-secreted NGF may not be the enhancement of neuronal survival, since administration of antiserum against NGF did not lead to an increase in neuronal death beyond the level induced by injury alone (Yip et al., 1984).

The proliferation of Schwann cells probably paves the way and acts as a scaffold for the incoming, regenerating axons as their daughter cells line up to form the "bands of Bungner" within the intact basement membrane tubes, through which regenerating growth cones extend (Hall 1986, 1997). In fact, the outgrowth of neurites may be dependent on the presence of Schwann cells since axons cannot regenerate through a nerve graft in which the Schwann cells have been eliminated by

repeated thawing, unless the axons are accompanied by Schwann cells from the host proximal segment (Scaravilli et al., 1986; Gulati, 1988).

Molecularly, Schwann cells may provide appropriate adhesion for the extending neurites by increasing expression of adhesion molecules like NCAM and L1/Ng-CAM on the cell surface (Martini and Schachner, 1988) and of laminin into the extracellular matrix (ECM) (Kucherer-Ehret et al., 1990). In culture, Schwann-cell secreted laminin has been shown to be chemotactic and highly permissive to growth cones (Lander et al., 1985). In vivo, the highly increased amout of laminin present in the distal segment of injured nerve may help direct regenerating neurites to migrate to the right direction (Siironen et al., 1996).



The age-old puzzle of PNS versus CNS regeneration

Since the time of Ramon y Cajal, scientists had noticed that while the peripheral nervous system (PNS) is endowed with impressive ability to regenerate, the central nervous system (CNS) regenerates very poorly, if at all, after injury (Perry et al., 1987). The severed CNS axons begin sprouting for a very short distance and then abort with their terminals transformed into club-shaped, dystrophic endings. Interestingly, these "sterile end balls", as Ramon y Cajal described, can remain in this state near the wound site for many months, without further advancement or contact with target end-plate (Ramon y Cajal, 1913).

Therefore, the paralysis or blindness caused by accident injuries to the spinal cord or the optic nerve, respectively, is often irrecoverable. Due to its important

implication in clinical medicine, the explanation for the tremendous difference in regenerative potential between the PNS and CNS has been a central quest of neurobiology. Much of the information accumulated to date can be categorized into three models: (1) differences in the nerve environment, (2) differences in the intrinsic state of the neurons, and (3) differences in secondary events following injury.

Model 1: differences in the extracellular environments of PNS and CNS

By the 1980's, the idea that CNS neurons were innately incapable of regeneration was disproved by a series of elegant experiments (Bray et al., 1987; Vidal-Sanz et al., 1987) in which a predegenerated section of sciatic nerve (from PNS) was grafted to an injured rat optic nerve (CNS) and, subsequently, long distance regeneration of retinal ganglion cell axons to the superior colliculus and even transmission of action potentials on illumination of the eye were achieved. Clearly, when given a favorable environment, CNS neurons are capable of axon regeneration.

Indeed, the PNS environment has a much higher matrix-to-cell ratio than the CNS (Carbonetto, 1991). Wrapping around the Schwann cells and the axons are the basement membrane tubes, which are rich in type IV collagen, laminin and heparan sulfate proteoglycan. Beyond the basement membrane of Schwann cells is the endoneurium, which contains abundant fibronectin and type I collagen. All of the above ECM molecules are known to be favorable substrates for neurite outgrowth (Rogers et al., 1983). It has also been shown that several of these ECM molecules become highly up-regulated after nerve injury, including type I and type III collagen

(Siironen et al., 1996), fibronectin (Vogelezang et al., 1999), laminin (Cornbrooks et . al., 1984; Siironen et al., 1996) and tenascin (Martini et al., 1990). These extracellular molecules, together with up-regulated cell-surface adhesion molecules NCAM and L1 on proliferating Schwann cells, provide a favorable path for neurite regeneration (Martini and Schachner, 1988).

In the CNS, the above-mentioned ECM molecules are largely absent. On the other hand, CNS axons are surrounded by oligodendrocytes (the functional counterpart of Schwann cells) and astrocytes. The myelin of oligodendrocytes has long been recognized as an inhibitory substrate for neurite outgrowth (Berry, 1982) and may account for the bulk of the inhibitory effect within the post-injury CNS before the formation of scar tissue. In fact, when mice were immunized with myelin to induce the production of antibodies against myelin-associated inhibitors, at least ten times more axons were able to regenerate in the injured corticospinal tract, often over long distances that allow recovery of motor functions (Huang et al., 1999). In the last five years, several cell-surface proteins of myelin have been identified as potent inhibitors of neurite outgrowth and the two best characterized are Nogo and myelin-associated glycoprotein (MAG) (Qiu et al., 2000).

Nogo

Nogo's were first identified (Chen MS et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) as the antigens of a function-blocking monoclonal antibody (IN-1) raised against a protein fraction of CNS myelin that exhibited strong inhibitory

effect (Caroni et al., 1988a, 1988b). These oligodendrocyte-associated antigens, originally named NI-35 and NI-250 (neural inhibitors of 35kd and of 250kd) can stop the migration of neurites by causing growth cone collapse (Cadelli et al., 1992) and when they were inactivated by the antibody IN-1, about 5-10% of the axons of a severed spinal cord were able to regenerate much better than those of untreated control (Schnell and Schwab 1990; Brösamle et al, 2000).

Three forms of Nogo mRNA have been identified (Nogo-A, -B and -C) and are believed to be isoforms produced by alternative splicing of the Nogo gene. Nogo-A is identical to NI-250 and is the only isoform that is expressed exclusively in neural tissue, implying that Nogo-B and -C may have functions unrelated to the nervous system, perhaps in affecting cytoskeleton dynamics (Caroni and Schwab, 1988a). Oddly enough, Nogo does not have any N-terminal signal sequence, implying that it may not be transported to the cell surface by the conventional protein secretory pathway. It does have two putative transmembrane domains and topographical mapping showed that the only part of Nogo that is exposed to the extracellular environment appears to be a short 66-amino acid loop (Nogo-66) between the two transmembrane domains (GrandPre et al., 2000). A Nogo receptor (NgR) which binds the Nogo-66 peptide has been cloned (Fournier et al., 2000) and, due to its linkage to cell-surface glycosylphosphatidylinositol (GPI) lipid, is believed to be only part of a larger signaling receptor complex. It is interesting that while Nogo is expressed in CNS but not in PNS, the Nogo-66 receptor is expressed in the brain (CNS) and dorsal root ganglia (PNS), as well as the heart and kidney.

Nogo shows homology to a family of proteins of unknown function, called reticulons, so named because of their localization in the endoplasmic reticulum (ER). Like the reticulons, Nogo is also retained in the ER and how it reaches the inner and outer leaflets of myelin (GrandPre et al., 2000; Chen MS et al., 2000) is still unclear. It should be pointed out that Nogo is not found on the outer membrane of myelin sheaths (Qiu et al., 2000) and it has been suggested that Nogo plays its role as an inhibitor of CNS axonal regeneration only after damaged myelin releases it to the extracellular environment (Goldberg and Barres, 2000a).

Myelin-associated glycoprotein (MAG)

The second myelin-associated protein that has been well-characterized as a potent inhibitor of neurite outgrowth is called myelin-associated glycoprotein (MAG) (Shen et al., 1998; McKerracher et al., 1994). MAG belongs to the immunoglobulin (Ig) super family of cell-surface transmembrane proteins and is present both in CNS and PNS myelin. In the post-injury CNS, MAG becomes exposed to regenerating axons either as a cell-surface form due to the damage of myelin or as a soluble form due to the proteolytic release of its entire extracellular domain (dMAG), which is highly inhibitory to axonal regeneration in vitro (Tang et al., 1997a, 2001). Interestingly, in the post-injury PNS, expression of MAG in Schwann cells is down-regulated along with other myelin proteins (Martini and Schachner, 1988; Fawcett and Keynes, 1990) while axonal regeneration does not take place until existing myelin debris is cleared during Wallerian degeneration (Brown et al., 1991).

This expression pattern of MAG probably accounts for its lack of inhibitory effect, despite its presence, in the injured PNS.

The best in vivo evidence for the inhibitory effect of MAG on axonal regeneration probably came from two different mutant mouse lines. First, in MAG-knockout mice, which maintain normal myelin until demyelination occurs at 8 month of age, injury to the CNS is followed by the regeneration of longer and more abundant axons when compared with wildtype (Li et al., 1996), although the difference is relatively subtle and not overwhelming probably due to the fact that other inhibitory factors (besides MAG) also exist in the CNS, such as Nogo (Qiu et al., 2000). Second, in C57BL/Ola mice, the post-injury PNS undergoes a delayed and slow Wallerian degeneration, subsequently failing to clear the myelin debris and resulting in very poor nerve regeneration (Brown et al., 1992; Bisby and Chen, 1990). However, in C57BL/Ola mice that are also MAG-deficient, greatly improved regeneration with twice as many regenerating axons as in regular C57BL/Ola mice could be observed (Schafer et al., 1996). Thus MAG is likely to play a role in influencing nerve regeneration in both CNS and PNS.

The receptor for MAG has not been identified yet, but is believed to be a sialoglycoprotein (Kelm et al., 1994; DeBellard et al., 1996) since MAG binds to sialic acid on neurons utilizing its Arg118 amino acid residue, which when mutated abolishes both binding and the inhibitory effect of soluble MAG on extending axons (Tang et al., 1997b).

Axon guidance molecules are possible inhibitors of CNS regeneration

Over the past five years, there has been an explosion of information regarding the molecular mechanisms of embryonic axonal guidance. A number of new guidance molecules have been identified in the developing CNS and, while some act primarily as chemoattractants (e.g. netrin), a much longer list of them act primarily as chemorepellants (e.g. semaphorins, ephrins, Ephs, slits and RGM "repulsive guidance molecule"), even though many of them can either attract or repel growing axons depending on the context of the neurons and the presence of other molecules involved (Dickson et al., 2001). To date, direct in vivo evidence is still lacking, but circumstantial evidence is supporting the possibility that those chemorepellants that either maintain a significant level of expression in adulthood or become up-regulated following injury, can play a major role in inhibiting successful nerve regeneration.

Semaphorins

The one family of guidance molecules that has been most often implicated to play a role in nerve regeneration is the semaphorins (Pasterkamp and Verhaagen, 2001; Gavazzi 2001), initially identified as oligodendrocyte-expressed collapsin due to its potent ability to cause growth cone collapse in embryonic chick sensory neurons (Luo et al., 1993). This family includes at least 20 members that are divided into 8 classes according to their structural characteristics (Raper, 2000)—some secretory (classes 2, 3 and viral), some transmembrane (classes 1, 4, 5 and 6) or membrane-anchored (class 7). The receptors for the secreted form of semaphorins,

neuropilin-1 and -2, are members of the immunoglobulin superfamily (He and Tessier-Lavigne, 1997), but due to the lack of a substantial cytoplasmic signaling domain, another protein partner is required to effect signal transduction. The protein partner is likely to be one from the family of large receptor tyrosine kinase called plexins (Takahashi et al., 1999; Rohm et al., 2000), themselves being the receptors for the transmembrane form of semaphorins (Tamagnone et al., 1999).

Most of the data relevant to the role of semaphorins on nerve regeneration has been obtained from studies on semaphorin 3A (Sema3A), the original secretory collapsin-1 (Pasterkamp and Verhaagen, 2001; Gavazzi 2001). While it is known that Sema3A knockout mice (Behar et al., 1996) and neuropilin-2 knockout mice (Chen H et al., 2000) display abnormalities in CNS and PNS wiring during embryonic development, studies on their response to nerve injury is still underway. Neuropilin-1 knockouts, which exhibit even more severe phenotypes than Sema3A knockouts, unfortunately, are embryonic lethal around E12.5 (Kitsukawa et al., 1997). However, according to a number of studies on normal mice, the general patterns of expression of Sema3A and neuropilin-1 appear to correspond to the degree of success of nerve regeneration in the particular systems.

First of all, in all the cases examined, the expression of neuronal neuropilin-1 persists into adulthood and is, following lesion, either up-regulated (as in CNS neurons of rubrospinal tract (Oschipok et al., 1999) and DRG neurons of sciatic nerve (Gavazzi, 2000)) or maintained (as in olfactory receptor neurons of olfactory nerve (Pasterkamp et al., 1998a) and spinal motor neurons in sciatic nerve (Gavazzi,

2000)). Although the significance of such expression pattern of neuropilin is unclear, it may be interpreted as a step toward heightened sensitivity by regenerating neurons to Sema3A in the environment, possibly to avoid sprouting into inappropriate Sema3A-positive areas (Gavazzi, 2001).

Second, the expression of Sema3A mRNA (as antibody to Sema3A was not available), is high at the lesion site in cases where regeneration usually fails to occur and is low or absent in cases where regeneration usually succeeds. For example, a stab lesion of the adult spinal cord (failed regeneration) induces the expression of Sema3A at the wound site while a similar lesion of the neonatal spinal cord (successful regeneration) does not (Pasterkamp et al., 1999a). In the adult PNS, which can successfully regenerate, sciatic nerve crush does not induce the expression of Sema3A at the lesion site (Pasterkamp et al., 1998b). As for the olfactory nerve, a peculiar part of the CNS where neurons continuously undergo vigorous mitosis and remodeling, lesion to the olfactory bulb (bulbectomy) leads to progressive increase of ema3A expression by the cells at the wound site, which, apparently, prevents generating axons from growing pass the lesion site (Pasterkamp et al., 1998a). On other hand, severing of the olfactory nerve (axotomy) only induces a transient ression of Sema3A by the cells at the wound site, which align the regenerating s but do allow the axons to grow around them and beyond the wound site. All observations are consistent with the interpretation that Sema3A acts as an tory factor of axonal regeneration where regeneration is unsuccessful.

Netrins

Vertebrate netrins were first identified as soluble chemoattractants secreted by floor plate cells in the embryonic spinal cord, in which commissural neurons were guided from the dorsal spinal cord towards the ventral midline (Serafini et al., 1994; Kennedy et al., 1994). Netrins may be considered related to laminin as it shows homology to the N-terminal portion of the laminin γ-subunit. Two netrin receptor families have been identified: DCC (Deleted in Colorectal Cancer, or UNC-40, or neogenin) and UNC-5 (or UNC-5H in vertebrates) (Fazeli et al., 1997; Leonardo et al., 1997). It is now clear that netrins can be attractive (when bound to DCC alone) or repulsive (when bound to a complex of DCC and UNC-5H) depending on the kinds of receptor present on the growth cone (Hong et al., 1999). Furthermore, a low intracellular cAMP level in the growth cone of *Xenopus* spinal neurons can switch the attraction by netrin into repulsion (Ming et al., 1997). Certain extracellular molecules, such as laminin and slit, when simultaneously bound to growth cones, can also reverse (Hopker et al., 1999) or silence the effect of netrin (Stein and Tessier-Lavigne, 2001).

Is netrin expression recapitulated in nerve regeneration as in embryonic evelopment? In the development of the eye, retinal ganglion cells (RGCs) have to end out of the retinal area and into the optic nerve. It has been shown that this cess requires the expression of netrin-1 in the optic nerve head region where C axons enter the optic nerve and the expression of the netrin receptor DCC on RGC axons (Deiner et al., 1997). Interestingly, no netrin-1 expression could be

found at the optic nerve head in adult rats before or after lesion (Petrausch et al., 2000) and the expression of DCC and UNC-5H2, which are both expressed by adult RGCs, were down-regulated to undetectable within 2 days after lesion (Petrausch et al., 2000; Ellezam et al., 2001). This appears to imply that part of the failure of RGCs to regenerate towards the optic nerve head may be due to a lack of netrin attraction from the target and a concurrent lack of sensitivity to netrin by the RGC axons. On the other hand, in adult goldfish optic nerve, which does successfully regenerate, both expression of netrin-1 by the optic nerve head and of netrin-receptor by the RGCs were observed before and after lesion (Petrausch et al., 2000). Apparently, the netrin guidance system continues to be functional in goldfish throughout adulthood.

In the adult rat sciatic nerve, netrin-1 mRNA is normally expressed at a low level but, by 2 weeks after transection, dramatically increases by 40-fold (Madison et. al., 2000). The source of post-lesion netrin-1 is localized to Schwann cells in the injured nerve, which are in the position to guide regenerating axons towards their target. Information about DCC or UNC-5H expression is not yet available, but the above data is consistent with a possible role of netrin in determining the regenerative capacities of various neural repair system.

Ephrins and slits

Very little information has emerged regarding the relevance of other axon guidance cues (besides semaphorins and netrins) to nerve regeneration in adults. However, considering their role as attractants or inhibitors of neurite extension in

embryonic development, one cannot help but speculate their playing similar roles in adults, where the developmental expression of at least some chemorepellants (e.g. semaphorins in systems mentioned above and ephrinA's in the superior colliculus after optic nerve injury) are recapitulated during nerve repair (Pasterkamp et al., 1999b; Knoll et al., 2001a). Therefore, it is imperative that we are keenly aware of their developmental significance.

The ephrins are a family of cell-surface ligands that are either GPI-anchored (ephrinA1-A5) or transmembrane (ephrinB1-B3), and they bind to a family of receptor tyrosine kinases called EphA's (which, in general, bind ephrinA's) and EphB's (which bind ephrinB's) (Klein, 2001). Binding of ephrin (expressed by glial cells) to Eph (expressed by an axon) causes a contact-dependent repulsion of the growth cone. This molecular repulsive mechanism accounts for the precise retinocollicular mapping in rats, where the nasal retinal ganglion cells (RGCs, low EphA5 and -A6 expression) project to the posterior superior colliculus (SC, high ephrinA2 and -A5 expression) while the temporal RGCs (high EphA5 and -A6 expression) project to the anterior SC (low ephrinA2 and -A5 expression) (Frisen et al., 1998). In short, this arrangement of wiring allows the more sensitive neurons to avoid the ephrin chemorepellant and, in effect, the ephrin gradient in the SC has painted a topographic map for the incoming RGCs to find their appropriate targets. Such graded expression of ephrinA's is known to reappears in the SC following optic nerve injury.

Similarly, for vertebrates with binocular vision, the nasal RGCs cross the optic chiasm to the contralateral side while the temporal RGCs do not cross and stay on the ipsilateral side. In *Xenopus* undergoing metamorphosis, it was shown that ephrinB is expressed by glial cells at the chiasm to repel the EphB-positive RGCs, which turns out to be those destined to stay on the ipsilateral side (Nakagawa et al., 2000).

However, ephrins have been shown to act as chemoattractants or adhesives in other systems, such as the vomeronasal system (in which axons from the vomeronasal organ project to the accessory olfactory bulb in a topographical manner consistent with ephrin attraction) (Knoll et al., 2001b) and the closing of the neural tube (which requires adhesion mediated by ephrinA5 and a truncated form of EphA7) (Holmberg et al., 2000).

Furthermore, ephrinB can not only act as a ligand but also a signaling receptor. Thus cells engaged in ephrinB/EphB interaction can possibly have forward or reverse signaling, or even both (bidirectional signaling) (Bruckner et al., 1997). Situations like this has been studied in the acallosal double knockout mice (EphB2- and EphB3-deficient) in which the ablated genes are normally expressed by the forebrain midline glial cells instead of the crossing neurons. (Orioli et al., 1996)

Slit is a secreted ECM protein that primarily acts as a chemorepellent (Broude et al., 1999) but can also act as a positive regulator of sensory axon elongation (Wang et al., 1999). Both netrin and slit are expressed by the midline glial cells in the developing spinal cord, while the receptor for slit, called Robo (roundabout), is up-regulated in commissural neurons only after crossing the midline (Stein and

Tessier-Lavigne, 2001). This special arrangement accounts for the extension of the axons towards the midline due to netrin attraction and then away from the midline due to slit repulsion. The axons lose responsiveness to netrin at the midline after crossing because the slit-bound Robo receptor forms a receptor complex with DCC (the netrin receptor) and causes a silencing of netrin attraction. Thus the axons only cross the midline once and only once, unlike the robo-deficient mutant which is not able to silence the attraction by midline netrin (Kidd et al., 1999).

Slit has also been shown to repel axons of spinal motor neuron, olfactory neuron and hippocampal neurons, causing growth cone collapse in vitro (Nguyen Ba-Charvet et al., 1999; Li et al., 1999). It probably plays a role in axonal guidance at the optic chiasm, since a Robo2-deficient zebrafish mutant "astray" exhibits abnormal midline crossings and wandering growth cones at this region (Fricke et al., 2001). However, virtually no published data are available regarding the expression patterns of slit and robo in adult nervous tissue before and after lesion. At present, its significance in nerve regeneration is unknown.

Model 2: differences in intrinsic neuronal state

Returning back to the question of differential regenerative capabilities of CNS and PNS, we next explore the possibility that CNS neurons themselves are fundamentally different from PNS neurons in several aspects relevant to regeneration. Undoubtedly, retinal ganglion cell (RGC) axons are capable of regenerating through a sciatic nerve graft, but according to David and Aguayo (1981), only 5-10% of the

RGCs actually regenerate, mostly those with cell bodies close to the graft site, and the axonal growth is clearly inferior to PNS neurons regenerating through the same environment. Furthermore, in vitro experiments showed that while both embryonic DRG and RGC neurons can grow long axons on laminin and astrocytes, RGCs of more mature and adult stage lose much of this ability while adult DRG neurons remain capable of growing (Bates and Meyer 1994; Bahr et al., 1995). GAP-43 (growth-associated protein of 43kd), a protein thought to be required (but not sufficient) for axonal growth (Benowitz et al., 1997) and a reliable indicator of regenerative ability, remains expressed in adult PNS neurons and is up-regulated after axotomy. In the adult CNS, such as the rubrospinal tract and RGCs, GAP-43 expression is absent and, after axotomy, is either not induced or induced only in the few neurons that regenerates into a PNS graft (Tetzlaff et al., 1991; Schaden et al., 1994). Apparently, CNS neurons experience an age-dependent decrease in regenerative potential much more than PNS neurons.

But what cellular and molecular mechanism account for such dramatic differences between CNS and PNS neurons? It appears that the intrinsic states of these two neuronal types differ in at least three ways: (1) the requirements for neuronal survival, (2) the ability to respond to trophic factors and (3) the responses to inhibitors of axonal growth (Goldberg and Barres, 2000b; Qiu et al., 2000; Fawcett 1997). Interestingly, these three issues may be tied together under one common theme: endogenous neuronal cAMP level.

The requirements for neuronal survival

Following axotomy of the optic nerve, most (about 65%) of the RGCs die by apoptosis, which can be rescued by overexpression of Bcl-2, a potent inhibitor of programmed cell death (Cenni et al., 1996; Bonfanti et al 1996) while some more die by necrosis due to mechanical or inflammatory damage (Thanos et al 1993; Cui and Harvey 1995). Most PNS neurons, however, do not die after axotomy (Meyer-Franke et al., 1995). Since it is known that the survival of neurons is dependent on trophic factors supplied by neighboring cells, which then suppress the intrinsic suicide program of the neurons (Raff 1992; Jacobson et al 1997), it is thought that axotomized RGCs die because of deprivation of target-derived neurotrophic factors which are normally transported retrogradely to the cell bodies (Bhattacharyya et al., 1997; Senger & Campenot 1997). Therefore, it came as a surprise when attempts to rescue axotomized RGCs by delivery of neurotrophic factors, including NT-4/5 (Clarke et al., 1998), BDNF (Di Polo et al., 1998) and GDNF (Koeberle and Ball, 1998), had resulted in only weak or transient effect. What indeed is required to maintain CNS neuronal survival?

Studies of neurons in culture suggest that simultaneous presence of multiple trophic factors are required to keep alive spinal motor neurons (Hanson et al., 1998) and RGCs (Meyer-Franke et al., 1995). In addition, electrical activity, provided either by depolarization or by cAMP elevation with CPT-cAMP (chlorphenylthio-cAMP, a non-degradable analog of cAMP), is also necessary to induce the survival effect of the neurotrophic factors. Thus, RGC survival can be maintained by a combination of

BDNF, CNTF and IGF-1, if they are also depolarized or elevated in intracellular cAMP (Meyer-Franke et al., 1995, 1998). It has been proposed that depolarization can activate ras through a calcium-dependent mechanism (Farnsworth et al., 1995) and the activation of ras can then activate MAP kinase and PI-3 kinase, leading to trophic response of the neurons (Datta et al., 1999; Grewal et al., 1999). On the other hand, PNS neuronal survival requires only a single trophic factor, even in the absence of cAMP elevation, to inhibit the cell death program. For example, NGF is sufficient to maintain sympathetic neurons and DRG neurons, while CNTF is sufficient to maintain ciliary neurons in vitro (Hamburger, 1993; Barde, 1994). In this sense, the requirement for survival is much less demanding for PNS neurons and, considering that neurotrophins can be obtained from dedifferentiating Schwann cells (after axotomy) (Matsuoka et al., 1991; Meyer et al., 1992) or from themselves (as an autocrine source) (Acheson et al., 1995), PNS neurons have a very good chance of staying alive after injury.

The ability to respond to trophic factors

There appears to be yet another reason why electrical activity is required for CNS neuronal survival. CNS neurons in culture, such as RGCs and small motor neurons, normally express only a low-level of cell-surface TrkB, the receptor for BDNF. Following depolarization or cAMP elevation, the percentage of neurons with high level of cell-surface TrkB greatly increases and the level of cell-surface TrkB increases by an average of fourfold. This phenomenon apparently occurs without the

synthesis of new protein, but rather by the rapid recruitment of vesicular TrkB to the cell-surface (Meyer-Franke et al., 1998), which is a mechanism well-documented in a number of other cases where hormonal elevation of cAMP causes a transient appearance of ion pumps or transporters on the cell surface, such as the insertion of the water channels aquaporin into the apical surface of collecting duct cells in response to ADH-induced (anti-diuretic hormone) cAMP surge (Katsura et al., 1997). In the contrary, PNS neurons, such as nodose ganglion neurons, display a high level of cell-surface TrkB regardless of the level of intracellular cAMP.

Hence, when electrical activity ceases following CNS axotomy, the intracellular level of cAMP drops, resulting in a low level of neurotrophin receptors on the cell surface, which accounts for the lack of responsiveness to trophic stimulation. In vivo data is also consistent with this hypothesis: the lost of response to BDNF and CNTF (measured by the ability to translocate MAP kinase to the nucleus) in axotomized RGCs in intact retina can be restored by elevation of cAMP (Shen et al., 1999). In conclusion, CNS neurons die after axotomy because, first, they lose the source of target-derived neurotrophic factors which are not replenished by CNS glial cells as they are in the PNS, and second, even if the trophic factors are present, they lack the receptors to receive the stimulation (Goldberg and Barres, 2000b).

The responses to inhibitors of neurite outgrowth

Several recent in vivo experiments had managed to produce extensive axonal outgrowth into "inhibitory" CNS tissue using various manipulations, but without the

use of neutralizing agents against inhibitors of neurite outgrowth. For example, Berry et al. (1996) were able to produce extensive RGC axonal regeneration into the severed optic nerve distal to the site of lesion by placing a piece of PNS tissue explant into the vitreous body of the eye. By implanting a piece of embryonic nerve tissue in the lesion site of an injured rat spinal cord and artificially filling the implant with BDNF or NT-3, Bregman (1998) was able to induce extensive axonal growth, not only into the implant, but also into the white matter of the host rat. Finally, Neumann and Woolf (1999) showed that, if the peripheral branch of the sciatic nerve is transected one week ahead of time (preconditioning lesion), extensive axon outgrowth of DRG neurons into the spinal cord will occur following a transection of the dorsal column. In these three cases, how can the axons regenerate into the CNS tissue, filled with myelin-associated and extracellular inhibitors of axon outgrowth?

It turns out many of the "inhibitors" and guidance cues we mentioned before can either be attractive, repulsive or neutral, depending on the state of the neurons, such as cytosolic cAMP levels and stimulation by neurotrophins. For example, the attraction of netrin-1 to *Xenopus* spinal neurons, can be switched to repulsion by a reduction of intracellular cAMP level (Ming et al., 1997). The repulsion of Semaphorin 3A (collapsin-1) can be switched to attraction by altering the level of cGMP, which occurs during neuropilin-1-L1 complex formation following homophilic binding of extracellular L1 (Castellani et al., 2000). Furthermore, BDNF sensitizes and NGF desensitizes the response of DRG neurons to Sema3A in terms of growth cone collapse, probably via the ligand-dependent formation of trkA/B-neuropilin-1

complex (Tuttle and O'Leary, 1998). More significantly, the inhibition of DRG and RGC axonal outgrowth by MAG and myelin fragments (which presumably contain Nogo) can be blocked by either a pre-exposure to neurotrophins (priming) or artificial elevation of cAMP (Cai et al., 1999). This phenomenon probably accounts for the developmental switch from growth promotion to inhibition of DRG (at P3-4) and RGC axons (at birth) by MAG, which occurs with a concomitant fall in endogenous cAMP level (Cai et al., 2001).

Thus, the insertion of a PNS explant into the eye (Berry et al., 1996), the pumping of BDNF or NT-3 (Bregman, 1998), and the pre-conditioning lesion of the peripheral branch of the sciatic nerve (Neumann and Woolf, 1999) probably worked by elevating the intracellular cAMP level of the regenerating axons and causing them to interpret the many CNS inhibitors as non-inhibitory (Qiu, 2000). The neurotrophins widely available in the injured PNS, therefore, serve not only as survival factors but also as blockage to any possible inhibitors that the regenerating axons may encounter on their tracks, probably by elevating the intracellular cAMP level of the axons. If this principle can be clinically applied to the CNS, it has the potential of blocking multiple axon outgrowth inhibitors with a single treatment.

Model 3: differences in the secondary events following axotomy

Astrocytic scarring

Following injury of the CNS but not PNS, reactive astrocytes at the lesion site form a "glial scar", presumably an attempt to preserve the blood-brain barrier by

quickly sealing off the wound site. However, inside this astrocytic scar are dense matrix materials such as chondroitin sulfate proteoglycan (CSPG), tenascin-R, phosphacan, neurocan and semaphorin III (Levine 1994; Canning et al., 1996; Laywell et al., 1992; McKeon and Buck, 1997; Pasterkamp et al., 1998a), many of which have been shown to be inhibitory to neurite outgrowth and are believed to have the same effect on CNS regenerating axons (Fidler et al., 1999; Snow et al., 2001, 1990; Becker et al., 2000; Lochter et al., 1991; Faissner and Kruse, 1990). Moreover, the processes of astrocytes surrounding the scar tissue become interlinked with tight junctions, possibly forming a physical barrier to growing neurites (Reier et al., 1983).

In fact, some researchers argue that the astrocytic scar tissue may be an even more significant inhibitor to CNS regeneration than myelin-derived inhibitors. Davies et al. (1999, 1997) used a microinjection technique to transplant small number of adult rat DRG neurons into the dorsal column of the spinal cord between C1 and C2 vertebrae and, at the same time or 2 weeks ahead of time, transected the dorsal column between C4 and C5 vertebrae. They observed that when care was taken not to traumatize the injected tissue, no astrocytic scar was formed at the injection site and the DRG axons regenerate extensively through a territory full of astrocytes and degenerated white matter, stopping only when the growth cones become dystrophic on contact with the glial scar matrix at the center of the lesion site, which is particularly rich in chondroitin sulfate proteoglycan. Why the regenerating axons were not stopped by the degenerating myelin was not fully explained by the authors,

but other researchers suggested that under these conditions the myelin was not damaged and, therefore, the myelin inhibitors were not exposed to the axons (Cai et al., 2001). It should also be pointed out that the transplanted neurons which Davies et al. used was peripheral (as in PNS) in origin. Thus the response of these neurons may not be completely applicable to axonal regeneration from a CNS source.

Macrophage invasion

In the PNS, however, no scarring occurs following nerve transection but a different kind of secondary events occur: invasion of peripheral macrophages in the distal segment. These inflammatory macrophages are instrumental in triggering the cascade of events that follow PNS injury (Lunn et al., 1989): when macrophages were prevented from entering a piece of excised rat sciatic nerve placed under the kidney capsule by means of a small-pore filter, the nerve remained intact and Wallerian degeneration did not occur (Beuche and Friede, 1984, 1986). However when the pores of the filter used were large enough for macrophages to pass through, the nerve readily undergo Wallerian degeneration as in an actual nerve injury. Furthermore, in C57BL/Ola mice where macrophage invasion is delayed due to a genetic mutation (Bisby and Chen, 1990) and in mice intravenously injected with antibody against the complement type 3 receptor (which interferes with macrophage migration across endothelium) (Rosen and Gordon, 1987), nerve regeneration was greatly impaired.

What important functions are macrophages performing in the post-injury PNS? Brown et al. (1991) has shown that it is the Interleukin-1 (IL-1) secreted by the invading macrophages that actually induces the Schwann cells to shed their myelin and to proliferate. Macrophages as professional phagocytes are important in clearing away the islands of degenerated axons and shed myelin (Beuche and Friede,1986), which may carry inhibitor of axonal growth, such as MAG (Schafer et al., 1996). As in other examples of wound healing, the macrophages probably play a role in remodeling the damaged extracellular matrix, evidenced by their up-regulation of certain matrix metalloproteinases and metalloproteinase inhibitors (La Fleur et al., 1996). All of these events are believed to facilitate successful nerve regeneration.

In the optic nerve of the CNS, however, macrophage invasion following lesion is very limited and is restricted to the immediate wound site (Perry et al., 1987), possibly due to the lack of expression of ICAM-1 in the endothelium of the post-lesion optic nerve (Castano et al., 1996). Some researchers regard this characteristic as an adaptation of the "immune-privileged" CNS to prevent any invading cells of the immune system from disrupting its intricate neuronal network (Schwartz and Moalem, 2001) but they also suggest that such characteristic may have contributed to the failure of the CNS to regenerate (Lazarov-Spiegler et al., 1998a). To test this possibility, Lazarov-Spiegler et al. (1998b) pre-incubated peripheral blood macrophages with PNS tissue and applied them to the lesion site of optic nerve or spinal cord. They observed successful infiltration of the implanted macrophages into the distal segment of the injured nerve, speedy clearance of myelin within, and much

improved axonal regrowth. For the transected spinal cord, partial locomotor activity was recovered. Thus, infiltration of macrophages into the CNS can indeed facilitate its success in regeneration.

Conclusion

It is clear that the cellular and molecular events involved in nerve regeneration are multidimensional. As more and more scientists began to realize, in order to effect successful repair of the injured CNS, a combination of strategies are likely to be necessary to address the many obstacles we have described. As we have been doing in this review, to better understand these obstacles, we need to examine the events that lead to the failure of CNS regeneration as well as those that lead to the success of PNS regeneration. It is the latter that this thesis project will be focusing on.

CHAPTER 2 :

Characterization of DRG Neurite Outgrowth in Normal and Pre-injured Nerve Cryosections of Adult Mouse

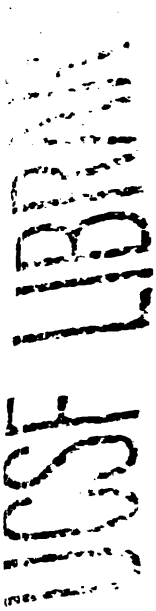
INTRODUCTION

It is well known that the mammalian peripheral nervous system (PNS) is capable of repairing itself after injury. This phenomenon, called nerve regeneration, has been the subject of research for over a century since the time of Ramon y Cajal (1913). In addition to its important implications to medicine, the nerve regeneration system also presents many intriguing questions to the field of cellular neurobiology in basic research. For example, neuroscientists have been trying to find a way to explain the fact that, in mammals, while the PNS can successfully regenerate, the central nervous system (CNS) completely lacks this capability (Perry et al., 1987).

In the 1980s, a series of nerve grafting experiments by Aguayo (Bray et al., 1987) brilliantly showed that axons of the CNS neurons are perfectly capable of re-growing when given the right environment. Since then, the typical hypotheses to explain the PNS versus CNS disparity have generally fallen into one of two forms: (1) "The mammalian CNS cannot regenerate because there exist certain *negative* factors in the CNS that are sufficient to inhibit regeneration. However, these negative factors are absent in the PNS," or (2) "The mammalian PNS can regenerate because there exist certain *positive* factors that are necessary to promote regeneration. However, these positive factors are absent in the CNS." Subsequently, depending on their

favorite experimental systems, the CNS or the PNS, the researchers would proceed to look for factors that fit hypothesis (1) or (2), respectively.

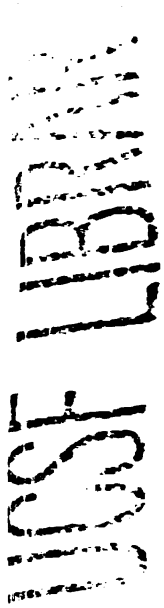
With an identified candidate factor, however, researchers generally have two major experimental approaches: in vivo and in vitro. The in vivo approach is to somehow alter this factor in vivo and see if that alters the regenerative behavior of the nervous system. For example, to test whether collagen promotes PNS regeneration, scientists used silicone tubing filled with collagen to connect the severed ends of the sciatic nerve and observed that the neurites could grow through the tube (Ohbayashi et al., 1996). This was also the case when two proteins found on myelin of oligodendrocytes, NI-35/200 (now called Nogo), were thought to play a role in inhibiting regeneration in the spinal cord (Schnell and Schwab, 1990). A pellet of hybridoma cells secreting a monoclonal antibody (IN1) against the proteins were implanted near the site of injury of the spinal cord and nerve regeneration could proceed to a much greater length than control. Thus NI-35/200, in their native forms, act as a negative factor to CNS regeneration.



The in vitro approach is to present purified neurons or neuronal cell lines in vitro with the candidate factor and see if the neurite outgrowth is affected. For example, Snow et al. (1990) plated PNS or CNS neurons onto dishes that were coated with laminin on one side and chondroitin sulfate on the other, and observed that the neurites originally growing on laminin would abruptly turn away when they encountered the laminin-chondrotin sulfate boundary. Thus it was concluded that chondroitin sulfate was inhibitory to nerve regeneration. Similarly, Martini and

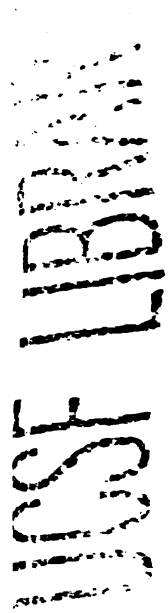
Schachner (1988; also Bartsch et al., 1995) observed extensive neurite outgrowth on the surface of COS cells that had been transformed to express MAG (Myelin-associated glycoprotein) and concluded that MAG-1 promoted neurite regeneration. Ironically, years later, also using the in vitro approach, another group found a fraction of CNS protein that inhibited neurite outgrowth in vitro to be MAG and concluded that it was a major inhibitory factor in CNS (Shen et al., 1998).

Unfortunately, each approach has its advantages and drawbacks. The in vivo approach allows one to gather data that are more directly applicable to the question on hand because the conditions more closely mimic that of actual nerve regeneration. However, it is often very difficult to specifically manipulate and control the intended conditions in vivo, not to mention the fact that it is time-consuming and expensive to prepare large number of animals to obtain a large data set. On the other hand, the in vitro approach allows researchers to reduce a complicated system into a simpler setup that can be easily manipulated and tested, making it more convenient to achieve a large sample size. However, for this very same reason, in vitro setups are often so simplistic that it fails to take into account some of the important aspects of the in vivo conditions. As a result, individual neurons taken out of their native environment may behave very differently in the culture dish than they would usually behave in the intact animal.



More recently, a compromised in vitro approach using unfixed longitudinal nerve cryosections has been used by several groups with a high degree of success with respect to their relevance with in vivo observations (Sandrock and Matthew,

1987; Carbonetto, 1987; Savio and Schwab, 1989; Crutcher, 1999; Watanabe and Murakami, 1990). Thick cryosections of a nerve do not contain any living cells but do contain all the native molecules of a nerve in their native configuration. These nerve cryosections could be presented to dissociated neurons or ganglion explants as a substrate of neurite outgrowth in culture dishes. Very often, by observing the behavior of the regenerating neurites, useful information about the permissiveness of the substrate could be obtained. For example Carbonetto et al. (1987) found that neurites of embryonic chick DRG (dorsal root ganglion) explants could extend on cryosections of adult rat sciatic nerve, goldfish optic nerve and embryonic rat spinal cord, but could not grow on adult rat optic nerve and spinal cord. It was then noted that the neural tissues of the earlier group can regenerate in vivo, while those of the latter group cannot. Thus the behavior of neurites in the cryosection system is believed to closely mimic that in vivo.



The current project began as a follow-up of one of such results reported by Bedi et al. (1992), who found that the adult rat sciatic nerve, initially a non-permissive substrate for neurites of dissociated adult dorsal root ganglion (DRG) cells, became increasingly permissive by 6 days after injury, implying that some sort of changes had to take place within the sciatic nerve to allow neurites to grow through. As a follow-up of Bedi et al.'s experiment, we presented cryosections of adult mouse sciatic nerve, both uninjured and pre-injured, to intact adult mouse DRG explants. We characterized the response of the DRG neurites, which, interestingly, turned out to be quite different from the observations by Bedi et al. We

found that, instead of a simple change in permissiveness, what actually changes after nerve injury is the ability of the nerve environment to allow regenerating neurites to remain stable and healthy. The implications of our observations are also discussed in this chapter.

METHODS AND MATERIALS

Operation of the mouse sciatic nerves

CF1 adult female mice between 2 to 4 months of age, unless otherwise stated, were used for the operation of sciatic nerves. The mouse was anesthetized by peritoneal injection of Avertin (2% tribromoethanol and 2.5% tertiary amyl alcohol in water) used at 0.13 ml per 10 g of body weight. After the fur of the legs was rinsed with 70% alcohol, a small (0.5 cm) incision of the skin was made at the mid-thigh level. From this point on, all surgical work was done under a dissecting microscope. Through this incision, a longitudinal white line of connective tissue which demarcated two pieces of thigh muscles was located and another small incision was made in the white line. When the two pieces of thigh muscles were separated apart, the sciatic nerve could then be located, very often found associated with a sizable blood vessel. To avoid excess bleeding during transection, the sciatic nerve was traced proximally to the point where the blood vessel deviated from the nerve. Still under the dissecting microscope, a pair of iris scissors was then used to transect the nerve with minimal disruption of the surrounding connective tissue. After the nerve

was observed to have been clearly cut apart, the two stumps were gently positioned such that the ends closely apposed each other. The skin was closed with a single wound clip. Usually, the sciatic nerve on the contralateral side was then similarly operated. After the operation, the animal was allowed to recover under monitoring for the next two hours.

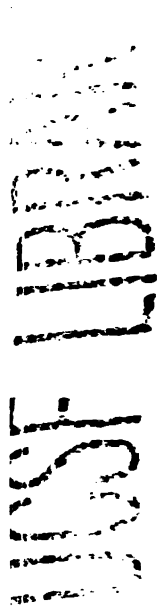
Preparation of cryosections of sciatic nerve

The sciatic nerve was excised in one piece from either unoperated or operated mice after they are sacrificed by cervical dislocation. The nerve was then placed in a 2 cm by 2 cm plastic mold with the "distal" and "proximal" ends labeled. The mold with the nerve inside was snap-frozen in liquid nitrogen for 2 minutes, after which it was temporarily stored in an ice bucket with dry ice. For long-term storage of up to 6 months, the molds and nerves were stored in Ziploc bags at –80°C.

Just before sectioning, the frozen sciatic nerve was embedded in the associated plastic mold with frozen section embedding medium (Histoprep from Fisher Scientific). 10 μm thick cryosections were picked up with 12-mm round glass cover slips (Fisher Scientific) that had been acid-washed, autoclaved and pre-coated with 0.1 mg/ml poly-D-lysine hydrobromide (43-100 kd, Sigma) in sodium borate solution. A dot was marked on the cover slip with a permanent marker to signify the distal end of the sciatic nerve. The cryosections were dried in air at room temperature for five minutes and, subsequently, stored for up to 2 days at –20°C until plating of dorsal root ganglia explants.

Dissection of adult mouse dorsal root ganglia (DRGs)

CF1 adult female mice between 2 to 4 months old were used for the source of adult DRGs. Before dissection, the mouse was sacrificed by cervical dislocation. The skin on the dorsal side was cut open at the mid-line in the anteroposterior direction. To isolate the vertebral column, two longitudinal cuts were then made in the musculature just lateral to the entire length of the vertebral column. The muscles dorsal to the vertebral column were cleaned away as much as possible to expose the bony vertebrae. The vertebral column, from neck to sacrum, was then completely removed from the rest of the body and placed on a piece of paper towel. Under a dissecting microscope, remnants of muscle attached on the dorsal side of the vertebrae were further teased away. Two longitudinal cuts were made on the lateral sides of the bony vertebrae using a pair of scissors with relatively thick beaks. The top bony plate was then lifted up, exposing the creamy white spinal cord underneath. The spinal cord was then slowly and gently lifted, leaving behind the round, button-like DRGs which sit in small sockets between every two vertebrae. After cutting the roots of the DRGs with a pair of fine scissors, the DRGs were then removed with a pair of fine forceps and placed in a 35-mm petri dish with 2 ml of Leibovitz-15 (L-15) culture medium at room temperature. In this way, 25 to 30 DRGs can be collected from the lumbar and thoracic areas of a single mouse within one hour.



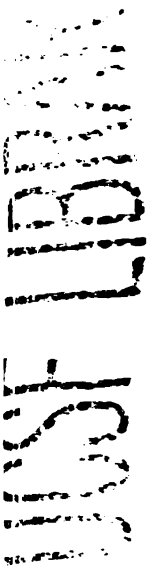
Preparation of adult mouse DRGs for in vitro reconstitution of nerve regeneration

After the dissection, all the DRG explants and the 2 ml of medium were transferred to a Falcon round-bottomed, snap-capped, polypropylene bacterial culture tube. Then 25μl stock collagenase D solution (100 μg/ml in PBS, lyophilized solid purchased from Boehringer Mannheim) was added to the medium to make a final concentration of 0.125% collagenase. Assuming the specific activity of the collagenase to be 0.5 U/mg, the test tube was incubated in 37°C for 1.0 hour, with one change of collagenase-L15 medium at midpoint of the incubation. For batches of collagenase with weaker activity, the time of incubation was proportionally prolonged. This formula was successfully applied on batches of collagenase D ranging from 0.17 to 0.52 U/mg in specific activity.

At the end of the incubation period, the medium was withdrawn and the DRGs were washed three times with 5 ml of calcium-magnesium-free PBS with 0.4% EDTA at room temperature. The purpose of the EDTA was to stop the collagenase activity. After washing, the DRGs were resuspended in culture medium (DME-H21, 10% fetal bovine serum, 50 ng/ml NGF (7s), 20 μM cytosine arabinoside, penicillin, streptomycin and gentamycin) and transferred to a clean 35-mm petri dish with a wide-mouth plastic dropper. The DRGs could be stored in 37°C incubator for up to three hours until plating.

Dissection of neonatal mouse DRGs

Newborn mice between 1 to 4 days of age were obtained from crossing CD1 males with CF1 females. After the newborn mice were sacrificed by decapitation, the dorsal skin was cut longitudinally at mid-line and sufficient musculature was removed to expose the backbone. Under a dissecting microscope, the jelly-like spinal cord was gently lifted away from within the backbone. A pair of round, semitransparent DRGs could be seen in the sockets between every two vertebral plates. By grabbing at the roots of the ganglia, the DRGs were then plucked off their sockets with a pair of fine forceps. Once a DRG was removed from the animal, it was transferred to a petri dish with DME-H21 medium and the roots of the DRG were trimmed off as much as possible with a scalpel. This step is both effective and critical in preventing Schwann cells from later overrunning the culture during the assay. Unlike the adult DRGs, the neonatal DRGs were immediately plated on the cryosections without storage or collagenase treatment.



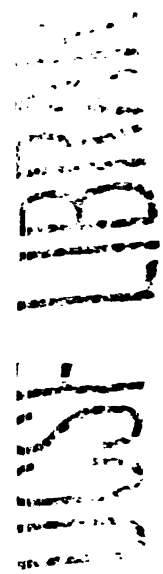
Setting up the in vitro reconstitution of nerve regeneration

Prior to plating of the DRGs, the glass cover slips carrying the sciatic nerve cryosections were placed in Nunc 4-well plates (one cover slip per well) and washed three times with PBS. After the last wash, 165 μl of culture medium (DME-H21, 10% fetal bovine serum, 50 ng/ml NGF (7s), 20 μM cytosine arabinoside, penicillin, streptomycin and gentamycin) was added to each well.

Under clean but non-sterile conditions at room temperature, a collagenase-treated adult DRG (in the petri dish of culture medium) was cleaned by teasing off as much as possible the associated irregular connective tissue with the aid of a dissecting microscope. This step turns out to be critical because if too much connective tissue remains with the DRG, the DRG neurites preferentially grow within the connective tissue and never extend onto the intended nerve cryosection or cover slip. It is also critical that this cleaning step is done with minimal manipulation because if the DRG loses it physical integrity, it will likely extend very few neurites.

The cleaned DRG was then placed in the middle and on top of the sciatic nerve cryosection on the cover slip in the Nunc 4-well plate. In this way, the DRG neurites would fall on both sides of the DRG and onto the cryosection. The cleaning and the plating of the 25 to 30 DRGs usually take 1.5 hours.

After all the DRGs were plated, the plates were then incubated in a moisture-saturated, 37°C incubator with 5% carbon dioxide for the desired period of time. The standard duration of incubation was 6 days. Feeding of the tissue cultures involved replacing 100 μl of the 165 μl culture medium in each well with fresh medium once every three days.

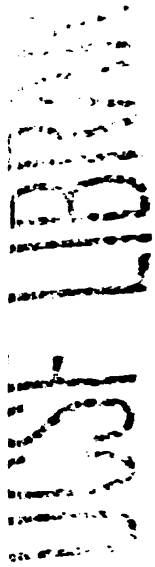


Immunostaining of DRG neurites

At the end of the incubation period, neurite growth within the cryosection substrate was visualized by immunostaining against GAP-43, a marker for rapidly growing neurites. The DRG culture was first fixed with 250 μl of 4%

paraformaldehyde, 0.075% saponin and 5% sucrose in PBS for 20 to 30 minutes at room temperature. The loading of the reagent was achieved by using two 1-ml syringes with 25G needles, one slowly injecting the fixative at one side of the well while the other withdrawing the culture medium from the other side at the same rate. The purpose was to avoid disturbing the delicate neurites by minimizing the change in the liquid level of the culture well. If there was too much liquid in the well, the DRG explant would float and tear off many neurites with it.

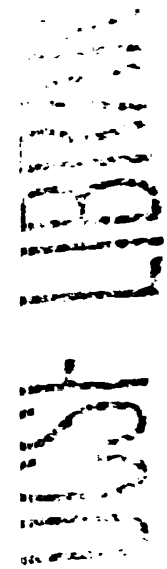
After fixation, the cover slip with the DRG explant was taken out of the Nunc 4-well plate and placed on a piece of dental wax. The DRG was rinsed with two drops of PBS dispensed from a 25G needle. This was followed by treatment with two drops of 0.1M glycine in PBS for 30 minutes to inactivate any remaining paraformaldehyde. Then the neurites were permeabilized with two drops of 0.1% saponin in PBS for 10 minutes. After permeabilization, the sample was pre-blocked with two drops of DME with 10% fetal bovine serum for 15 minutes. Dual staining was done with mouse IgG against rat GAP-43 (1:500, mouse monoclonal, clone 91E12, Boehringer #1379011) and rabbit IgG against bovine neurofilament-200 (1:200, rabbit polyclonal, Sigma #N-4142) diluted in DME with 10% fetal bovine serum. This was allowed to incubate inside a chamber with wet paper towel overnight at 4°C.



The following day, the samples were washed three times with 2 drops of PBS for 5 minutes each. Dual staining was done with lissamine rhodamine (LRSC)-conjugated donkey anti-mouse IgG antibodies and fluorescein

(DTAF)-conjugated donkey anti-rabbit IgG antibodies (both had been pre-adsorbed to prevent cross-species reaction, Jackson Immunoresearch Laboratories). These secondary antibodies were used at 1:100 dilution in DME with 10% FBS. This was allowed to incubate in the same moist chamber for 2 hours at room temperature.

Finally, each sample was washed with PBS for 5 minutes, washed with PBS with Hoechst 33258 (0.25 μg/ml, a fluorescent stain for nuclei) for 10 minutes and washed with PBS for 5 minutes. The Hoechst stain localizes non-neuronal cells such as Schwann cells and fibroblasts. The cover slip was then mounted upside down on a glass microscope slide with gelvatol (8 g polyvinyl alcohol (Monsanto) dissolved in 40 ml 0.2M Tris at pH 8.5 by warming to 50-60°C, then mixed with 20 ml glycerol after cooling). A Zeiss Axiophot compound microscope equipped with epifluorescence was used to view the samples.

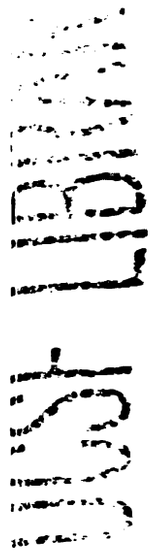


Other primary antibodies used (all at 1:200 dilution) in this studies include: anti-L1 (rat monoclonal, clone 324, Boehringer #1450 999), anti-MAPs (microctuble associated proteins) (rabbit polyclonal, Sigma #M-7273), anti-NCAM (rabbit polyclonal , Chemicon #AB1505), anti-synaptophysin (mouse monoclonal, clone SY 38, Boehringer #902314), anti-β-tubulin (mouse monoclonal, clone 2-28-33, Sigma #T-5293) and anti-ubiquitin (rabbit polyclonal, Sigma #U-5379).

RESULTS

Issues with using adult dorsal root ganglia explants

Besides more labor-intensive to obtain than embryonic DRGs, adult DRGs also require special attention during tissue culture. First, unlike embryonic DRGs, adult DRGs do not adhere to polylysine-coated glass and tend to float in culture medium, causing the neurites to not extend onto the substrate. To keep them stranded on the substrate without floating, we had to use a limited amount of culture medium just enough to cover the DRGs. Second, we found that freshly-extracted adult DRGs very rarely extend neurites onto the substrate. Scanning electron microscopy showed that the DRG neurons were vigorously growing neurites but the neurites remained within the DRG, probably due to its high content of connective tissue (data not shown). To overcome this problem, we carefully removed the connective tissue that ensheathed the DRGs, first chemically with collagenase digestion and then mechanically with fine forceps under microscope. These treatments, however, must not be excessive: over-incubation with collagenase or physically breaking the integrity of the DRGs would almost certainly cause the DRGs to fail to grow neurites.



Neurites of adult DRGs growing on cryosections of uninjured and pre-injured sciatic nerves are comparable in length

We first investigated the ability of adult DRG explants to extend neurites on uninjured versus pre-injured sciatic nerve cryosections and compare with that observed in the experiments of Bedi et al. (1992), who used adult DRG dissociated neurons. Therefore, we placed mouse DRG explants on 10 μm-thick cryosections of these two types of tissues and incubated in a serum- and NGF-containing medium for 6 days. The neurites of the DRG were then visualized by GAP-43 immunostaining and epifluorescence.

In agreement with the result of Bedi et al. (1992), we observed that adult DRGs grew extensive neurites on the distal segment of the 10-day post-transection sciatic nerve cryosections (fig. 2-1a, b), indicating that the post-injury mouse sciatic nerve was a permissive substrate for neurite regeneration.

However, unexpectedly, we also observed extensive neurite outgrowth of adult DRGs on cryosections of normal, uninjured sciatic nerve (fig. 2-1c, d), a very consistent result that has been observed in over 90% of DRGs cultured in more than a hundred experiments since then. In addition, the length of these neurites (16.5 $\pm$ 2.3 mm, mean $\pm$ standard error; n = 8, where n is the number of sample outgrowths) was comparable with that of neurites on 10-day post-transection sciatic nerve cryosections (14.8 $\pm$ 1.4 mm; n = 4), as measured by the length of the longest neurite in a sample using a microscope eyepiece fitted with calibrated reticule. The lengths of the neurites in the two cases were not significantly different.

This appears to be in contradiction with the observations of Bedi et al. (1992), who reported that dissociated DRG neurons were not able to extend neurites longer than three times the size of the cell body when plated on uninjured sciatic nerve cryosections. A comparison of the experimental conditions reveal the following three major differences between the two research groups: (1) the conditions of neurons: DRG explants versus dissociated DRG neurons, (2) the animal species: mice versus rats; (3) and the culture medium: 10% fetal bovine serum (FBS) versus 2% fetal bovine serum plus Bottenstein's N2 supplement (which is a mixture of insulin, transferrin, selenite, bovine serum albumin and NGF).

We tried to resolve the discrepancy by repeating the experiment but changing one or more of the above culturing conditions at a time. This included culturing (1) *dissociated* mouse DRG neurons on mouse uninjured sciatic nerve cryosections in 10% FBS (fig. 2-2a), (2) or in N2 plus 2% FBS (fig. 2-2b); (3) *rat* DRG *explants* on rat uninjured sciatic nerve cryosections in 10% FBS, (4) or in N2 plus 2% FBS; (5) *rat dissociated* DRG neurons on rat uninjured sciatic nerve cryosections in 10% FBS, (6) or in N2 plus 2% FBS. (data not shown for (3) to (6)) Notice that case (6) represents an exact duplicate of Bedi et al.'s experimental conditions. However, in all cases, we observed extensive outgrowth of neurites on adult uninjured sciatic nerve cryosections.

To date, we have been unable to explain the apparent difference between our results and that of Bedi et al., but it is not likely to be caused by the difference in our choice of DRG explants, animal model or culture medium. It is possible that the

care in washing and fixation that we took to preserve highly fragile neurites gave rise to the difference in our results. Nevertheless, the fact that we were able to produce extensive outgrowth, even in an exact duplicate of Bedi et al.'s conditions, argued in favor of the conclusion that adult DRG neurons are capable of outgrowing on adult uninjured sciatic nerve cryosection. However, as shown below, the neurite outgrowths on injured and uninjured nerve are qualitatively different.

Regenerating neurites degenerate in cryosections of uninjured nerve but not in cryosections of distal segment of pre-injured nerve

While extensive neurite outgrowths were observed in cryosections of both normal and pre-injured nerve, there was a qualitative difference in the outgrowths. On *uninjured* (but not post-transection) nerve cryosections, we consistently observed DRG neurites that were necrotic and hazy in appearance. In addition, there was a large amount of GAP-43-positive, globular, bead-like debris appearing along and near the neurites (fig 2-1c and d). We believe that the debris is the result of beading and fragmentation of degenerating neurites for several reasons: first, the debris is never present in areas of the cryosections where neurites are not in the vicinity; second, the cryosections themselves are completely GAP-43 negative and the neurites are the only materials that stained for GAP-43; and third, the size of the debris is always about the width of the neurites.

Did the neurites degenerate because of poor culture conditions? Two observations argued against this possibility. First, these beads of debris were only

found associated with the neurites located on the nerve cryosection, but not with the neurites located on the polylysine-coated glass cover slip in the same culture well (fig. 2-3a and b). Therefore, the degeneration is due to a certain property of the nerve cryosection with which the neurites were in contact but not with other factors such as culture medium or incubation temperature. Second, on cryosections of 10-day post-transection sciatic nerve, the DRG neurites were healthy and solid in appearance, while the beads of debris were present only in very small amount (fig. 2-1a and b). Since both types of samples were incubated with the same culture medium and conditions, it is not likely that any difference in the degree of neurite degeneration was caused by the culture conditions.

In order to further ascertain that the debris is not an artifact due to the fixation and immunostaining process, we had utilized a different and simpler vital staining method to visualize the debris and the neurites. The vital stain used, CFDA-SE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, Molecular Probes) can enter cells by diffusing through the cell membrane and, when cleaved by a cytoplasmic esterase, emitted green fluorescence under epifluorescence microscopy (fig. 2-4a and b). The fact that hazy neurites and beads of debris can be observed by this procedure, which took only five minutes and required only two changes of the medium and no fixation, supported the idea that the debris was not produced by the immunostaining procedure. Since CFDA-SE is a vital stain, the results also showed that the debris was once part of living cells, i.e. the neurites. (Note: In spite of its convenience, CFDA-SE vital staining was not usually used in our experiments

because the selective staining of neurites lasted only for about 10 minutes. After that, the cryosection, which also contained low esterase activity, became fluorescent as well.)

Using immunostaining and confocal microscopy co-localization, we found that the GAP-43-positive debris was also positive for several neural proteins, including NCAM, MAPs (microtubule-associated proteins), synaptophysin, tubulin and ubiquitin (fig. 2-5a to e). The debris, however, was negative or very weak for L1 (fig. 2-5f), neurofilaments (200-kd heavy subunit) (fig. 2-5g) and actin microfilaments (stained with FITC-conjugated phalloidin) (not shown). The absence of staining for the last two proteins could potentially be used as a marker for the degenerative debris. Moreover, since neurofilament is expressed relatively late compared to GAP-43, these data suggest that the neurites degenerate before becoming completely mature.

The following table summarizes the markers used to characterize DRG neurites cultured on sciatic nerve cryosections:

Markers	DRG neurites	Debris	Cryosection
GAP-43	+	+	–
NCAM	+	+	–
MAPs	+	+	–
synaptophysin	+	+	–
tubulin	+	+	–
ubiquitin	+	+	–
L1	+	–	–
neurofilaments (200kd subunit)	+	–	+
actin filaments	+	–	–

The time course of neurite degeneration on uninjured nerve cryosection in vitro

In our DRG-cryosection tissue culture, neurites first emerged from the DRG explant between 2 to 3 days after plating. Since the presence of debris was previously observed at 6 days after plating, we asked what was happening to the neurites before 6 days in culture. Did the degeneration process began as soon as the neurites came in contact with the uninjured nerve cryosections? Or did the neurites remain healthy on the cryosections for a period of time before degenerating? The answer could be important in understanding the nature of the interactions between the neurites and the nerve environment during in vivo regeneration.

In a timed-series experiment, we compared the conditions of the DRG neurites on uninjured nerve cryosections after 3, 4, 5 and 6 days in culture. This experiment was performed twice, with 4 samples each. At 3 days in culture, the outgrowth of neurites was sparse and they had been in contact with the cryosection for only 1 day or less. We found that the neurites were brightly and evenly stained, well defined and healthy in appearance. However, even at this early stage, many of the neurites were associated with globular debris (fig. 2-6a), unlike the debris-free neurites that grew on the glass coverslip. This seems to suggest that the debris begin to form almost as soon as the neurites contact the nerve cryosection. By comparison, DRG neurites growing on 10-day post-transection sciatic nerve cryosections (3 days in culture) were almost free of globular debris (fig. 2-6f).

Between 3 to 6 days in culture, the length and quantity of the neurites increased with time, while the amount of globular debris also increased proportionately. The globular debris did not appear all of a sudden at a certain time point, but rather, appeared simultaneously with the appearance of the neurites (fig. 2-6a to d), which also gradually became more discontinuous and more hazy. The fact that the neurites increased in length while degeneration was underway probably indicates that a significant portion of the neurites were able to continue vigorous growth while others were in the process of degeneration.

The DRG neurites on uninjured nerve cryosections continued to deteriorate beyond 6 days in culture. By 9 days after plating, the vast majority of the neurites appeared highly degenerated, very fuzzy, beaded and fragmented (fig. 2-6e). The degeneration, however, was restricted to neurites in contact with the nerve cryosection while those growing on the glass coverslip remained debris-free and solidly-stained for GAP-43.

In comparison, DRGs plated on 10-day post-transection nerve cryosections remained relatively free from debris and maintained a healthy GAP-43 staining from 3 to 9 days in culture (fig. 2-6f to i), demonstrating that the degeneration of the neurites on uninjured nerve cryosection was likely caused by certain intrinsic properties of the uninjured nerve, which are absent in pre-injured nerve.

Taken together, these observations suggested that whatever properties of the normal nerve that cause regenerating neurites degenerate disappeared from the

sciatic nerve within the first 10 days after a transection injury such that regenerating neurites could grow stably, at least in our culture system.

A semi-quantitative scoring method for measuring the degree of neurite degeneration

As we proceeded to examine DRG neurite outgrowth on nerve cryosections under various conditions, it became apparent that a uniform system for measuring the degree of neurite degeneration was necessary to compare different groups of samples within an experiment, as well as samples from one experiment with those of another. For this purpose, we devised a "degeneration score" for neurite outgrowths with a scale of 1 to 6 ("1" representing extremely degenerated while "6" representing completely healthy neurite outgrowth). Figure 2.7 displays typical neurite outgrowth from each point scale of the 6-point scoring system.

The two major criteria of scoring are (1) the health condition of the neurites and (2) the amount of globular debris relative to the amount of neurites. In general, a sample with a low score tended to exhibit necrotic neurites with hazy, discontinuous and poorly-defined GAP-43 staining, and a lot of globular debris along the neurites. On the other hand, a sample with a high score tended to have neurites with solid, continuous and well-defined GAP-43 staining, with few globules of debris. Since this scoring method works best when the samples being compared exhibit comparable amount of neurite outgrowth, we restricted its use to DRG neurite outgrowths that were in culture for 6 days, but not other duration.

The ability of the sciatic nerve to support stable neurite regeneration peaks at 8 days after transection injury

Some kind of changes happen within the first ten days following nerve transection, turning the distal segment into a substrate that supports stable neurite outgrowth. We wanted to examine the time course of these in vivo changes, as measured by the DRG-cryosection assay method. How much time after injury did it take for the transformation of the nerve environment to happen? How long does the nerve environment remain supportive of stable neurite outgrowth?

To do this, we cultured adult mouse DRGs on cryosections from mouse sciatic nerves that were harvested 0 (uninjured), 4, 6, 8, 10, 16 and 20 days after transection. Six days later, we visualize the neurite outgrowths by GAP-43 immunostaining as before and blind-scored the condition of the outgrowths using the semi-quantitative method described above.

This experiment was repeated twice with 8 samples per time point, with qualitatively similar results. The result of one such experiment is shown in a series of seven graphs in Fig. 2-8, each representing the scores from DRG-cryosection cultures using sciatic nerve cryosections from one particular time point after injury. The gray vertical bar in each graph denote the range of degeneration scores assigned to the median 75% of the samples, which practically means all the samples except the one with the highest score and the one with the lowest score. The vertical bars are placed in the graphs only as a cue to clarify the trend of the scores.

The results show that the samples from any one particular time point encompassed a range of three scores. The uninjured sciatic nerves (t=0 day) yielded a low median score of about 2, meaning that the native sciatic nerve environment did not support stable neurite outgrowth (fig. 2-8a), as we had shown earlier. By 4 days after transection, the median score had moved to between 3 and 4, meaning that the nerve environment had changed considerably in just 4 days in response to the injury, resulting in a substrate that could support neurites to grow noticeably more stably than uninjured sciatic nerve could (fig. 2-8b). Sciatic nerves from 6 days after transection performed very similarly to that from 4 days post-transection, both producing a median score of between 3 and 4 (fig. 2-8c).

For a more rigorous way of analyzing the data, we performed a statistical test called Wilcoxon's two-sample test on the set of scores from t=0 day and those from t=4 days. The Wilcoxon's test, like Student's t-test, can be used to tell if two sets of data are significantly different from each other but is based on ranks instead of parametric values. The computed p-value (significance level) is 0.0029 ($p < 0.05$), which, by the 5% level criteria, means that the two sets of scores are significantly different, in agreement with our previous statement that the neurites grew noticeably more stably at t=4 days. The p-value for t=4 days versus t=6 days is 0.13, which is above 5%, meaning that the two sets of scores are not significantly different.

By 8 days post-transection, the peak of the curve had shifted further to the right such that the median score had increased to about 5. The neurites of this group were distinctly more healthy and solidly stained than those of any other time-points

(fig. 2-9d). The clean cryosection background also showed a lack of globular debris, implying that degeneration of neurites on such cryosections were at a minimum. Wilcoxon's test yields a p-value of 0.0009 between t=6 days and t=8 days, implying that the scores at t=8 days are significantly higher than those at t=6 days.

Interestingly, the ability of the sciatic nerve environment to support stable neurite outgrowth did not remain for long. By 10 days after transection, just two days after the nerve environment was supporting strong outgrowth of neurites, the degeneration score shifted back to the left to a median score of between 3 and 4. The neurites outgrowing on such cryosections were no longer as healthy as those on 8-day post-transection nerve cryosections, but were nonetheless distinctly more healthy than those on uninjured nerve cryosections (fig. 2-8e). Wilcoxon's test also verifies that the scores at t=10 days are significantly lower than those at t=8 days ($p = 0.0015$).

From this point on, the performance of the sciatic nerve was more-or-less maintained at the same level until at least 16 days post-transection (fig. 2-8f) (Wilcoxon's p=0.31 for t=10 days versus t=16 days: no significant difference). By 20 days after transection, however, the median score finally dropped back visually to the level of uninjured nerve (Wilcoxon's p=0.0030 for t=16 days versus t=20 days). For the regenerating neurites, the nerve environment at this point had practically returned to its previous uninjured state, since they could no longer grow in a stable manner (fig. 2-8g).

Eight days after transection correspond to the penetration of the first regenerating neurites into the distal segment of the sciatic nerve in vivo

What is the significance of this pattern of increase and decrease of the ability to support stable neurite outgrowth? Does this particular time scale correspond to any important events in nerve regeneration in vivo? In order to visualize the progress of the natural nerve regeneration at different time points, we immunostained adjacent longitudinal sections of the same sciatic nerves used in the previous time-course experiment for neurofilaments (NF-200) or L1, which are markers that disappear from the distal segment by 1 to 2 days after transection (data not shown). Therefore any staining that reappears after 3 days indicates the appearance of newly regenerated neurites from the proximal segment of the transected nerve.

The results, based on two experiments with one sciatic nerve per time-point, showed that by 4 days and 6 days after transection of the sciatic nerve, abundant regenerating neurites amassed at the proximal stump of the wound site but had not crossed the wound site into the distal stump (fig. 2-9b) These new regenerating neurites, when immunostained for NF-200, appeared as long, slender, wavy, threadlike lines in the longitudinal nerve sections, unlike the thick, short bundles that are typical of mature neurites. Such threadlike lines were completely absent in the distal segment of the injured nerve at these time points.

By 8 days after transection, however, regenerating neurites could be seen present in the distal stump for the first time (fig. 2-9c). On one immunostained cryosection, we could observe about 5 to 10 of these long, threadlike lines appearing

on a dark background, indicating the first sign of successful penetration of neurites into the distal segment at a time, according to our previous DRG-cryosection assay, when the distal nerve environment was optimal in its ability to support stable neurite outgrowth. It follows that the neurites entering the distal nerve segment at this time point are very likely to remain stable and continue to extend through the rest of the nerve.

As expected, as the regeneration process continued to 10, 16 or 20 days after transection, NF-200 staining became more and more abundant in the distal segment (fig. 2-9d to f). These latter time points also correspond to a period when the ability of the distal segment to support stable neurite outgrowth was declining, despite the fact that more new neurites were arriving each day. It is conceivable that once the first wave of regenerating neurites had established themselves in the distal segment, subsequent neurites could use them as a substrate for migration and therefore were not adversely affected by the decline in permissiveness of the nerve environment.

Thus, by as early as four days after transection, large number of regenerating neurites have reached the proximal stump but do not penetrate into the distal segment until eight days after transection, which happened to be the time when the substrate is transformed to its optimal state. The coincidence in the timing of neurite penetration and of substrate transformation may be due to the necessity for substrate transformation to precede incoming neurites. It also suggests that the process of substrate transformation may ultimately determine the speed of advancement of regenerating neurites.

The subsequent decline in the ability to support stable outgrowth occurs regardless of the presence of regenerating neurites

But what causes the decline in the ability to support stable outgrowth beyond 8 days post-transection, right after the regenerating neurites successfully penetrate the distal segment? Since the decline occurs shortly after regenerating neurites extend into the distal segment, one intriguing possibility is that these neurites are somehow causing the nerve environment to return to a non-permissive nature once they themselves have traveled through.

To test this hypothesis, we repeated the previous time-course experiment except that this time, after the sciatic nerve was transected, a 1-cm section of the tip of the proximal segment was excised to create a gap of about 1.5 cm between the proximal and distal stumps. This effectively prevented the neurites from entering the distal segment and, at the time of harvesting of the nerves, we also verified that the proximal and distal segments were not physically connected by connective tissue. In this situation, the regenerating neurites could not physically affect the events happening in the distal nerve environment. This experiment was performed once, with 16 samples per time-point.

The result of the experiment is shown in fig. 2-10. It shows that the distal nerve segment was highly supportive of stable outgrowth at 8 days after transection, which was also the case previously when regenerating neurites were allowed to enter. Furthermore, on subsequent time points (11 days, 15 days, and 18 days after transection), the ability to support stable outgrowth declined in a similar pattern as

before, even though the neurites were prevented from entering the distal segment. Wilcoxon's test verified that the scores of t=11 days are significantly lower than those of t=8 days (p=0.0001), while the scores of t=11 days versus t=15 days (p=0.67) and those of t=15 days versus t=18 days (p=0.49) are not significantly different.

Apparently, the regenerating neurites, which were absent in the distal segment, are not the cause of the decline in substrate permissiveness. This implies that the small time window (between 8 days and 10 days post-transection) of optimal support for stable outgrowth is programmed to close regardless of whether neurite regeneration had occurred.

Development versus regeneration: Neonatal peripheral nervous system

Nerve regeneration in adults is sometimes described as a recapitulation of neural development in embryos and neonates. It is well known that, at birth, the nervous system of a mouse is not yet fully developed and neurites are still in the process of extending through nervous tissue to their intended targets. We may ask: why is it possible for neonatal neurites to grow in the neonatal nerve environment?

From our previous experiments, we have learned that adult regenerating neurites can grow in the sciatic nerve because the nerve environment is one that supports stable neurite outgrowth. Extending the analogy to the neonatal system, we may hypothesize that the neonatal sciatic nerve is innately capable of supporting stable neurite outgrowth, just like an adult sciatic nerve after post-injury transformation.

However, there is a second possibility. We may hypothesize that the neonatal developing neurites are programmed to simply ignore the nerve environment it encounters and to continue to extend without degenerating, perhaps due to the lack of a sensing mechanism for the non-supportive nature of the substrate. In fact, experiments by Carbonetto et al. (1987) and Savio and Schwab (1989) in which embryonic or neonatal DRG neurons were seeded on normal (i.e. uninjured) adult sciatic nerve tissue and extensive regeneration of neurites was subsequently observed, probably argue for this hypothesis.

In our next series of experiments, we tested the validity of these two opposite, but not mutually exclusive, mechanisms which enable stable neurite outgrowth in the neonatal nervous system.

The neonatal nerve environment can support stable outgrowth of adult DRG neurites

We have shown that the adult sciatic nerve environment is non-permissive for stable neurite outgrowth and that adult DRG neurites do react to the non-permissive nature of the nerve environment. Therefore, we can use these two tissues as references to test the properties of the neonatal counterparts.

First, we plated adult DRGs on longitudinal cryosections of neonatal (10 days after birth) sciatic nerve. After 6 days of culture, we observed that the adult DRGs consistently extended large number of very stable neurites (fig. 2-11a), in parallel with the length of the cryosections, implying that the extracellular matrix materials within

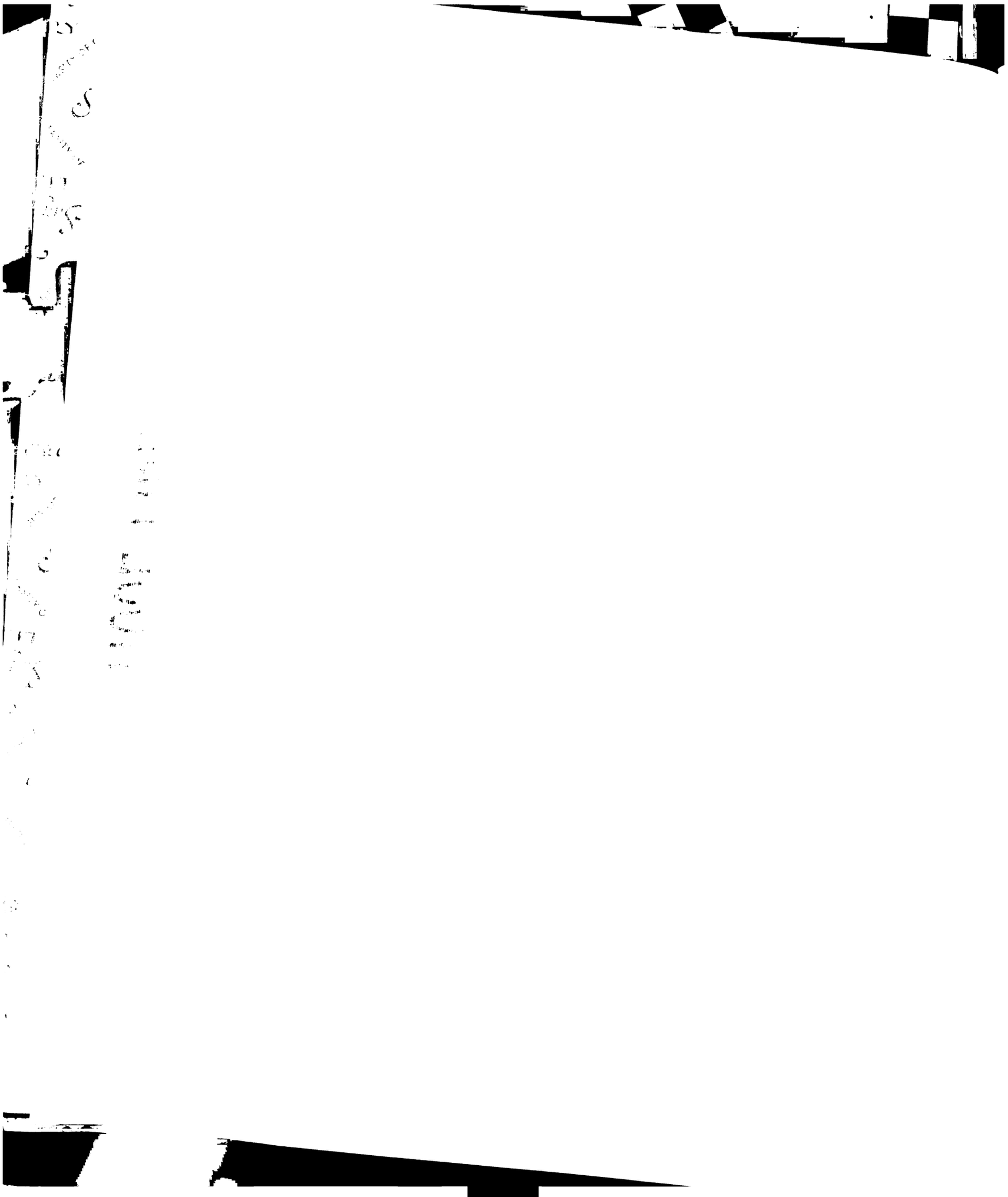
the nerve were already well aligned. The remarkable absence of globular debris was obvious when compared to the negative control (fig. 2-11b) in which adult uninjured nerve cryosections were used as the substrate for outgrowth. This experiment was duplicated twice with 8 samples each for neonatal and adult nerve cryosection.

This result shows that the neonatal sciatic nerve environment is similar to that of post-injury adult sciatic nerve in that they both support stable neurite outgrowth of adult DRG neurites.

Neonatal DRGs can grow stable neurites in a normal adult sciatic nerve environment

Next we plated neonatal (3-day old) DRGs on longitudinal cryosections of normal adult sciatic nerve (4 samples, one experiment). After 4 days in culture, we invariably observed profuse neurites growing healthily and stably on the cryosections, also well-aligned with the length of the nerve cryosections (not photographed). The absence of globular debris was very apparent. This result indicates that neonatal neurites are not affected by the non-permissive nature of adult sciatic nerve, which usually causes adult neurites to degenerate.

Thus in the neonatal nervous system, two mechanisms are simultaneously at work to ensure stable outgrowth: the nerve environment supports the neurites to remain healthy and, at the same time, the neurites are not sensitive to the kind of environment they are growing in. It may simply be a manifestation of the immature nature of the neonatal nervous system.



DISCUSSION

Discrepancy with published results

We used an in vitro system of culturing adult DRG explants on top of unfixed cryosections of sciatic nerve to study the behavior of neurites during regeneration. Our observation that adult DRGs grew extensive neurites on adult normal sciatic nerve cryosections does not disagree with a previous report (Bedi et al., 1992) that dissociated adult DRG neurons fail to extend neurites on the same substrate. At first, to account for this discrepancy, we hypothesized that the cells within the intact DRG explants may provide special neurotrophic factors to the neurons, resulting in more vigorous outgrowth. We also considered the possibility that the neurites from DRG explants may be able to fasciculate more easily into thicker cable of neurites and therefore were less fragile and more likely to be preserved and observed in the immunostaining process.

While these explanations may still be valid, our own experiments using dissociated DRG neurons had produced extensive neurite outgrowth on cryosections of normal adult sciatic nerve. Moreover, we could not determine if the different results were due to a meaningful biological reason or a trivial technical reason. However, it would be safe to conclude that adult dissociated DRG neurons are capable of growing on adult nerve tissue at least under certain conditions. As a result, we did not pursue any further effort to obtain a more definitive answer. We

did continue to use DRG explants because we thought that the integrity of the DRG was more relevant to the in vivo conditions.

The physiological significance of degeneration following outgrowth

Although adult DRG explants are able to extend neurites on both normal and pre-injured sciatic nerve cryosections, it was soon clear that those in contact with normal sciatic nerve cryosections later undergo degeneration. The process of degeneration appeared to begin soon (i.e. within one day) after the neurites make contact with the normal nerve cryosection, but the breakdown of the neurites took several more days to complete. It is interesting to note that while some neurites were undergoing degeneration, a large number of other neurites were able to continue to lengthen, possibly using other established neurites as the substrate.

What is the functional significance of neurite degeneration in response to contact with normal sciatic nerve? In the mature CNS, it has been proposed that the normal function of the many inhibitors of neurite outgrowth, such as Nogo, MAG, collapsin (semaphorin III) and chondroitin sulfate proteoglycan, is to maintain the stability of the established neural circuitry by restricting inappropriate rewiring and by preventing excessive sprouting (Goldberg and Barres, 2000a). It has been shown that if Nogo in the rat cerebellum is neutralized by application of the monoclonal antibody IN-1, the Purkinje cells will spread profuse neurites that cover much of the granular layer within a week (Buffo et al., 2000). These aberrant neurites, however, eventually disappear within a month as the effect of the antibody disappears.

In the PNS, however, the extracellular matrix is loaded with molecules that are favorable for neurite outgrowth, such as laminin and collagen IV. Therefore, a similar mechanism is not likely to be functioning in the PNS. It is tempting to speculate that the degenerative response we observed may work as an innate mechanism of the mature PNS to eliminate inappropriate axonal sprouting after it occurs. If this is the case, it is notable that such mechanism does not operate by the immediate repulsion or collapsing of growth cones, as conventional inhibitors and negative guidance cues do. Instead, the response is one of delayed withdrawal: neurites are briefly allowed to step into the nerve environment and later induced to degenerate if the they find themselves in an inappropriate location. The ultimate effect, similar to that of CNS inhibitors, is still the maintenance of the established neural configurations between the sensory (or motor) neurons and their peripheral targets.

"Degeneration signal" versus "permissive signal"

Apparently, the sciatic nerve environment itself has to carry the necessary information to induce the in-coming neurites to degenerate. It can be in the form of a signaling molecule located in the adult nerve environment, which tells the inappropriate neurites to degenerate. Thus, one may interpret that a possible function of Wallerian degeneration after nerve injury is to clear away such "degeneration signal" so that regenerating neurites can remain stably established in the distal nerve segment. On the other hand, it can also be in the form of a signaling molecule that is usually absent in the adult nerve environment but is required to

permit neurites to remain. In this case, one may interpret that Wallerian degeneration serves to prepare the distal nerve segment by paving it with a "permissive signal".

There is no strong experimental data that directly supports one of the two scenarios. However, one can make an argument against the possibility of a "permissive signal". Recall that the neurites growing extensively on the cover slip showed no sign of degeneration. Yet the surface of the glass cover slip has only polylysine and the molecules brought in by the culture medium. If a "permissive signal" molecule is required to support stable outgrowth of neurites, this molecule would have to be polylysine or some molecule present in the culture medium. Polylysine is an artificial molecule and, as such, is not believed to be present in an injured nerve. The molecules that came from the culture medium, however, were presumably also available in the cryosection (which is in contact with the culture medium) and, yet, the neurites on the cryosection could not remain stable. Thus it appears that the neurites can grow healthily with any special "permissive signal", which, in turn, implies that the neurites on the cryosection degenerate because they encounter a "degeneration signal".

Of course, such an argument may be flawed. One may counter-argue that the "permissive molecule" is not polylysine, but one that is polylysine-like with multiple positive charges. Or one may counter-argue that the "permissive molecule" indeed came from the culture medium; however, it selectively adheres to the glass coverslip but not to the cryosections.

Degeneration as a mode of neural pathfinding

Neural pathfinding is generally thought of as directional movement of growth cones in response to environmental cues such as chemoattractants or chemorepellents (by contact or at a distance) (Tessier-Lavigne and Goodman, 2000; Krull and Koblar, 2000; O'Leary and Wilkinson, 1999). Here, our observation of degeneration following neurite outgrowth may represent a different mode of neural pathfinding. Although not very common in research literature, it is by no means without precedence, as in the development of the retinocollicular projection in rat (Simon et al., 1994).

In this system, the retinal ganglion cells (RGCs) first extend long neurites to establish overlapping, diffuse connections with the superior colliculus (SC) but with a fair amount of topographical specificity: nasal RGCs mainly extend axons to the posterior SC and temporal RGCs mainly extend axons to the anterior SC. This process is now understood to be mediated by a gradient distribution of ephrinA in the superior colliculus and a gradient expression of EphA, the receptor of ephrinA, in the retinal ganglion cells (Klein, 2000). However, many of the axons make initial targeting errors and the final map is obtained, after birth when ephrinA has been down-regulated, as a result of remodeling by retraction of aberrant branches and cell death (O'Leary et al., 1986; Mark et al., 1993).

Furthermore, it was reported that the cells of posterior tectum (chick equivalent of superior colliculus) produce a secreted factor that causes withdrawal of temporal RGCs, but not nasal RGCs (Ichijo and Bonhoeffer, 1998). When temporal

RGCs were grown on a substrate consisted of the processes of posterior tectal cells (the incorrect target for temporal RGCs), extensive neurite outgrowth were observed by 3 days in culture but these neurites were mostly "withdrawn" by 7 days in culture, leaving behind what appeared to be globules of debris on the substrate. The same "withdrawal" response can also be induced by a conditioned medium of the posterior tectal cells, showing that a diffusible factor produced by these cells is involved. The secreted factor is known to be heat-labile, distinct from ephrinAs and expressed by the posterior tectum at a much later stage when ephrinAs have been down-regulated.

Perhaps a molecule functionally similar to the secreted factor of posterior tectum is also present in the uninjured PNS, causing the withdrawal of aberrant DRG neurites in adult normal sciatic nerve.

The timing of neurite regeneration and environment transformation

Our data showed that the distal nerve environment gradually increased its ability to support stable outgrowth after nerve transection, reaching an optimum at about 8 days post injury. During this period, the regenerating neurites in the proximal nerve segment reached the tip of the proximal stump by 4 days after injury. However, the first regenerating neurites were not observed in the distal segment until 8 days after injury. What happened to the neurites during the four days when they seemingly did not make any progress?

By 4 days after injury, the proximal and distal stumps are already physically connected with an observable buildup of connective tissue. One possibility is that this connective tissue mass requires a long time for the neurites to traverse. This may not, however, completely account for the delay of four days, which seems long for such a short distance. Another possibility is that some neurites did actually penetrate into the distal segment, but because of the non-supportive nature of the nerve environment, they quickly degenerated and therefore were not observed. It is therefore tempting to speculate that the transformation of the post-injury nerve environment may be a rate-limiting step in the overall progress of nerve regeneration at this early stage. This may also explain why in cases where Wallerian degeneration, which presumably includes the remodeling of the nerve environment, such as in the C57BL/Ola mice (Brown, 1991; Bisby and Chen, 1990), the progress of neurite regeneration into the distal segment was also delayed.

The significance of a temporal window for optimal support of neurite outgrowth

Interestingly, the optimal support of stable neurite outgrowth by the distal segment is only temporary, as it already begins to decrease just two days after the first neurites arrive and completely disappears by 20 days after injury. We at first hypothesized that the entering neurites or associated cells were changing the nerve environment once again, perhaps by laying down a "degeneration signal" molecule behind them, so that other neurites would not take the exact same course. However,

this hypothesis is not supported by our data because, even when regenerating neurites are prevented from entering, the distal segment still undergoes the same schedule of rise and fall in the ability to support stable outgrowth. This observation also rules out the possibility that remyelination in the distal segment may be causing the decline since, without any neurites, the Schwann cells would not be induced to make myelin.

In any case, the decline in ability to support stable outgrowth is probably effected by cells residing within the distal segment. But what could possibly be the function of such decline? Perhaps some insight can be gleaned from the observation that during the regeneration of injured motor neurons toward the gastronemius muscle in adult rats, terminal Schwann cells near the neuromuscular junctions are induced to express semaphorin 3A, generally a chemorepellent to growth cones (De Winter et al., 1999, 2000). It was further suggested that the presence of Sema3A may be critical in slowing down the growth of regenerating axons as they approach the proximity of the targets and eventually act as a stop signal.

By analogy, the decline in the ability to support stable outgrowth by the injured sciatic nerve may also be a result of the distal segment signaling to the in-coming neurites to prepare to stop. Consistent with this idea, it is known that DRG neurons indeed up-regulate the expression of neuropilin-1, the receptor for Sema3A, upon injury, presumably leading to an increase in the sensitivity to Sema3A in the environment. The localization of Sema3A protein in the distal nerve has not been reported, however, due to the lack of an antibody against Sema3A.

It is conceivable that the pioneering neurites may have only a narrow window of time to successfully establish themselves in the distal segment. However, once established, even though the nerve environment is no longer optimal, the neurites apparently remain stable and do not degenerate, as there is an increase in the density of neurites from 10 to 16 days after injury. One may hypothesize that once the growth cones have traversed a certain part of the nerve environment and the axons become established, they are no longer sensitive to the non-supportive nature of the environment. Furthermore, these established neurites may be used by late-coming neurites as the substrate for outgrowth.

Neither the sensing nor signaling mechanism is installed in the immature neonatal peripheral nervous system

Peripheral nerve regeneration in adult is often compared with neural development in neonate and embryo. Here we may have described one major difference between the two processes. In contrast to adult neurites, our data shows that neonatal neurites do not recognize the non-supportive (degenerative) nature of an "inappropriate" (adult) nerve environment. At the same time, the neonatal nerve environment is not perceived by adult neurites as inappropriate. In other wordw, neither the "sensing" mechanism nor the "signaling" mechanism is functional in the neonatal PNS. This can be interpreted as a simultaneous absence of the "sensing receptor" in the neurons and of the "signaling molecule" in the nerve environment, the genes of which are probably expressed only after maturation.

This phenomenon may work as a "double insurance" to ensure that the neontal PNS can grow vigorously without impediment while the adult PNS cannot sprout aberrantly into inappropriate areas. A similar theme of "double insurance" is also revealed by a survey of the developmental responses to myelin-associated inhibitors, which are not expressed in embryonic rats before myelination occurs, a process which takes place later after birth. Therefore, rat early embryonic (E14) retinal ganglion cells (RGCs) are able to grow on neonatal CNS tissue, but not on adult CNS tissue (Shewan et al., 1995). Thus, CNS tissue is permissive prior to adult stage. On the other hand, rat embryonic RGCs do not respond to Nogo, one of the well characterized myelin-associated inhibitors, and do not exhibit the long-lasting, large increase in intracellular calcium that is typical in Nogo-induced growth cone collapse of adult RGCs (Bandtlow and Loschinger, 1997). The Nogo-66 receptor is also not expressed in chick early embryonic (E7) RGCs and DRG neurons, until a much later stage (E13; Note: the E numbers in rat and chick may not be comparable) (Fournier et al., 2001). As for myelin-associated glycoprotein (MAG), another known CNS inhibitor, the embryonic RGCs actually interpret it as a promoter of neurite outgrowth in vitro (Turnley and Bartlett, 1998). The response, however, is switched to one of inhibition by adult RGCs at about the time of birth, together with a concomitant decrease in intracellular cAMP level, which is believed to be a determinant of how MAG is interpreted by the neurons (Cai et al., 2001). Thus the embryonic CNS neurons either cannot detect the myelin-associated inhibitors or fail

to interpret them as inhibitory. As we can see, both the "signaling" and "sensing" mechanisms are absent in the early embryonic CNS.

CHAPTER 3:

THE CELLULAR AND MOLECULAR MECHANISMS UNDERLYING THE TRANSFORMATION OF THE NERVE ENVIRONMENT AFTER INJURY OF THE MOUSE ADULT PERIPHERAL NERVE

INTRODUCTION

The mammalian peripheral nervous system is highly capable of regenerating after a transection injury (Ramon y Cajal, 1913). It has been well demonstrated that a series of cellular and molecular events which happen shortly after nerve injury is essential to the success of subsequent regeneration (Fawcett and Keynes, 1990). These events, collectively known as Wallerian degeneration, have been the focus of a great many studies on peripheral nerve regeneration.

During Wallerian degeneration, myelin sheaths of Schwann cells which wrap around conducting axons degenerate in the nerve segment distal to the injury site (Beuche and Friede, 1984). Another cellular hallmark of Wallerian degeneration is the arrival of a large number of macrophages recruited from the periphery (Perry et al., 1987). These macrophages are believed to perform many important functions, such as the phagocytosis of the myelin debris to make space for the regenerating neurites and the rebuilding of the damaged extracellular matrix (ECM). They have also been shown to induce the growth of neurites, probably indirectly by inducing Schwann cells to proliferate and secrete neurotrophic factors such as NGF (Heuman et al., 1987). In addition, they are probably important in recrafting the undamaged

ECM of the distal segment into one that is appropriate for the outgrowth of regenerating neurites (La Fleur et al., 1996).

The importance of macrophages has been implicated by researchers studying the injured central nervous system where macrophages fail to infiltrate and nerve regeneration is subsequently very poor (Avellino et al., 1995; Hirschberg et al., 1995; Perry et al., 1987). By implanting blood macrophages that have been co-cultured with PNS tissue into the lesion site of the spinal cord, some researchers had achieved improved CNS regeneration and partial motor function recovery in rats (Lazarov-Spiegler et al., 1998). Other researchers have shown that optic nerve cryosections, which are non-permissive for embryonic DRG neurite outgrowth, can be turned into a permissive substrate by pre-incubation with live macrophages from injured brain (David et al., 1990).

In the previous chapter, we have demonstrated a possible novel contribution of Wallerian degeneration to the success of peripheral nerve regeneration. By using an in vitro organ culture system, we showed that in the environment of an uninjured sciatic nerve, regenerating neurites can initially extend but they cannot remain healthy and quickly degenerate. However, by 8 days after injury when Wallerian degeneration is well under way, the environment of the distal nerve has been transformed into a substrate that is capable of supporting stable outgrowth of neurites. The specific cell types that contribute to the above effect could be any of the several cell types that emerge in the distal nerve following injury, the most prominent ones being macrophages, Schwann cells, and fibroblasts.

We have hypothesized two working models to account for the molecular mechanism leading to the above-mentioned transformation of the distal nerve environment. The first one involves the laying down of a positive "permissive signal" in the nerve environment during Wallerian degeneration, which the incoming neurites require in order to remain stable. Thus, the normal nerve cannot support stable neurite outgrowth because it lacks this "permissive signal" and the neurites proceed to degenerate. Candidate molecules that fall into this category include neurotrophic factors (e.g. NGF, BDNF, GDNF, CNTF, etc.), adhesion and ECM molecules that promote neurite outgrowth (e.g. laminin and fibronectin) and attractive axonal guidance cues (e.g. netrin).

The second working model involves the removal or inactivation of a negative "degeneration signal" that innately resides within an adult normal nerve and causes neurites to degenerate on contact. When this "degeneration signal" molecule is removed during Wallerian degeneration, the incoming neurites are not induced to degenerate and thus continue to outgrow stably. At present, endogenous molecules that are capable of causing neurite degeneration have not yet been identified, although an unidentified factor produced by the chick posterior tectum has been demonstrated to cause neurites of temporal retinal ganglion cells (RGCs) to "withdraw" (Ichijo and Bonhoefer, 1998). On the other hand, other known molecules have been shown to cause instantaneous growth cone collapse and failure of neurite outgrowth: inhibitors of axonal outgrowth (e.g. chondroitin sulfate proteoglycan, myelin-associated glycoprotein "MAG", tenascin-R) and repulsive

axonal guidance cues (e.g. semaphorins, ephrins, slits, etc.). These are the next most likely group of candidate "degeneration signal" molecules for our working model.

In this chapter, we shall present a series of experiments aimed at identifying the cell types and the molecules involved in the transformation of the nerve environment after injury.

METHODS

In vitro reconstitution of nerve regeneration using unfixed cryosections of adult sciatic nerve and adult intact DRG explants

The procedure for nerve injury operation, harvesting of injured nerves, cryosectioning of unfixed nerves, extraction of DRG explants from adult mice, the culturing of DRGs on cryosections, fixation and immunostaining of neurites were identical with that described in the previous chapter. Briefly, the sciatic nerves of 2-month old mice were operated at mid-thigh level. After a specified period of time, the mice were sacrificed. The nerves were harvested, then snap frozen in liquid nitrogen. They were then embedded in frozen section embedding material and cryosectioned longitudinally. The nerve cryosections were placed on poly-D-lysine-coated glass circles and a DRG explant from another adult mouse was cultured on top of each cryosection in a medium containing serum, NGF, cytosine arabinoside and penicillin. After 6 days in culture, the DRGs were fixed in

paraformaldehyde and immunostained with anti-GAP-43 antibodies for the visualization of the growing neurites.

Transgenic mice used in nerve injury experiments

Op/op osteopetrotic mice deficient in CSF-1 were raised from several breeder pairs of op/op males and op/+ females purchased from Jackson Laboratory. They are maintained in our clean-room animal facility on a diet of soft slurry made of milk powder and baby cereal, changed three times per week. Stromelysin-1 knock-out mice were provided by John Mudgett (Mudgett et al., 1998) and bred in our clean-room facility.

Immunostaining of cryosections with F4/80 antibodies, a mouse macrophage marker

Nerve cryosections mounted on glass slides were first fixed for 30 minutes with a small puddle (0.5 ml) of 4% paraformaldehyde held together by a waxy ring drawn with a Pap Pen. The cryosections were then washed with two drops of 0.1M glycine in PBS for 30 minutes twice to inactivate any remaining paraformaldehyde. After washing, the cryosections were pre-blocked with two drops of DME with 10% fetal bovine serum for 1 hour. Primary staining was done overnight with F4/80 monoclonal antibody (a mouse macrophage marker; undiluted hybridoma supernatant) at 4 °C. After three washes with 2 drops of DME with 10% fetal bovine serum for 5 minutes each, secondary staining was done with alkaline

phosphatase-conjugated rabbit anti-rat IgG antibodies (at 1:100 dilution in DME with 10% FBS) for 2 hours at room temperature.

To perform the color reaction, the cryosections were first washed three times with 0.5 ml of reaction buffer (100 mM Tris, 100 mM NaCl, 50 mM $CaCl_2$, pH 9.5) with levamisole (20 µl per ml) for 15 minutes each. Then the slides were put upside down on a 0.5-ml puddle of staining solution on a piece of parafilm. (The staining solution was prepared by mixing 22.5 µl NBT (stock: 75 mg/ml in 70% dimethylformamide), 17.5 µl X-phosphate (stock: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide), 0.1 ml levamisole and 5 ml reaction buffer). The reaction was stopped by washing with PBS with EDTA when the experimental slide was fairly darkened while the negative control slide (with the F4/80 antibody omitted in the primary staining) was only lightly darkened.

The slides were finally mounted with cover slips with gelvatol (8 g polyvinyl alcohol dissolved in 40 ml 0.2M Tris-HCl buffer at pH 8.5 by warming to 50-60°C, then mixed with 20 ml glycerol after cooling).

Preparation of conditioned media

To prepare a batch of macrophage-conditioned medium, six 2-month-old CF1 mice were each injected intraperitoneally with 1 ml of 3% Brewer's thioglycollate broth. Three days later, the mice were sacrificed by cervical dislocation. The skin covering the peritoneum was sterilized with alcohol and cut open, exposing the intact peritoneum. 5 ml of calcium, magnesium-free PBS (phosphate buffered saline)

was injected into the peritoneum with a 20-gauge needle. The peritoneum was gently massaged and the calcium, magnesium-free PBS was withdrawn with a sterile Pasteur pipet, making sure that no visible bleeding occurred. The cells in the collected PBS were then spun down at 700 RPM for five minutes and resuspended in DME with 10% fetal bovine serum to a concentration of 1 million cells per ml. 10 ml of cells were plated per T75 cell-culture flask. Two to three hours after plating, the medium was switched to DME with 0.2% LH (lactalbumin hydrolysate) with or without 10 ng/ml of LPS (lipopolysaccharide). From then on, conditioned medium was collected and replenished with fresh medium once every four days for a total of 12 days. This conditioned medium, as well as all others, were stored frozen in 1-ml aliquots until use.

To prepare Schwann cell-conditioned medium, Schwann cells were collected from one-day old neonatal mouse sciatic nerves. Twenty of such nerves were placed in 2 ml of DME and incubated with 0.1% bacterial collagenase for 30 minutes at 37°C. After that, the tissue was further incubated in 2 ml of DME with 0.1% collagenase and 0.25% trypsin for 30 minutes at 37°C. After replacing the medium with 5 ml of DME with 10% fetal bovine serum, the tissue was spun down at 1500 RPM for 5 minutes. The pellet was then resuspended in 2 ml of DME with 10% fetal bovine serum and triturated with Pasteur pipet for 30 times. The remaining tissue was broken up by trituration with a fire-polished narrow-mouth Pasteur pipet. The cells were then plated in a T75 flask. The next day, the medium was changed to DME

with Bottenstein's N2 supplement (purchased from Gibco BRL, Inc.) and 10 μM cytosine arabinoside. The conditioned medium was collected four days later.

To prepare fibroblast-conditioned medium, fibroblasts were collected from synovium of a rabbit and cultured in DME with TPA (tetradecanoylphorbol acetate) at a density of 1 million cells per milliliter (the cells were collected and cultured by Patrice Tremble, a member of the lab). The conditioned medium was collected four days after culture.

P388D1-conditioned medium was prepared by incubating the cells for 4 days at near-confluence in T-75 flasks with 10 ml of DME with 0.2% LH, 100 ng/ml LPS and antibiotics. U937-conditioned medium was a gift from the lab of Michael Banda and was prepared by 4-day incubation of the cells in DME with 10% serum. IC-21-conditioned medium was prepared by 4-day incubation of the cells in RPMI with 10% serum.

Preincubation of nerve cryosections

Longitudinal nerve cryosections mounted on glass cover slips were incubated at 37°C overnight with different conditioned media, enzymes, growth factors (in DME) or neurotrophic factors. The enzymes used in the pre-incubation of nerve cryosections (all at 37°C) included trypsin (from 0.002% to 0.05%, purified crystalline, 3 minutes), collagenase (from Clostridium, crude preparation that contained proteases like dispain, 0.125%, 30 minutes), liver transglutaminase (125 nM and 1.25μM, 1 hour), chondroitinase ABC (0.2 U/ml, 1 hour),

phosphatidylinositol phospholipase c (PI-PLC, 20 mU/ml and 200 mU/ml, 1 hour), hyaluronidase (1 mg/ml, 30 minutes), and heparinase (0.5 U/ml, 30 minutes).

The growth factors used include IL-1α (4 ng/ml), IFN-γ (40 ng/ml), TGF-β1 (10 ng/ml), LIF (2000 U/ml), bFGF (100 ng/ml), EGF (50 ng/ml), insulin (2 μg/ml), HGF (50 ng/ml), PDGF-BB (200 ng/ml) and IGF-1 (100 ng/ml).

The neurotrophic factors used include NGF (50 ng/ml), BDNF (50ng/ml), NT-3 (50 ng/ml), CNTF (ciliary neurotrphic factor, 50 ng/ml), GDNF (glial-derived neurotrophic factor, 50 ng/ml) and combinations of the above. In experiments where neurotrophic factors were used to pre-incubate nerve cryosections, the same neurotrophic factors were also added to the DRG explant culture medium during the DRG-cryosection assays.

Inactivation of macrophage-conditioned medium with specific classes of protease inhibitors

The protease inhibitors we used were PMSF (phenylmethylsulfonyl fluoride, 4 mM, for serine proteases), E-64 (10 μg/ml, for cysteine proteases) and pepstatin (10 μg/ml, for aspartic proteases) and EDTA (10 mM, for metalloproteases).

The macrophage-conditioned medium was first concentrated 8-fold by applying polyethylene glycol (PEG) on dialysis tubings containing the conditioned medium. Except for EDTA (see below), the protease inhibitor(s) was mixed with the concentrated macrophage-conditioned medium in Eppendorf tubes at the stated concentrations and allowed to stand for one hour at 37°C. Then the mixtures were

dialyzed (Spectropore tubing with cut-off point of 14 kd) against 2 L of PBS at 4°C overnight, and against another 2 L of PBS at 4°C for six hours. The resulting dialysates were used to pre-incubate nerve cryosections as before.

EDTA, however, was added to the conditioned medium after PEG concentration and dialysis, but one hour before it was used to pre-incubate the nerve cryosections.

RESULTS

Macrophages secrete a diffusible molecule that is capable of transforming the normal nerve environment

We first asked whether any of the major cell types present during Wallerian degeneration — fibroblasts, Schwann cells and macrophages, can transform the distal nerve environment after sciatic nerve transection. To answer this question, we raised conditioned media from these three cell types. Normal sciatic nerve cryosections were then incubated overnight in these conditioned media and adult mouse DRG (dorsal root ganglia) explants were cultured on the cryosections after the removal of the conditioned media. If the neurites from the DRGs grew stably, we inferred that a factor capable of transforming the nerve environment was secreted by the cells. If the neurites did not grow stably, we inferred that the cells did not secrete any such factor. This experiment was performed twice, each time with 8 samples for each of the 3 types of conditioned media.

We found that neurites growing on cryosections pre-treated with Schwann cell-conditioned medium (fig. 3-1a) or fibroblast-conditioned medium (fig. 3-1b) degenerated after six days of culture. The appearance of the neurites was similar to that of neurites growing on cryosections pre-treated with unconditioned medium (negative controls, fig. 3-1c). However, neurites growing on cryosections pre-treated with macrophage-conditioned medium (fig. 3-1d) did not degenerate and remained stable after 6 days of culture. The appearance of such stable outgrowth was similar to that of neurites growing on cryosections of 8-day post-transection sciatic nerve, which we reported in the previous chapter.

To verify more definitively the effect of macrophage-conditioned medium on the sciatic nerve environment, we prepared a large number of DRG neurite outgrowths on cryosections pre-treated with macrophage-conditioned medium (experimental outgrowths, n=18) and DRG neurite outgrowths on cryosections pre-treated with DME culture medium (controls, n=20). We then blind-scored all the samples according to the appearance of the outgrowths using the semi-quantitative scoring method described in the previous chapter. A score of 1 represents extremely degenerative outgrowth and a score of 6 represents extremely stable outgrowth.

We found that, on a graph of frequency vs. degeneration score (fig. 3-2), the peak of the histogram for the experimental outgrowths (at a score of 3) was one full point above the peak of that for the controls (at a score of 2) and that the range of scores for the median 70% of the two groups did not overlap. Subsequently, we performed Wilcoxon's two-sample test (two-sided), a nonparametric statistical

analysis based on ranks, and found the Wilcoxon's p-value to be 0.0024. By the 5% level criteria, this is strong evidence that the degeneration scores of the experimental outgrowths are significantly higher than that of the controls.

Hence we concluded that macrophages in vitro are capable of secreting a diffusible factor that transforms the normal nerve environment, and this factor was not detectable from Schwann cells and fibroblasts in our experiments. This raises the possibility that the same factor may be involved in vivo during Wallerian degeneration in the transformation of the post-injury nerve environment.

The transforming activity is also secreted by macrophage cell line

Since primary peritoneal macrophages express the nerve-transforming activity, we wanted to test if cell lines with monocyte-macrophage characteristics also express a similar activity. The confirmation of such behavior in macrophage cell lines will further support our observation in primary macrophages. Furthermore, once such a cell line is identified, a convenient source of the activity will be available to us for further biochemical studies.

We prepared conditioned media from P388D1 (mouse monocyte-macrophage cell line), U937 (human monocyte-like histiocytic lymphoma) and IC-21 (mouse SV-40 transformed peritoneal macrophage cell line). After pretreating normal nerve cryosections with the conditioned media, we tested their ability to support stable DRG neurite outgrowths as before. This experiment was repeated twice, each time with eight samples per conditioned medium.

The result is shown in fig. 3-3. Among the three cell lines tested, P388D1 produced a transforming activity above the level of DME control (Wilcoxon's $p = 0.003 < 0.05$). However, U937 and IC-21 did not produce any significant activity compared to control (Wilcoxon's $p = 0.82$ and 0.52, respectively).

Having identified P388D1 cell line as a source of the transforming activity, we wanted to compare the amount of activity produced by P388D1 cell line with that produced by primary peritoneal macrophages. To do this, we prepared 5-fold dilutions of macrophage-conditioned medium and P388D1-conditioned medium, and tested to see at what dilutions would the activity drop to the level of negative control. It should be noted that, since P388D1-conditioned medium was routinely prepared in the presence of lipopolysaccharide (LPS) as a stimulant, we had also added LPS to the culture medium of peritoneal macrophages for this experiment in order to make a fair comparison.

The result is shown in fig. 3-4. The p-values shown are derived from Wilcoxon's one-sided two-sample test between each dilution of conditioned medium and DME control. If we use $p=0.05$ as the cut-off level of significance, an above-background activity was displayed by macrophage-conditioned medium more concentrated than a 5x dilution ($p=0.056$ at 5x dilution). At dilutions over 5x, the level of activity was not significantly higher than that of DME control. On the other hand, the activity of P388D1-conditioned medium was detectable only at 1x (undiluted, $p=0.0003$) but appeared to quickly drop off to background by 5x dilution

($p=0.43$). Thus primary macrophages appeared to produce the active molecule at up to 5 times higher concentration than P388D1 cell line does.

Another interesting point can be deduced from the data of this experiment. Considering that the activity of the conditioned medium from 4-day, high-density culture of peritoneal macrophages could be reduced to background by a mere 5-fold dilution, we reasoned that there could be two possible scenarios: (1) that the molecule responsible for the transforming activity has a high specific activity but is probably produced in small quantity, or (2) that the molecule has a low specific activity but a high concentration is necessary to achieve physiological effect. In either case, a minimal dilution of the molecules would cause the activity to fall below the detectable level.

Secretion of transforming activity is dependent on the maturation state of macrophages

Tissue-specific macrophages are derived from circulating blood monocytes that exit the circulation and become residents of specific tissues. Upon the expression of phenotypes appropriate to the tissues of residency, these differentiated cells enter a more mature stage, ready to be activated by inflammatory agents. When stimulated by foreign substances (e.g. bacterial antigens) or cytokines (e.g. interleukins), these macrophages further mature into "activated" macrophages that display higher capacity for phagocytosis, increased metabolic activity (such as the production of

reactive oxide species and lysosomal enzymes) and enhanced tumor cell-killing ability (Junqueira et al., 1989).

We wanted to test if the level of transforming activity produced by macrophages is affected by their maturation stage. Since the macrophages responding to injury of the sciatic nerve indeed undergo a maturation process to an activated stage following infiltration of the tissue (exemplified by the expression of interleukin-1 and phagocytosis of myelin), we hypothesized that the nerve-transforming activity is accordingly up-regulated upon activation.

To test this idea, we activated thioglycollate-elicited (but non-activated) macrophages with lipopolysaccharide (LPS, 100 ng/ml) or cytokines (0.5 ng/ml IL-1 and 10 ng/ml IFN-γ) in culture and tested to see if their conditioned media contained an increased amount of the transforming activity using the DRG-cryosection assay.

The result is shown in fig. 3-5. We found that within one day of culture, LPS-activated macrophages (a) were already secreting a significantly higher amount of transforming activity than unactivated macrophages (b) ($p=0.019<0.05$, Wilcoxon's one-sided two-sample test). The degeneration score profile of unactivated macrophage-conditioned medium is very similar to that of DME medium alone in previous experiments, implying that macrophages at the less mature stage were producing nearly no activity. In longer culture (3 days), the amount of transforming activity produced by activated macrophages still exceeded that by non-activated macrophages in general ($p=0.052$ for LPS (d) vs. unactivated (c), $p=0.004$ for IFN-γ

(f) vs. unactivated (c)), but the difference may not be as significant statistically in some cases ($p=0.077$ for IL-1 (e) vs. (c)).

Apparently, even macrophages without exposure to activating agents began accumulating detectable level of transforming activity after the first day of culture. It is possible that the act of extracting the macrophages from the peritoneum might have the effect of activating at least some of the macrophages.

In summary, activated macrophages produce the nerve-transforming activity at a higher rate than non-activated macrophages, at least initially. The secretion of the transforming activity is therefore regulated with the maturation state of the macrophages.

In vivo evidence supporting the role of macrophages in transforming the post-injury distal nerve environment

If macrophages in vitro are capable of transforming the distal nerve environment, are they present in vivo in the distal nerve at a time frame consistent with such function? To find out, we did histochemical staining with the monoclonal antibody F4/80, a known marker for mouse macrophages, on cryosections of post-transection sciatic nerve of different time points (0, 4, 6, 8, 10, 16 and 20 days; from the same tissues used in the timed-series experiment described in chapter 2). We previously reported that the ability of the sciatic nerve to support stable neurite outgrowth gradually increased initially, peaking at 8 days post-injury, and gradually declined afterwards to the level of uninjured nerve by 20 days post-injury.

We found that macrophages first began to infiltrate by 4 days after injury, but only at the region immediately distal to the transection site (fig. 3-3a and b). They appeared more distally by 6 days after injury (fig. 3-3c) and were present throughout the length of the distal segment by 8 days after injury (fig. 3-3d). This implies that the completion of the macrophage infiltration coincides with the optimum support of stable neurites by the distal nerve environment. From 8 days to 20 days post-injury (fig. 3-3e and f), macrophages continue to be present throughout the length of the distal segment.

Thus in the four days before the distal nerve environment becomes capable of supporting stable neurite outgrowth, inflammatory macrophages infiltrate and progressively occupy the full length of the distal segment. This time-frame of macrophage infiltration is consistent with the hypothesis that macrophages are involved in the transformation of the distal nerve environment in vivo.

Use of the op/op osteopetrotic mice as a macrophage-deficient mouse model

Perhaps more definitive in vivo evidence of a possible role of macrophages in the transformation of post-injury nerve environment can be obtained by testing what happens to the injured nerve when macrophages are lacking or absent. If macrophages are indeed necessary for such transformation, one would expect the post-injury nerve of a macrophage-deficient mouse to remain non-supportive of stable neurite outgrowth.

To test such hypothesis, we turned to the op/op osteopetrotic mice, which is known to carry a frameshift mutation within the coding region of the macrophage colony-stimulating factor (M-CSF or CSF-1) gene, leading to a complete absence of functional M-CSF protein, important in the development of cell types of monocyte or macrophage lineage (Naito et al., 1997). As a result, op/op individuals have a much reduced number of macrophages in most tissues, including liver, kidney, lung, uterus, ovary, bone marrow, synovium and subcutaneous tissue. Indeed, the characteristic round snout, dome-shaped skull and failed rupturing of incisors in op/op mice are the result of a lack of osteoclasts, which are bone-specific macrophages responsible for the resorption and remodeling of bones. (Marks et al., 1984).

We purchased several breeder pairs of op/op male and op/+ female to breed a colony of op/op mice. However, op/op individuals were very sensitive to anesthesia and were often too fragile to survive the nerve transection operation. In addition, they have difficulties surviving through the weaning period before which they were still dependent on their mother for milk. We were able to improve the yield of op/op mice by switching them to an enriched diet of soft slurry made of powdered milk and baby cereal, instead of dry ground-up mouse pellets. Nonetheless, it took more than one year before we were able to obtain enough op/op individuals to experiment on.

We transected the sciatic nerve of op/op mice and harvest the nerve 10 days later. We then tested the ability of the post-injury nerve to support stable outgrowth using the DRG-cryosection assay with DRGs from normal CF1 mice (the op/op mice are of C57BL strain). The op/op mice used were 1-month (two epxeriments) or

2-month (one experiment) old females. Op/op uninjured nerve, normal post-injury nerve and normal uninjured nerve were also tested as controls.

The result of the experiment is shown in fig. 3-7 (n=8 for each treatment). To our surprise, just as wild type sciatic nerve (c and d) showed an increase in support of stable outgrowth 10 days after injury ($p=0.0021 < 0.05$), the op/op sciatic nerve (a and b) also showed a similar increase after injury ($p=0.0012 < 0.05$). This was contradictory to our prediction for a mouse that is deficient of macrophages and might further imply that macrophages were not required for the transformation of post-injury nerve environment.

Therefore, we did immunostaining of adjacent sections of the four sciatic nerve tissues with F4/80 antibodies to assess the level of macrophage infiltration (fig. 3-7). We found that while macrophages were absent in uninjured nerves of normal and op/op mice, abundant macrophages had infiltrated the 10-day post-injury nerves of both normal and op/op mice. The densities of macrophages in the normal and op/op post-injury nerve were fairly comparable by visual inspection. The op/op mice turned out not to be macrophage-deficient as far as inflammatory response to injury of peripheral nerve tissue is concerned.

As a result, the op/op mouse model has not yielded definitive information that confirms the possible role of macrophages in the transformation of post-injury environment as we had hoped, even though the data remained consistent with our hypothesis that macrophages are necessary for this process.

The activity in macrophage-conditioned medium is likely a protein

We now turn our attention to the biochemical nature of the transforming activity in the macrophage-conditioned medium. In order to elicit the macromolecular class of the active molecule, we performed several biochemical tests on LPS-activated macrophage conditioned-medium (fig. 3-8), showing that the active molecule behaves in ways that are consistent with that of a typical protein. Each of these experiments was performed at least twice, with 8 samples per experiment.

Firstly, the activity of the conditioned medium was destroyed by heating for 15 minutes in a boiling water bath (fig.3-8c vs. a, $p=0.001 < 0.05$), just as most proteins are denatured by similar heating while most carbohydrates and lipids remain stable.

Secondly, the activity of the conditioned medium was greatly reduced by treatment with purified crystalline trypsin (at 0.05% or 500 μg/ml) for 30 minutes at 37°C (and then stopped by 1% soybean trypsin inhibitor and 10% serum) (fig. 3-8d vs. a; $p=0.0011 < 0.05$). Since trypsin is a proteolytic enzyme, the decrease in activity was probably due to the digestion of certain protein molecules. It should be noted that, with trypsin at 0.05%, the activity of the conditioned medium was not completely eliminated since the trypsinized conditioned medium still exhibited activity above DME control (fig. 3-8d vs. e; $p=0.03 < 0.05$). We did not use a higher concentration of trypsin because it could not be completely inhibited by the soybean trypsin inhibitor and, when carried over to the cryosections, would directly react with the cryosections, affecting the result of the assay.

Thirdly, the activity was retained after the macrophage-conditioned medium was dialyzed (14 kd cut-off) against large volume of PBS overnight at 4°C (fig. 3-8f), since the activity was not significantly different from non-dialyzed conditioned-medium (fig. 3-8a) ($p=0.47$, f vs. a) but much higher than DME control (fig. 3-8b) ($p=0.002 < 0.05$). This indicates that the active molecule is not a small molecule below 14 kd, which is a size range more typical of soluble carbohydrates and lipids. Furthermore, the dialysis procedure could be preceded by concentration of the conditioned-medium using polyethylene glycol (PEG) without losing the activity (fig. 3-8g; $p=0.47$, g (PEG concentrated plus dialysis) vs. f (dialysis alone)).

The above three tests showed that the molecule responsible for the activity in the macrophage-conditioned medium has a size above that of typical soluble carbohydrates and lipids, is heat labile and is degradable by proteolytic digestion. Together they suggest that this molecule is very likely, or at least associated with, a protein.

Macrophage-conditioned medium activity can be mimicked by a general protease

Given that the active molecule in macrophage-conditioned medium is a protein, what type of protein can it possibly be? Based on one of our two working models (see Introduction of this chapter) for the molecular mechanism of post-injury nerve environment transformation, we hypothesize that the macrophage-secreted activity acts to remove or inactivate a "degeneration signal" molecule initially present

in the uninjured sciatic nerve, thus rendering the nerve environment supportive of stable neurite outgrowth.

To date, few endogenous molecules are known to be capable of causing existing axons to degenerate in a manner observed in our in vitro DRG-cryosection assays. However, Ichijo and Bonhoeffer (1998) reported the discovery of a "withdrawal factor" produced by the chick posterior tectum, which can cause the withdrawal of pre-existing temporal retinal axons, but not of nasal retinal axons. This unidentified "withdrawal factor" is distinct from the well known chemorepellents ephrin-A2 and ephrin-A5 but, like most typical proteins, is soluble, diffusible, heat-labile and over 5 kb in size. With this as precedent, it is therefore reasonable to further hypothesize that a similar protein exists in the uninjured sciatic nerve as a "degeneration signal" molecule.

To test this hypothesis, we treated normal sciatic nerve cryosections briefly with light trypsin, a general protease, and used the DRG-cryosection assay to see if the cryosections were rendered supportive of stable neurite outgrowth. If the nerve cryosections become supportive after trypsin treatment, we can deduce that a certain protein in the uninjured never environment can cause regenerating axons to degenerate.

The result is shown in fig. 3-9 and fig. 3-10. In fig. 3-9a, the adult injured sciatic nerve cryosection shown had been pre-treated with 0.01% trypsin at 37 °C for three minutes. Under this condition, the cryosection remained intact and its morphology remained unaltered by visual inspection under microscope. GAP-43

immunostaining showed that the DRG neurites growing on the pre-treated cryosection were solid, healthy, uniform in width and free of debris. This is contrary to the degenerative appearance of DRG neurites growing on DME-treated cryosections (fig. 3-9b, control), and reminiscent of DRG neurites growing on 8-day post-injury nerve cryosections (fig. 2-8d) and on neonatal nerve cryosections (fig. 2-11a). The solid appearance of the neurites on trypsin-treated cryosections was highly replicable among all the samples in multiple experiments (see fig. 3-10a and b for statistical data).

We further treated adult uninjured nerve cryosections with serial dilutions of trypsin at 37 °C for 3 minutes and found that the transforming effect could be seen with trypsin concentrations as low as 0.002% (fig. 3-10c, $p=0.007 < 0.05$ when compared with 0% trypsin control). Judging from the degeneration score profile, one may infer that the level of transforming activity elicited by 0.002% trypsin at 37 °C for 3 minutes is comparable to that of 5x diluted LPS-activated macrophage-conditioned medium at 37 °C for 24 hours (compare fig. 3-10c with fig. 3-4c).

However, if the temperature of trypsin treatment was dropped to room temperature, the activity of 0.01% trypsin for 3 minutes was insufficient to effect the transformation of nerve cryosections (fig. 3-10e, $p=0.67$ compared to 0% trypsin). Furthermore, 0.125% bacterial collagenase crude preparation (which contains a small amount of non-specific proteases like dispain) for 30 minutes at 37 °C was also effective in transforming nerve cryosections (fig. 3-10f, $p=0.006 < 0.5$ compared to 0% trypsin), showing that the transforming effect is not specific to trypsin.

Use of the stromelysin knock-out mouse model to test the role of a specific protease in post-injury nerve transformation

Since trypsin can mimic the effect of macrophage conditioned-medium, we hypothesized that the active molecule involved might be a proteolytic enzyme. Among the many extracellular proteinases known to be produced by macrophages, stromelysin-1 (matrix metalloproteinase-3 or MMP-3) seemed to fit the profile of the active molecule involved in nerve transformation. First, stromelysin-1 is indeed highly expressed in the distal segment of the injured sciatic nerve (LaFleur, 1996). Second, stromelysin-1 is typically involved in tissue repair and remodeling events, including wound healing, branching morphogenesis in mammary gland and inflammatory response of alveolar macrophages. (Chang and Werb, 2001; Sternlicht and Werb, 2001; Trask et al., 2001). Third, stromelysin-1 is generally not expressed by tissue macrophages at basal state but its expression drastically increases following cell differentiation, such as in LPS-activated or surfactant-D-stimulated alveolar macrophages (Campbell et al., 1991; Trask et al., 2001). Thus, stromelysin-1 expression is tightly regulated with the maturation state of macrophages in a manner similar to that of the nerve-transforming activity in our system.

To test the possibility that stromelysin-1 plays a role in the transformation of nerve environment after injury in vivo, we employed a transgenic mouse line in which the stromelysin-1 gene has been inactivated by targeted mutagenesis (Mudgett et al., 1998). These "stromelysin knock-out mice" are viable, healthy and reproductively normal. Outwardly, they are indistinguishable from the wild type mice

and the phenotypes due to deficiency of stromelysin tend to be subtle. These mice had been shown to exhibit impaired wound contraction (Bullard et al., 1999), lack of contact hypersensitivity to cutaneous irritant (Wang et al., 1999) and reduced branching morphogenesis in developing mammary gland (Alexander et al., 2001).

We transected sciatic nerves of stromelysin knock-out mice and harvested them 10 days after the operation to test if cryosections of the post-injury nerve can support stable outgrowth using the DRG-cryosection assay. We also tested 10-day post-transection nerve from wild type mice, as well as uninjured nerve from stromelysin knock-out and wild type mice for comparison. This experiment was repeated three times and the one with the largest sample size is reported in fig. 3-11.

We found that both stromelysin knock-out mice (a) and wild type mice (b) had been transformed to support stable neurite outgrowth by 10 days after transection when compared to their respective uninjured control (c and d) ($p=0.0001 < 0.05$ for both knock-out and wild type). In this respect, a deficiency of stromelysin-1 did not eliminate the ability of knock-out mice to transform their nerve environment after injury. If we compare the degeneration scores of post-injury stromelysin knock-out mice (a) and those of post-injury wild type mice (b), they are not significantly different from each other at the 0.05 level ($p=0.07$, 2-sided 2-sample test). Therefore, we conclude that stromelysin is not necessary to effect transformation of the post-injury nerve environment in vivo.

Preliminary evidence that proteases may be involved with the macrophage-conditioned medium activity

We also took a different approach to test the hypothesis that the active molecule in the macrophage-conditioned medium is a protease. We did so by testing if synthetic protease inhibitors specific to each of the four known classes of proteases (metalloprotease, serine protease, cysteine protease and aspartic protease) can inhibit the activity in the macrophage-conditioned medium. After much experimental complications, we finally collected preliminary data that support the hypothesis. But due to the preliminary nature of the results, we are presenting them in more details in the appendix following this chapter.

Macrophage-conditioned medium activity cannot be mimicked by non-proteolytic matrix-modifying enzymes tested

Since our discovery of the ability of trypsin in transforming the nerve environment, we have focused on the possibility that the presumptive "degeneration signal" molecule in the uninjured nerve is a protein. But theoretically, it can also be a carbohydrate, lipid or other types of non-protein molecule that happens to be associated with a protein. Our results of the trypsin experiment did not exclude this possibility.

In order to test this idea, we treated uninjured nerve cryosections with various enzymes that break down specific non-protein components of the extracellular matrix and used the DRG-cryosection assay to see if the pre-treated nerve

cryosections become supportive of stable neurite outgrowth. The list of enzymes tested include ones that break down chondroitin sulfate proteoglycan, phospholipid linkages, hyaluronic acid and heparan sulfate proteoglycan. Transglutaminase, which modifies proteins by cross-linking glutamine residues, is also included in the test. The experiment was performed twice with 8 samples per enzyme.

- liver transglutaminase (1.25μM, 1 hour, 37 °C)
- chondroitinase ABC (0.2 U/ml, 1 hour, 37 °C)
- phosphatidylinositol phospholipase c (PI-PLC, 200 mU/ml, 1 hour, 37 °C)
- hyaluronidase (1 mg/ml, 30 minutes, 37 °C)
- heparinase (from *Flavobacterium heparinum*, 0.5 U/ml, 30 minutes, 37 °C)

The nerve cryosections following treatment with the above enzymes did not show any physical sign of having been altered and were visually identical to untreated cryosections under light microscopy.

The result of the experiment is shown in fig. 3-12. We found that nerve cryosections pre-treated with transglutaminase (c), chondroitinase ABC (d), PI-PLC (e), hyaluronidase (f) or heparinase (g) did not support stable neurite outgrowth above the level of control (b) ($p >> 0.05$), implying that the "degeneration signal" molecule in the nerve environment is not a substrate of these enzymes and that the macrophage-conditioned medium activity is not likely to be acting on the substrates of these enzymes. The negative result with chondroitinase ABC was particularly interesting because chondroitin sulfate was a known inhibitor to neurite outgrowth in

the CNS (Snow et al., 2001) but its removal in the PNS apparently does not affect neurite outgrowth in our in vitro culturing system.

Macrophage-conditioned medium activity cannot be mimicked by growth factors known to be produced by macrophages

We now turn our attention to the "permission signal" model, the second of the two possible working models for the molecular mechanism of post-injury nerve transformation. In this model, we hypothesize that infiltrating macrophages secrete certain "permission signal" molecules, which become incorporated with the post-injury nerve environment and subsequently promote the outgrowth of stable neurites.

The classical molecules that promote neurite outgrowth include permissive matrix molecules (such as laminin and fibronectin) and neurotrophic factors (such as nerve growth factor family members and ciliary neurotrophic factor), neither of which is typically expressed by macrophages (Rappolee and Werb, 1989). However, macrophages secrete a wide variety of other growth factors that can possibly affect the survival and growth of neurons. Therefore, we want to test the possibility that macrophage-derived growth factors can transform the nerve environment in a manner similar to the activity of macrophage-conditioned medium.

To do this, we tested the ability of normal sciatic nerve cryosections to support stable outgrowth following 24-hour incubation with each of the many growth factors

listed below (in DME). The experiment was repeated twice with eight samples per growth factor.

- Fetal bovine serum (10%)
- IL-1α (4 ng/ml)
- IFN-γ (40 ng/ml)
- TGF-β1 (10 ng/ml)
- LIF (2000 U/ml)
- bFGF (100 ng/ml)
- EGF (50 ng/ml)
- Insulin (2 μg/ml)
- HGF (50 ng/ml)
- PDGF-BB (200 ng/ml)
- IGF-1 (100 ng/ml)

The result is shown in fig. 3-13. We performed Wilcoxon's two-sample test on the degeneration scores obtained from each growth factor treatment against those from DME treatment (control). We did not find any of the tested growth factors to be capable of inducing stable neurite outgrowth above the level of DME control, which macrophage-conditioned medium did ($p=0.002 < 0.05$). In fact, the neurites on growth factor-treated cryosections (see photos in fig. 3-13) appeared indistinguishable from those on DME-treated cryosections. Therefore, we concluded that the active molecule of macrophage-conditioned medium is not likely to be one of the growth factors tested.

Presence of neurotrophic factors in the cultures of DRG explants did not induce stable neurite outgrowth on normal cryosections

Neurotrophic factors are potent molecules that regulate the survival, growth, differentiation and neurite extension of neurons. Since macrophages are not commonly known to secrete neurotrophic factors except in anecdotal reports (such as Ikeda et al., 2001; Caroleo et al., 2000), it is not very likely that neurotrophic factors are present in the macrophage-conditioned medium. However, it remains possible that certain factors in the conditioned medium bind to or interact with the nerve cryosections in such a way that, when DRG explants are cultured on them, Schwann cells or fibroblasts from the DRG become induced to express neurotrophic factors, indirectly affecting the stability of neurite outgrowth. This would be an interesting possibility if neurotrophic factors can indeed induce stable neurite outgrowth.

To test if neurotrophic factors can induce stable neurite outgrowth, we both pre-incubated normal sciatic nerve cryosections with single or multiple neurotrophins (see list below) for 24 hours and cultured DRG explants on the cryosections in the presence of the same neurotrophin(s), in addition to the 50 ng/ml of NGF routinely added to the culture medium. For controls, we used macrophage-conditioned medium (positive control) or plain DME (negative control) for pre-incubation of cryosections but no extra neurotrophin for culturing of DRG explants besides the 50 ng/ml of NGF already present in the culture medium. The experiment was repeated

twice with eight samples per treatment. The following is a list of the neurotrophins tested:

- NGF (50 ng/ml)
- BDNF (50 ng/ml)
- NT-3 (50 ng/ml)
- CNTF (ciliary neurotrophic factor, 50 ng/ml)
- GDNF (glial-derived neurotrophic factor, 50 ng/ml)
- combinations of the above, including:
  - NGF + BDNF
  - NGF + NT-3
  - NGF + GDNF
  - NGF + BDNF + NT-3
  - NGF + BDNF +CNTF
  - NGF + NT-3 + CNTF
  - NGF + NT-3 + GDNF
  - NGF + BDNF + NT-3 + CNTF

The degeneration score profiles of the neurite outgrowths are shown in fig. 3-14. Wilcoxon's two-sample test shows that the health conditions of the DRG neurites in the presence of additional neurotrophins (c to n) was not significantly different than that with no additional neurotrophins (b) ($p >> 0.05$). Again, macrophage-conditioned medium induced stable outgrowth as before.

In conclusion, the stability of stable outgrowth on normal nerve cryosection does not appear to be enhanced by the neurotrophins tested even when the neurotrophins clearly have direct access to the neurites. This casts doubt on the hypothesis that the macrophage-conditioned medium exerts its effect directly or indirectly by means of neurotrophins.

DISCUSSION

Summary of results and conclusions

We found that elicited peritoneal macrophages secrete a diffusible factor that can transform an adult normal sciatic nerve cryosection into one that supports stable neurite outgrowth, an effect similar to that observed in vivo within the first eight days following a transection injury. This diffusible factor was also produced by P388D1 mouse monocyte-macrophage cell line, although only at about one-fifth the concentration produced by primary macrophages. The expression of the factor appeared to be dependent on the maturation state of macrophages: no expression in freshly collected peritoneal macrophages, higher expression by 3 days of culture, and even higher expression when activated by inflammatory agents such as lipopolysaccharide, IL-1 or IFN-γ.

We have also shown by immunohistochemistry that macrophages were present in the injured sciatic nerve in a time frame that is consistent with the hypothesis that they are responsible for transforming the post-injury nerve environment. They arrive at the distal stump of the transected nerve 4 days after injury, then progress distally until they are present throughout the distal segment by 8 days after injury, which was previously shown to be the time when the nerve environment is optimal for supporting stable neurite outgrowth. The fact that the macrophages were present four days before the nerve environment attains optimal support may suggest either that the transforming process requires four days of

macrophage activity or that it requires a critical concentration of macrophages to effect, which can be achieved only after four days of infiltration.

We had tried using the op/op CSF-1-deficient mice as a macrophage-less mouse model to elicit the role of macrophages in post-injury nerve transformation in vivo. Unfortunately, the op/op mice, although deficient in macrophages in some tissues, actually exhibited massive infiltration of macrophages in the distal post-injury nerve similar to that observed in wild type mice.

Biochemical analysis of the macrophage-conditioned medium indicated that the molecule(s) responsible for its activity is likely a protein by the criteria of size, heat denaturation and trypsin digestion.

We showed that when normal nerve cryosections are treated briefly with light trypsin, they become capable of supporting stable DRG neurite outgrowths, much like nerve cryosections pre-treated with macrophage-conditioned medium. Preliminary data from experiments using protease inhibitors also support the activity involved may be a protease. Since trypsin is a protease with broad specificity, we hypothesized that stromelysin-1, a protease produced by activated macrophage, might be involved in the nerve transforming process. However, when the sciatic nerves of stromelysin knock-out mice were transected, the nerve environment were able to support stable neurite outgrowth 10 days post-injury, implying that stromelysin-1 is not required for the transformation process.

We have tested the effect of different matrix-modifying enzymes on normal nerve cryosections and determined that if a "degeneration signal" molecule indeed

exists in the normal nerve environment, it is not likely to be chondroitin sulfate proteoglycan, heparan sulfate proteoglycan, hyaluronic acid or linked to a phospholipid. We also ruled out the possibility that certain macrophage-secreted growth factors can transform the normal sciatic nerve environment to one that support stable neurite outgrowths. Finally, we showed that even though NGF is absolutely required to promote the initial extension of neurites, their stability on nerve cryosections is likely to be determined by factors other than the five neurotrophic factors tested (NGF, BDNF, NT-3, GDNF and CNTF).

Lessons learned from the use of transgenic mice

Transgenic or "knock-out" mice are favorite tools for biologists to definitively demonstrate essential functions of a gene or protein of interest, based on altered phenotypes in the transgenic mice compared with wild type mice. However, in cases where altered phenotypes are not apparent, the data is harder to interpret into useful information.

We had used the CSF-1-deficient op/op mice and the stromelysin-1-deficient mice to test the role of macrophages and stromelysin-1 in post-injury nerve transformation. In the case of the op/op mice, macrophages, which were deficient in most tissues, actually successfully reacted to sciatic nerve injury and infiltrated the tissue at a density comparable to wild type mice. One interpretation is that these infiltrating macrophages came from the recovery of certain populations of macrophages under the influence of some other growth factors which may have

compensated for the effect of CSF-1. In fact, not only are GM-CSF (granulocyte-macrophage colony-stimulating factor) and IL-3, both involved in the regulation of macrophage differentiation, highly elevated in op/op mice (Wiktor-Jedrzejczk, 1993a), it was also shown that injection of exogenous GM-CSF can completely reverse the macrophage-deficiency phenotype in op/op mice, except for osteoclasts (Wiktor-Jedrzejczk, 1993a). Several groups of researchers have also reported spontaneous recovery of many macrophage populations with age, such as in alveoli of lungs (Shibata et al., 2001), bones and bone marrow (Nilsson et al., 1995). Furthermore, the observed recovery could be accelerated by an enriched diet (Wiktor-Jedrzejczak, 1993b), which could have happened in our colony of op/op mice fed with milk and cereal slurry.

Another possible interpretation is that infiltrating macrophages of injured nerve tissue are derived from a CSF-1-independent subpopulation of macrophages and therefore were not affected by the lack of CSF-1. Several researchers (Naito et al., 1997; Wiktor-Jedrzejczak et al., 1992) have hypothesized that, in normal mice, while a CSF-1-dependent subpopulation of macrophages is responsible for the regulation of immune cells by production of monokines, a separate CSF-1-independent subpopulation of macrophages is responsible for phagocytosis, antigen processing and presentation. The latter group might account for the inflammatory reaction following sciatic nerve injury.

In the case of the stromelysin-deficient mice, we found that the post-injury transformation of the nerve environment was not hampered by the absence of

stromelysin. It is interesting that while stromelysin is involved in so many different processes of tissue repair and remodeling, the phenotypes of the knock-out mice are generally mild. One interpretation is that two other closely-related isoforms of stromelysin exist (stromelysin-2 and -3), which in turn belong to a family of about 25 matrix metalloproteinases (MMP). It is conceivable that these other members of the MMP family may alter their pattern of expression and compensate for the lack of stromelysin-1. This is similar to the previous case of CSF-1 deficient mice, where GM-CSF and IL-3 became highly elevated, partially compensating for the effect of CSF-1 deficiency.

As a result, the lack of phenotype in our "knock-out" mice experiments does not automatically mean that the cell type or protein of interest is irrelevant to the biological process at hand. Compensatory events may have occurred that mar the expected phenotypes.

"Permissive signal model" versus "degeneration signal model" revisited

With the results described in this chapter, we now re-evaluate our two working models of nerve transformation. Although we still do not have definitive evidence to rule out either model, overall, it appears that our new data lend more support to the "degeneration signal model" than to the "permissive signal model" for several reasons.

First, we have identified the macrophages as the cell type capable of causing the transformation of nerve environment. As macrophages are involved in many

processes of tissue repair and remodeling by phagocytosis and expression of matrix-degradative enzymes, they are possible candidate to be involved in the removal of a "degeneration signal" molecule in the nerve environment after injury. On the other hand, macrophages do not typically express molecules classically involved in the promotion of neurite outgrowth, such as permissive matrix molecules and neurotrophic factors. Many of the growth factors produced by macrophages, however, are not capable of inducing the normal nerve environment to support stable outgrowth. Since we have further demonstrated that stable outgrowth of DRG neurites cannot be induced by direct access to neurotrophic factors, the support of stable neurite outgrowth is likely effected by molecular mechanisms other than one via neurotrophic factors.

Second, we showed that trypsin is capable of rendering normal sciatic nerve cryosections supportive of neurite outgrowth. This observation implies that the transformation of the nerve environment does not necessarily require the addition of any extra "permission signal" molecule. Rather, the proteolytic processing of certain yet unknown protein that pre-exists in the normal nerve is sufficient to allow stable neurite outgrowth on the nerve substrate. Such protein may possibly function as a "degeneration signal" molecule. While macrophages do not secrete trypsin, they typically secrete a wide range of other proteases during tissue remodeling events, some of which can possibly play the in vivo role of the same proteolytic processing of the nerve substrate exhibited by trypsin in vitro. Therefore, the "degeneration signal" model appears more consistent with the proteolytic mode of operation.

Furthermore, one may argue in favor of the "degeneration signal" model based on the fact that many of the matrix molecules abundant in the peripheral nerve, such as laminin, fibronectin and type I and type IV collagen, are known to be permissive substrate for neurite outgrowth. It follows that the environment of a normal nerve should favor stable neurite outgrowth, unless a special "degeneration signal" is present to tell the neurites not to grow stably. The "permissive signal" model, however, proposes that an additional molecule is to be laid down in an already permissive substrate after injury. Although possible, this model does not satisfactorily explain the fact that normal nerve fails to support stable outgrowth.

Still it is possible that neither of the two working models are entirely accurate as proposed. For example, a "degeneration signal" may not be inactivated by a degradative enzyme, but instead, by a capping protein—in which case, the addition of an extra molecule is called for by the "degeneration signal" model. Conversely, the nerve environment may be transformed by a "permissive signal" resulting from the modification of a protein present in the native nerve environment—in which case, a protein-processing event is called for by the "permissive signal" model. Therefore, when using the two working models as a guide to interpret experimental data and to plan for future experiments, it is important to keep in mind that other variations or even more tortuous models also exist.

CHAPTER 3 APPENDIX:

Preliminary evidence that proteases may be involved with the macrophage-conditioned medium activity

We have shown that trypsin, a general protease, can mimic the effect of macrophage-conditioned medium on uninjured sciatic nerve cryosections. However, the important question remained: is the activity in macrophage-conditioned medium really a protease?

To answer this question, we tested whether synthetic inhibitors for the four classes of proteases (E-64 for cysteine proteases, pepstatin for aspartic proteases, PMSF for serine proteases and EDTA for metalloproteases) can also inhibit the activity of macrophage-conditioned medium. If they do, we can infer that a protease of a certain class is involved with the activity.

E-64, pepstatin and PMSF are insoluble and irreversible protease inhibitors. As a result, we had to perform extra steps to ensure that the protease inhibitors never come in contact with the cryosections because, if they do, their presence can disrupt the outgrowth of the neurites since they are almost impossible to completely wash away.

Therefore, we first concentrated the macrophage-conditioned medium eight-fold with polyethylene glycol (PEG), treated the concentrated conditioned medium with the protease inhibitor(s) for one hour, and then dialyzed the mixture overnight against phosphate-buffered saline (PBS) to remove any free protease inhibitors before it is used to pre-treat the uninjured nerve cryosections overnight.

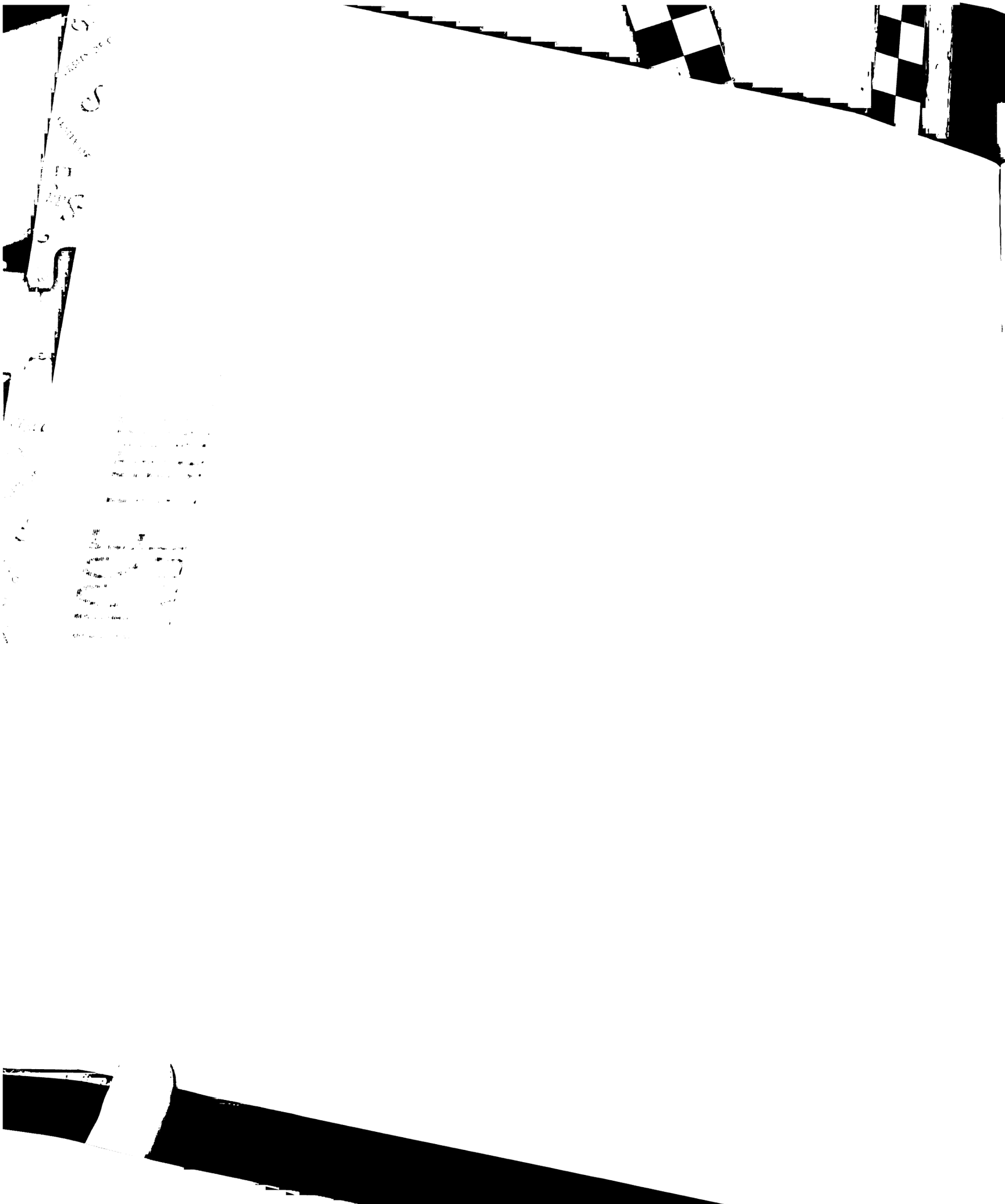
EDTA, however is a soluble, reversible inhibitor. Therefore it is added to the conditioned medium during the pretreatment of the cryosections, after PEG concentration and dialysis. Thorough washing with PBS later is sufficient to completely remove it from the cryosections.

It took many trials and adjustments to finally get all the positive and negative controls to work properly. The result of the final experiment is shown in the table below. Since this particular experiment was performed only once with four samples per treatment, the result is preliminary in nature and must be verified by duplicated experiments.

Sample #	Treatments before pre-incubation with nerve cryosection	DRG assay median score
1a	MØ CM → dialysis	4.5
1b	DME → dialysis	2.5
2a	MØ CM + PMSF → dialysis	3
2b	DME + PMSF → dialysis	2.5
3a	MØ CM + E-64 → dialysis	2.5
3b	DME + E-64 → dialysis	2
4a	MØ CM + Pepstatin → dialysis	2
4b	DME + Pepstatin → dialysis	2
5a	MØ CM → dialysis → + EDTA	6
5b	DME → dialysis → + EDTA	3
6a	MØ CM + PMSF, E-64, Pepstatin → dialysis	2.5
6b	DME + PMSF. E-64, Pepstatin → dialysis	1.5
7a	MØ CM + PMSF, E-64, Pepstatin → dialysis → + EDTA	4
7b	DME + PMSF, E-64, Pepstatin → dialysis → + EDTA	2.5

The scale of the degeneration score ranges from 1 to 6. A high score was assigned to highly stable neurite outgrowth while a low score was assigned to highly degenerative neurite outgrowth.

The controls tell us that our manipulations did not perturb the system in any unintentional way. For example, the neurites in sample 1a (positive control) was



much more stable than that of sample 1b (negative control), meaning that the macrophage-conditioned medium, after the processes of PEG concentration and overnight dialysis, still retained its activity. The median scores of sample 1b (negative control, without protease inhibitor) and samples 2b, 3b, 4b, 5b, 6b, 7b (negative controls, with various protease inhibitors) are very similar (with a difference of 1 or less), which tells us that the dialysis procedure had successfully removed any free protease inhibitors since the neurites would degenerate in the presence of residual protease inhibitor. Therefore, any changes in the stability of DRG neurites in the experimental samples can only be attributed to the protease inhibitor-treated macrophage conditioned-medium.

Two observations can be made from the results of the experimental samples. First, the activity of the macrophage-conditioned medium could be inhibited by any one of the following three protease inhibitors: PMSF (for serine proteases), E-64 (for cysteine proteases) or pepstatin (for aspartic proteases). For example, comparing samples 2a with 2b, 3a with 3b, and 4a with 4b, the activity of macrophage-conditioned medium dropped to about the level of the negative controls when the protease inhibitors were added. Second, rather unexpectedly, EDTA elevated, instead of inhibited, the activity of macrophage-conditioned medium. When EDTA was added to the conditioned medium (sample 5a), the subsequent neurites grew exceptionally stably (median score of 6) compared to negative control (sample 5b; median score of only 3). In addition, this elevation of activity occurred even when the macrophage-conditioned medium had already been

treated with PMSF, E-64 and pepstatin (sample 7a; median score of 4) compared with control (sample 6a; median score of 2.5). Thus EDTA appeared to at least partly compensate or overcome the inhibiting effects of PMSF, E-64 and pepstatin.

Discussion

The result showed that any one of three classes of protease inhibitor can inhibit the activity of the conditioned medium, making it difficult to definitively attribute the activity to one single class of protease. Moreover, it raises intriguing questions concerning the exact nature of the macrophage-secreted activity. It may be possible that the activity actually involves a cascade of several classes of proteases, similar to the plasmin-fibrin clotting cascade (Sidelmann, 2000), a phenomenon common to many proteases synthesized as pro-enzymes, which are dependent on other proteases for their activation. If this is the case, inhibiting any one member of the cascade may subsequently inhibit the activity.

The result showed that EDTA did not inhibit the activity of the conditioned-medium, so one may conclude that the activity is not attributable to a metalloprotease. But how could the activity be increased by EDTA? Perhaps the activity could be destroyed by a metalloprotease present in the conditioned medium and by inhibiting this metalloprotease, more of the activity was preserved. On the other hand, the activity may be inhibited by divalent cations like Mg^{2+} and Ca^{2+}, which can be sequestered by a chelator, such as EDTA, leading to an increase of the

activity. For example, cysteine proteases are inhibited by divalent cations and, therefore, can be activated by EDTA.

In conclusion, it appears that some kinds of protease activity are likely to be involved with the macrophage-conditioned medium activity, but the complete picture may be more complicated than the single-protease hypothesis.

CHAPTER 4: SUMMARY OF THE THESIS AND FUTURE RESEARCH DIRECTION

This current thesis project was first conceived based on two pieces of information about nerve regeneration: first, nerves capable of regeneration (e.g. sciatic nerve of peripheral nervous system) become permissive to the growth of neurites following injury while nerves incapable of regeneration (e.g. optic nerve of central nervous system) do not; second, nerves capable of regeneration display profuse infiltration of macrophages following injury while nerves incapable of regeneration do not. Thus we set out to test the hypothesis that the infiltrating macrophages after nerve injury cause the positive change in substrate permissiveness for neurite outgrowth.

However, as we proceeded with our experiments, we discovered that what changes in the sciatic nerve following injury is not the permissiveness to neurite attachment, since, under our experimental conditions, regenerating neurites were able to grow extensively on both uninjured and post-injury sciatic nerve substrate. What does change, however, is the ability to support these regenerating neurites to remain healthy after the initial growth. We observed that neurites growing on uninjured sciatic nerve substrate begin to degenerate shortly after contact with the substrate while neurites outgrowing on post-injury nerve substrate remain healthy for up to 9 days in culture. Such observations could imply a method of neural pathfinding, not by contact adhesion or long-distance chemoattraction, but by the trimming of inappropriate neurites based on the property of the substrate.

Hence, we interpreted the non-supportive nature of normal adult sciatic nerve as a safeguard against inappropriate sprouting of neurites. Consistent with this idea, we found that the neonatal nerve environment supports stable outgrowth of adult DRG (dorsal root ganglia) and that neonatal DRG neurites do not degenerate when growing on adult normal sciatic nerve environment. Taken together, the observations indicate that the neonatal nerve, a site of active neurite outgrowth, presents itself as an appropriate substrate for neurite outgrowth and that the neonatal neurites, already in a state of active growth, do not recognize the adult nerve environment to be inappropriate. In other words, the systems of substrate presentation and recognition are probably not installed before the nervous system matures.

Interestingly, the ability to support healthy neurites by the post-injury sciatic nerve is only temporary. According to our semi-quantitative blind-scoring system, the substrate is most capable of supporting healthy neurites around 8 days after injury but this capability gradually decreases from 10 days after injury until it disappears by 20 days after injury. We do not know what causes such decrease but apparently, the penetration of regenerating neurites into the distal post-injury nerve segment is not involved. The finding that the post-injury nerve environment is optimally supportive of healthy neurite outgrowth for only a brief time window may have far-reaching consequence in the treatment of nerve injury.

We then returned to the question whether macrophages play any role in the change of the nerve substrate to support outgrowth of healthy neurites following nerve injury. First, we found that macrophages in vivo begin to appear at the distal

stump 4 days after injury and complete the infiltration throughout the distal nerve segment by 8 days after injury, coincidental with the time of optimal support for stable outgrowth. Thus the time course of macrophage infiltration is consistent with the possibility that this cell type is involved in the change of the substrate property. Second, macrophages in vitro are capable of producing a diffusible factor that transforms normal sciatic nerve substrate into one that supports stable neurite outgrowth, a property formerly only observed in post-injury sciatic nerve substrates. Thus, we inferred that macrophages are capable of transforming the post-injury distal nerve segment to support stable neurite outgrowth.

The identity of this macrophage-secreted diffusible factor is not yet known. But it is likely a protein according to our biochemical characterization. This factor is also produced by P388D1 monocyte-macrophage cell line and is up-regulated when macrophages are activated by inflammatory agents such as lipopolysaccharide. It may be a macrophage-secreted protease that removes or otherwise inactivates a presumptive "degeneration signal" molecule existing in the nerve environment, since brief exposure of nerve cryosections to trypsin has the same transformation effect as macrophage-conditioned medium and synthetic protease inhibitors appear to interfere with the activity of the macrophage-conditioned medium.

On the other hand, enzymes that degrade non-protein matrix components, such as chondroitin sulfate proteoglycan, do not exert the same effect on nerve cryosections, implying that these components are not directly involved with the "degeneration signal". We have also shown that a battery of purified

macrophage-secreted growth factors tested do not mimic the effect of macrophage-conditioned medium, discounting the possibility that these molecules are involved. Finally, we found that DRG neurites do not grow on normal nerve cryosection in a stable manner even in the presence of exogenous neurotrophins, either singly or in various combinations, suggesting that the mechanism to induce stable outgrowth is probably by means other than neurotrophic factors.

Issues left unresolved in this project

Two major questions central to the understanding of the post-injury nerve transformation process are left unresolved by this project, making them possible targets of future research. First, what is the identity of the active molecule in macrophage-conditioned medium? Second, are macrophages causing the transformation of nerve environment in vivo?

Identification of the macrophage-secreted active molecule by purification or cloning

Certainly a major breakthrough to understanding how macrophages contribute to the transformation of the post-injury nerve will occur with the identification of the macrophage-conditioned medium activity. With the protein and gene of the activity identified, a whole host of other studies will be possible, including the expression of the molecule by macrophages using in situ hybridization,

immunocytochemistry and PCR. Furthermore, a plausible mechanism for the transformation process can be hypothesized and tested.

The active molecule can be identified by biochemical purification from macrophage-conditioned medium. To this end, a faster and simpler version of the DRG-cryosection assay will be very helpful for testing a large number of cryosection samples. It will also be useful to identify a cell line and an activating condition in which the same activity can be mass-produced cheaply. We have identified P388D1 as a possible source of the activity, but perhaps due to its early differentiation stage in the macrophage lineage, the secreted level of the activity is only a few fold above that of background. A cell line representing a more mature stage, coupled with activating agents, may be a better choice.

As for cloning of the gene encoding the macrophage-secreted activity, DNA microarray represents a new exciting approach. With this method, thousands of cDNA or ESTs (expressed sequence tags) can be screened in a single experiment without any preconceived function of the protein. This method is particularly suitable for picking out genes that are differentially expressed, such as, in our case, genes that are highly expressed by LPS-activated macrophages but not by freshly-collected macrophages. Since the identities of the cDNAs etched on the microarray are known, we can easily compile a list of tens of such differentially expressed genes and sort out the ones that encode secretory and proteolytic enzymes for further testing. The ability of these proteins to induce nerve transformation can then be confirmed by testing conditioned medium of CHO cells transfected with the identified genes. Thus, unlike

the approach of biochemical purification, using DNA microarrays allows us to clone and identify the macrophage-secreted activity without further investment in improving the DRG-cryosection assay protocol or the source of the macrophage-secreted activity.

Obtaining more in vivo evidence to support the role of macrophages in post-injury nerve transformation

The putative ability of macrophages to transform the post-injury nerve environment has been deduced from experimenting with molecules secreted by macrophages in vitro. Although we have shown that infiltrating macrophages in vivo appear in the injured nerve in a time frame consistent with a role in transforming the nerve environment, more direct evidence is still lacking. We have attempted to use the op/op macrophage-deficient mice to test the effect of absence of macrophages, but unfortunately, macrophages apparently independent of CSF-1 were able to infiltrate the injured nerve in a manner almost indistinguishable from wild type mice.

Undoubtedly, if the active molecule that causes the transformation effect is cloned, much in vivo information can be obtained by studying its expression by in situ hybridization or immunohistochemistry. For the mean time, other mouse lines that display deficiency in macrophage recruitment during Wallerian degeneration can be used, such as the C57BL/Ola mice (aka Wld^S mice; Bisby and Chen, 1990), tumor necrosis factor-α knock-out mice (Liefner et al., 2000) and the CSF-1 receptor knock-out mice (Dai et al., 2002).

However, as discussed before, knock-out mice often undergo compensatory events that hide the phenotypes. A more novel approach is to study mice in which macrophages are biochemically eliminated just before nerve injury. Several groups have reported success in using liposome-encapsulated dichloromethylene bisphosphonate (clodronate) to induce selective apoptotic death in monocytes and phagocytic macrophages (Popovich et al., 1999; van Rooijen, 1996). Such approaches can even allow for dose-adjustment in controlling the amount of macrophages available for the inflammatory responses.

Future direction of research on issues extended from this project

The discovery of the nerve transformation process after injury opens up a whole host of other related questions which were not even addressed in this project. Many of these questions can form the basis for future research, as new technology becomes available to explore them.

One such issue has to do with the exact manner in which neurites are withdrawn when they step on the substrate of normal uninjured nerve. So far, we were only able to take snapshots of the neurite outgrowth at different time points in culture. We understand that the degeneration happens rather quickly after contact with the cryosection but we also observed continued growth of neurites after contact. To understand the dynamics of these two simultaneous processes, it would be useful to be able to observe neurite outgrowth in real time. One promising approach is to utilize animals that carry a copy of the green fluorescent protein (GFP) gene coupled

with a constitutive promoter (actin promoter) (Davies et al., 1999). The extending axons from these animals, apparently unaffected by the GFP protein, are fluorescently labeled to the point where even growth cone morphology can be observed. Therefore, time-lapse cinematography can be used to observe the neurite degeneration process in real time, either on 2-dimensional nerve cryosection substrate or within a 3-dimensional nerve explant. It is possible that special behaviors of the neurites prior to degeneration can be identified, allowing the design of a shorter real-time assay using isolated neurons.

As far as the molecular mechanism goes, we have collected evidence favoring the "degeneration signal" model, which states that a certain molecule, possibly a protein, exists in the normal nerve environment, causing regenerating neurites to degenerate. So far, we have been focusing on the identity of the macrophage-secreted protein that possibly inactivate the "degeneration signal" molecule, but what about the identity of the "degeneration signal" molecule itself? Our trypsin experiment indicated that it is likely a protein but we do not know anything beyond that. A possible strategy to clone this molecule is to recognize that neonatal sciatic nerves do not induce neurite degeneration but do as the nerve matures. Using the DNA microarray method, we can look for genes that are not expressed in neonatal nerve but are highly expressed at the time when the nerve begins to become non-supportive of neurite outgrowth. These genes are potential candidates for the "degeneration signal" molecule. The function of candidate molecules can then be tested by coating the expressed proteins on a suitable

substrate and checking to see if neurites degenerate when they come in contact with the proteins.

Since macrophages appear to play a central role in modifying the nerve environment after injury, it will be very informative to compare the states of the macrophages before and after their infiltration of an injured nerve. To date, a good catalog of genes that change expression between these two states of macrophages has not been reported. Such a catalog will provide a clear picture as to what the macrophages are equipped to do once inside the injured nerve and what other actions by the macrophages may also contribute to the transformation of the nerve environment. Again, DNA microarray analysis that compares gene expression of circulating monocytes, versus early infiltrating macrophages and versus later-stage infiltrating macrophages may accomplish this goal.

Finally, is the transformation of nerve environment really required for successful regeneration? So far we have been studying the transformation environment in isolation without knowing whether this process actually affect the overall nerve regeneration. It appears that the obvious way to address this question is to prevent the transformation of nerve environment from taking place and see if subsequent nerve regeneration fails as a result. Unfortunately, at present, we do not have any tools that can selectively perturb the transformation process without also perturbing other events important for regeneration. This situation can be alleviated when we finally identify either the macrophage-secreted active molecule required for inactivating the "degeneration signal" or the "degeneration signal" molecule itself.

We will be able to generate transgenic mice deficient of the macrophage-secreted factor, which supposedly will not be able to inactivate the "degeneration signal". Alternatively, mice carrying a transgene encoding an uncleavable form of the "degeneration signal" molecule can be created. These mice will express a persistent version of the "degeneration signal" molecule, which may in turn affect their ability to regenerate their injured nerves.

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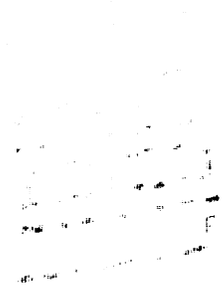

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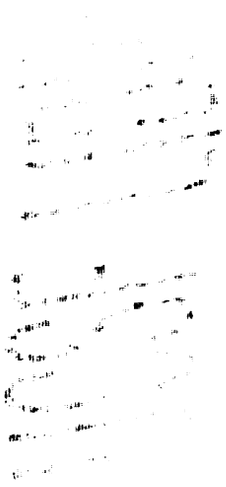

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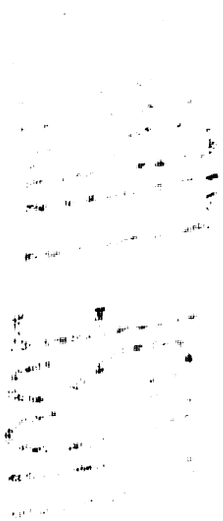

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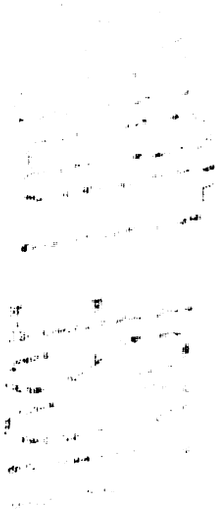

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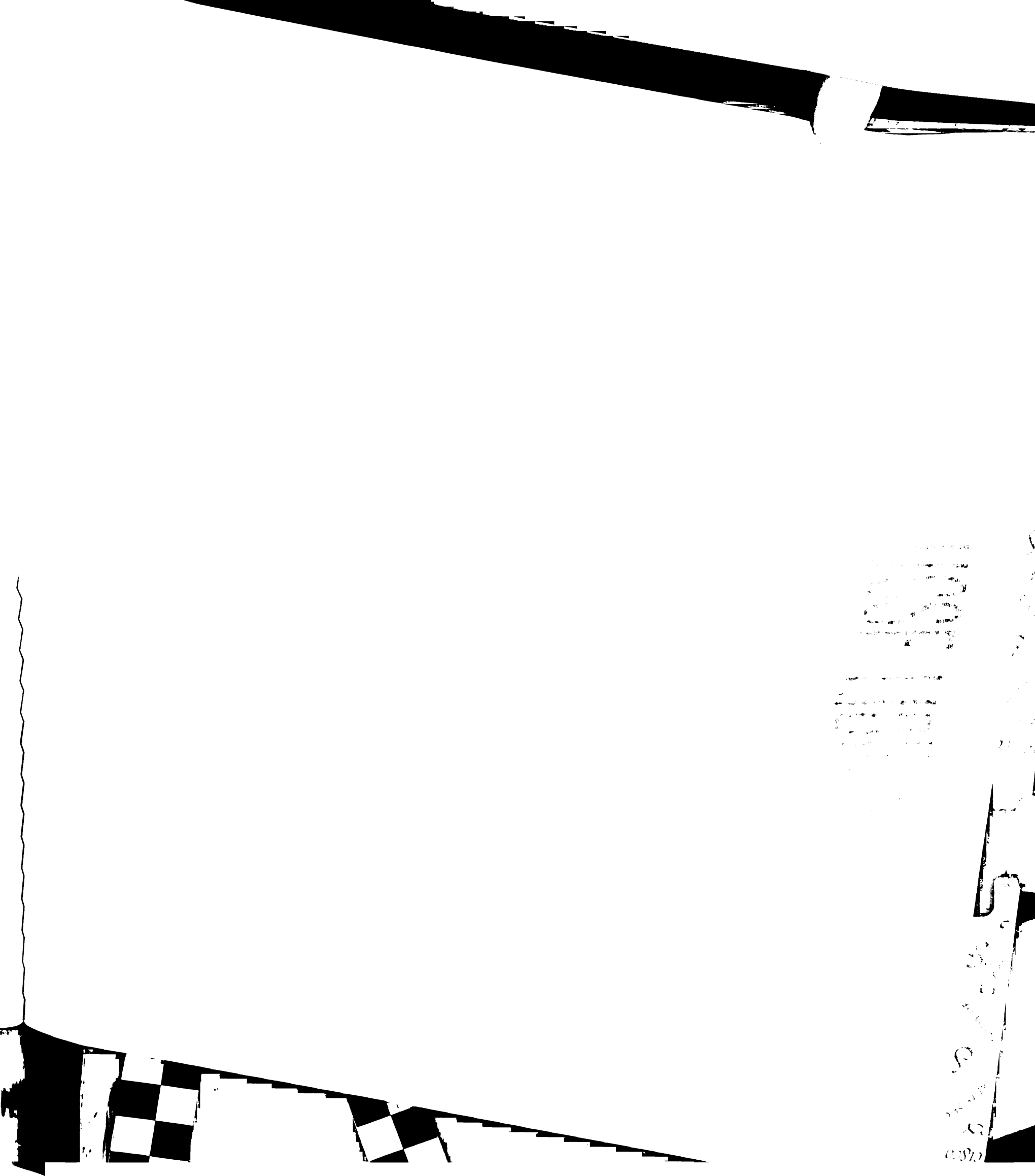

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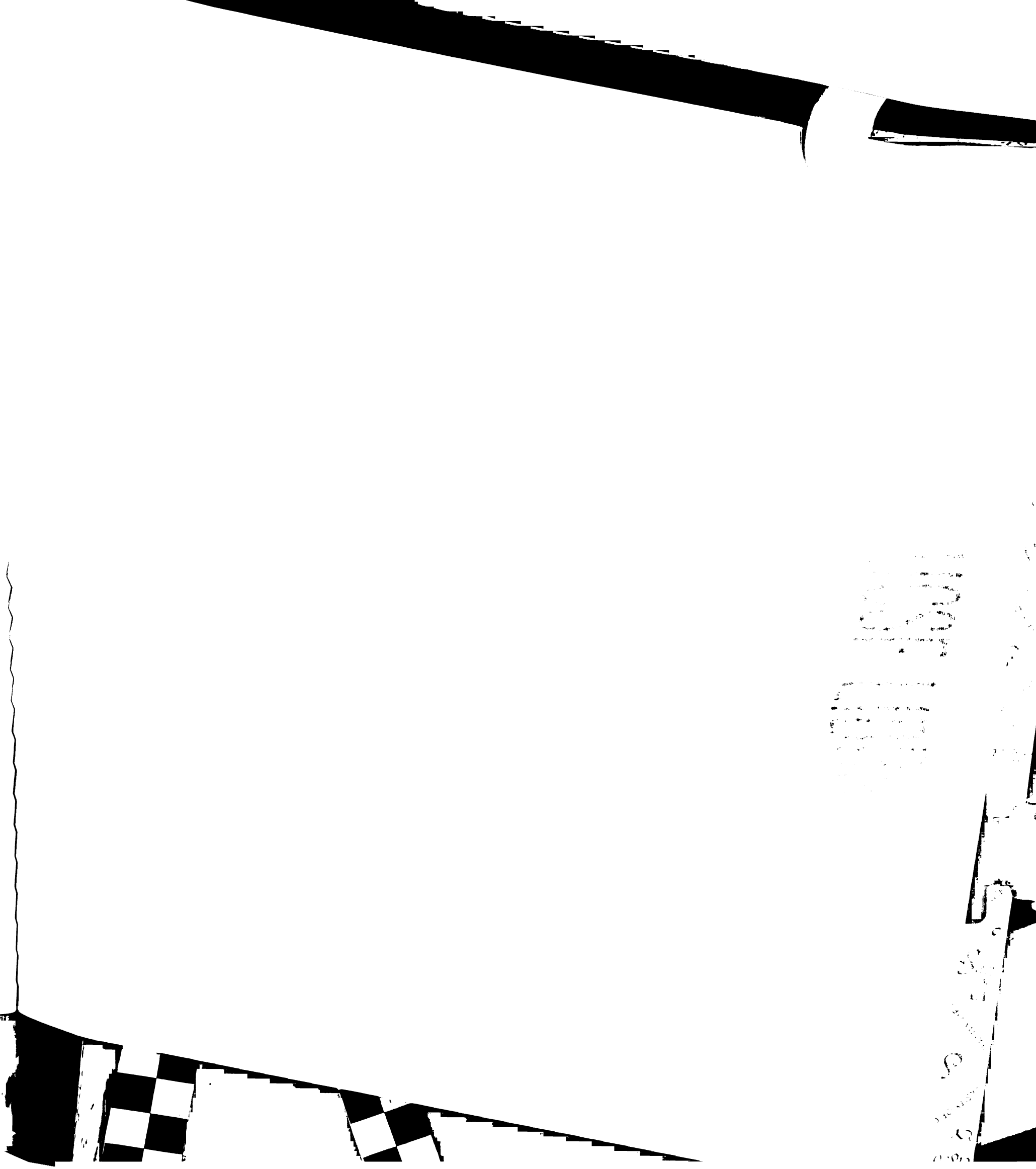

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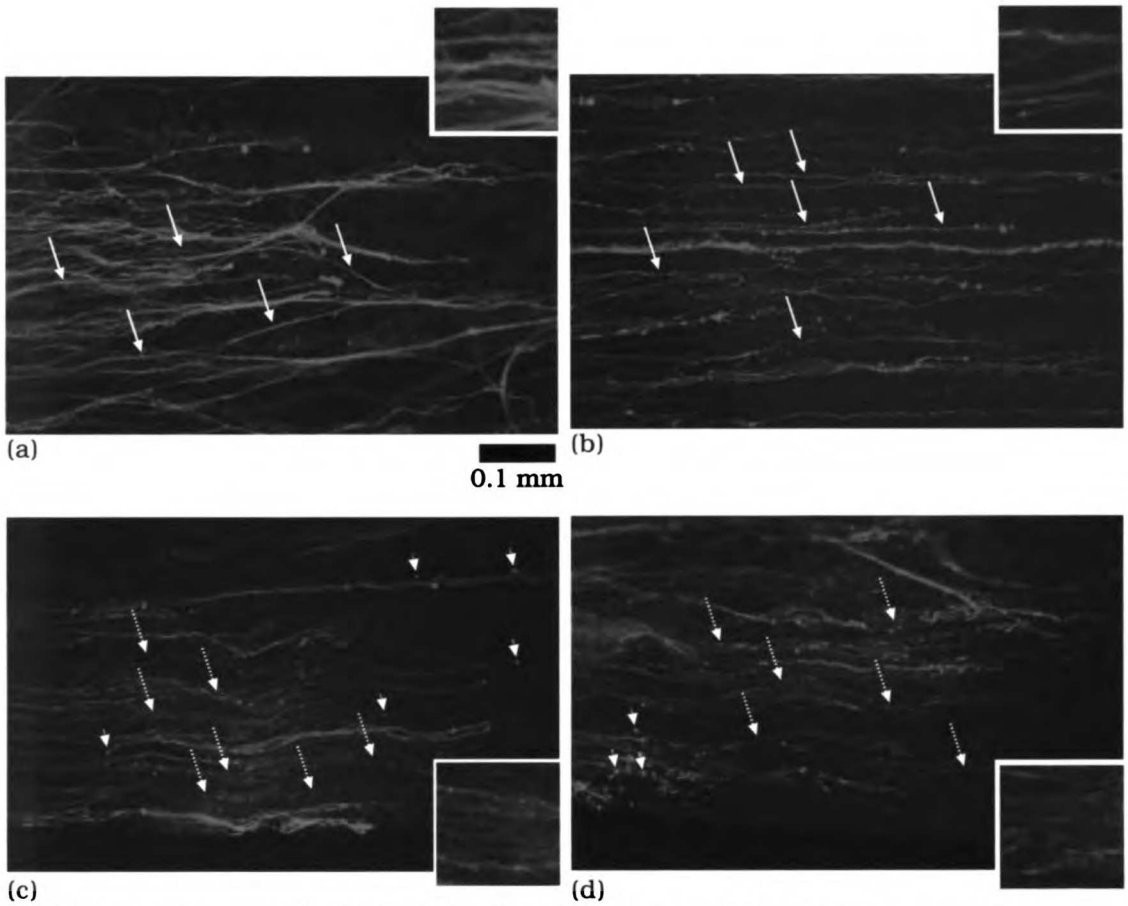


Fig. 2-1 Neurite outgrowths from adult mouse DRGs on unfixed cryosections of uninjured (a, b) and 10-day post-injury (c, d) sciatic nerves. GAP-43 immunostaining. Solid arrows (a, b) are pointing at examples of solid-staining, healthy neurites while dotted arrows (c, d) are pointing at examples of hazy-staining, degenerative neurites. Arrowheads in (c) and (d) are pointing at examples of debris blobs that are separated from neurites. The inserts show magnified view of the neurites. These pictures were taken with the neurites, not the debris, in the focal plane.

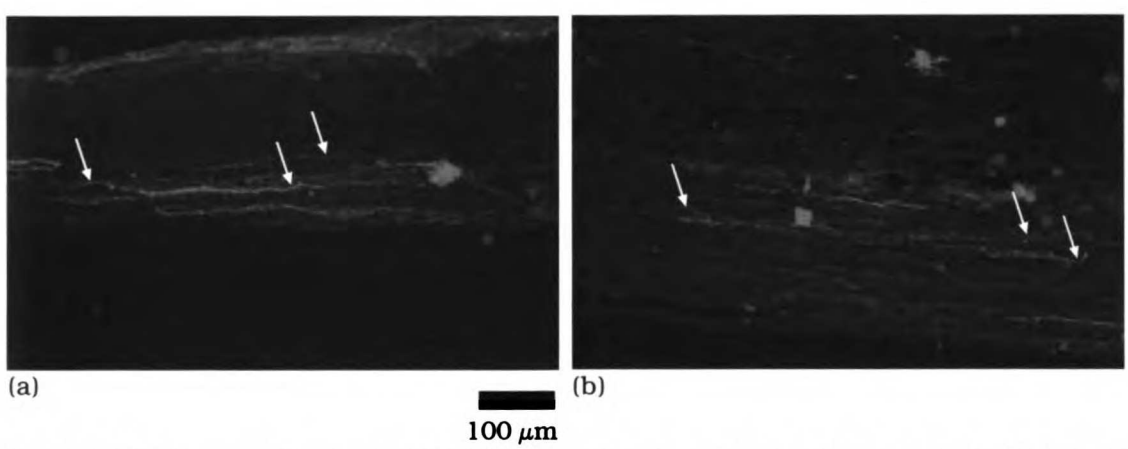


Fig. 2-2 Adult mouse dissociated DRG neurons with extensive neurite outgrowth (arrow) on unfixed cryosections of adult uninjured sciatic nerves. GAP-43 immunostaining. (a) Culture medium with 10% serum, (b) Culture medium with N2 / 2% serum.

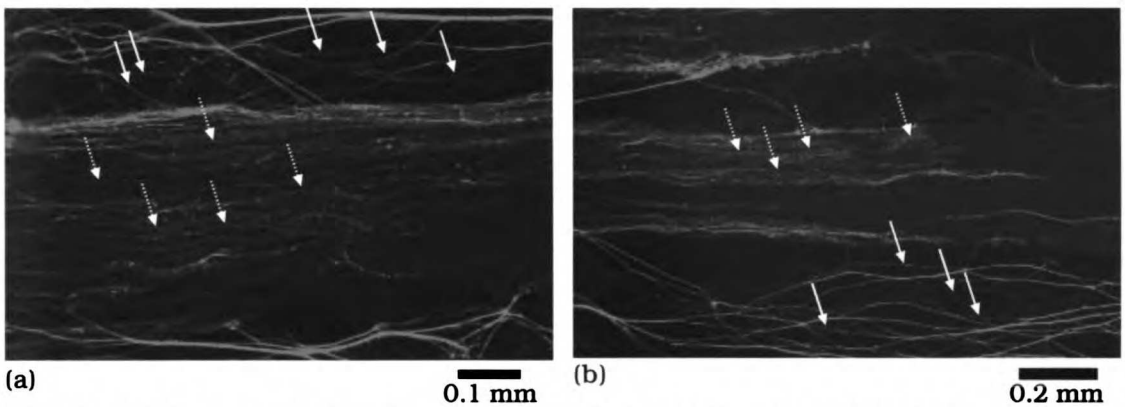


Fig. 2-3 Adult DRG outgrowths on cryosections of adult uninjured sciatic nerves after 6 days in culture. GAP-43 immunostaining. (a) and (b) are two separate examples showing degenerative neurites (dotted arrows) on the cryosections and solid neurites (solid arrows) on the glass coverslips. In these pictures, the focus is on the neurites, instead of the debris.

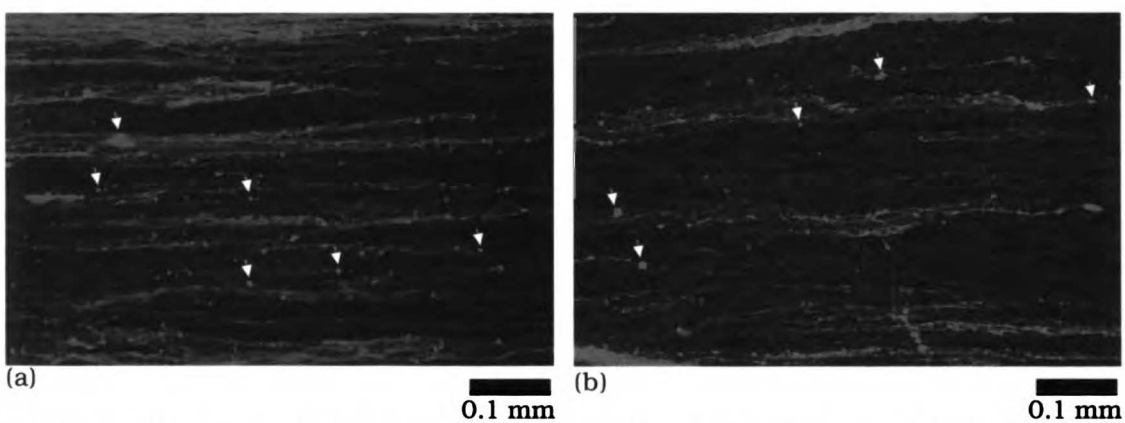
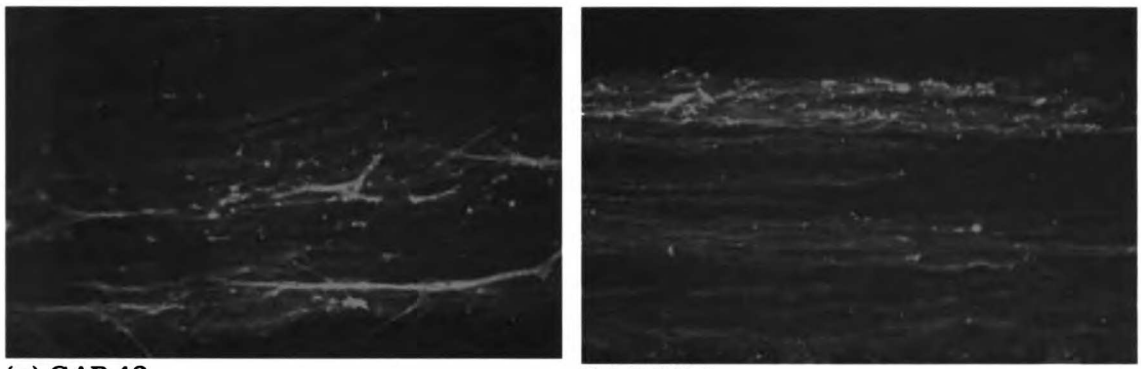
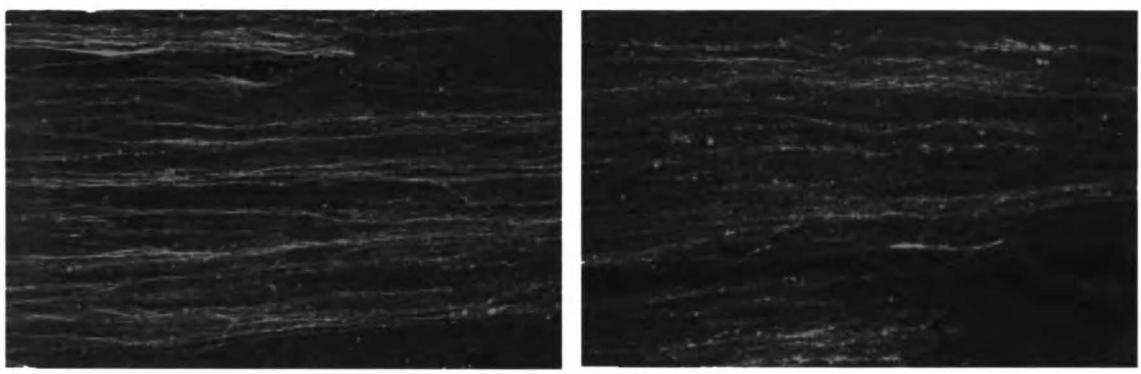


Fig. 2-4 Vital staining (carboxyfluorescein diacetate, succinimidyl ester) of adult DRG neurites on cryosections of adult uninjured sciatic nerves. (a) and (b) are two separate samples showing positive staining of debris and hazy staining of neurites, a pattern very similar to that with anti-GAP-43 antibodies. Since vital staining requires only two simple changes of medium, the appearance of debris and hazy neurites were not likely to be artifacts of the fixation and immunostaining procedure. The debris blobs are in focus in these pictures.



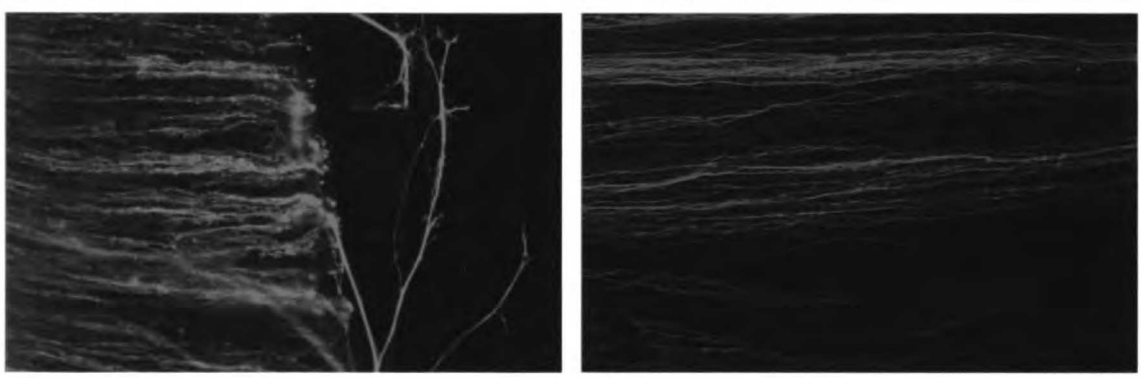
(a) GAP-43

(b) NCAM



(c) MAPs

(d) Synaptophysin



(e) Ubiquitin

(f) L1

(g) GAP-43 (top) and neurofilament 200-kd heavy subunit (bottom) dual-channel confocal vertical section

Fig. 2-5 Immunostaining of neurite outgrowth of adult DRGs on cryosections of adult uninjured sciatic nerves, 6 days in culture. Debris of neurites stain positive for GAP-43, NCAM, microtubule-associated proteins (MAPs), synaptophysin and ubiquitin (a to e) but negative for L1 and neurofilament 200-kd heavy subunit (NF-200)(f to g). Because NF-200 is also present in the cryosection substrate, confocal vertical sections were used to verify negative NF-200 staining of neurite debris.

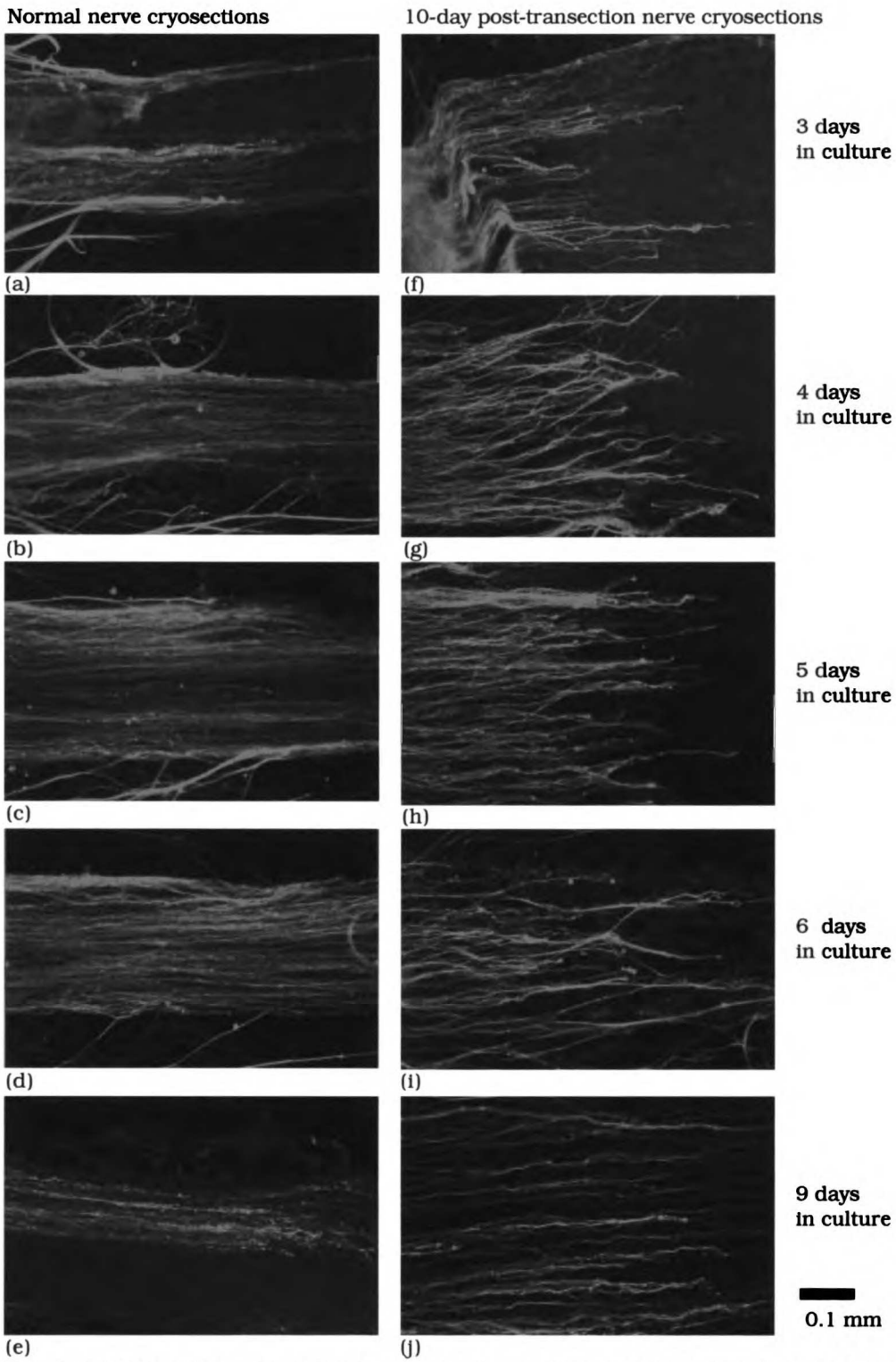
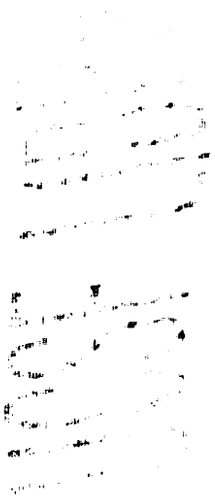


Fig. 2-6 DRG neurite outgrowths on normal nerve cryosections (a to e) and on 10-day post-transection nerve cryosections (f to j), in culture for 3 to 9 days. GAP-43 immunostaining. On normal nerve cryosections, debris formation and neurite degeneration could be seen as early as 3 days in culture. On post-transection nerve cryosections, however, DRG neurites remained healthy and solid.



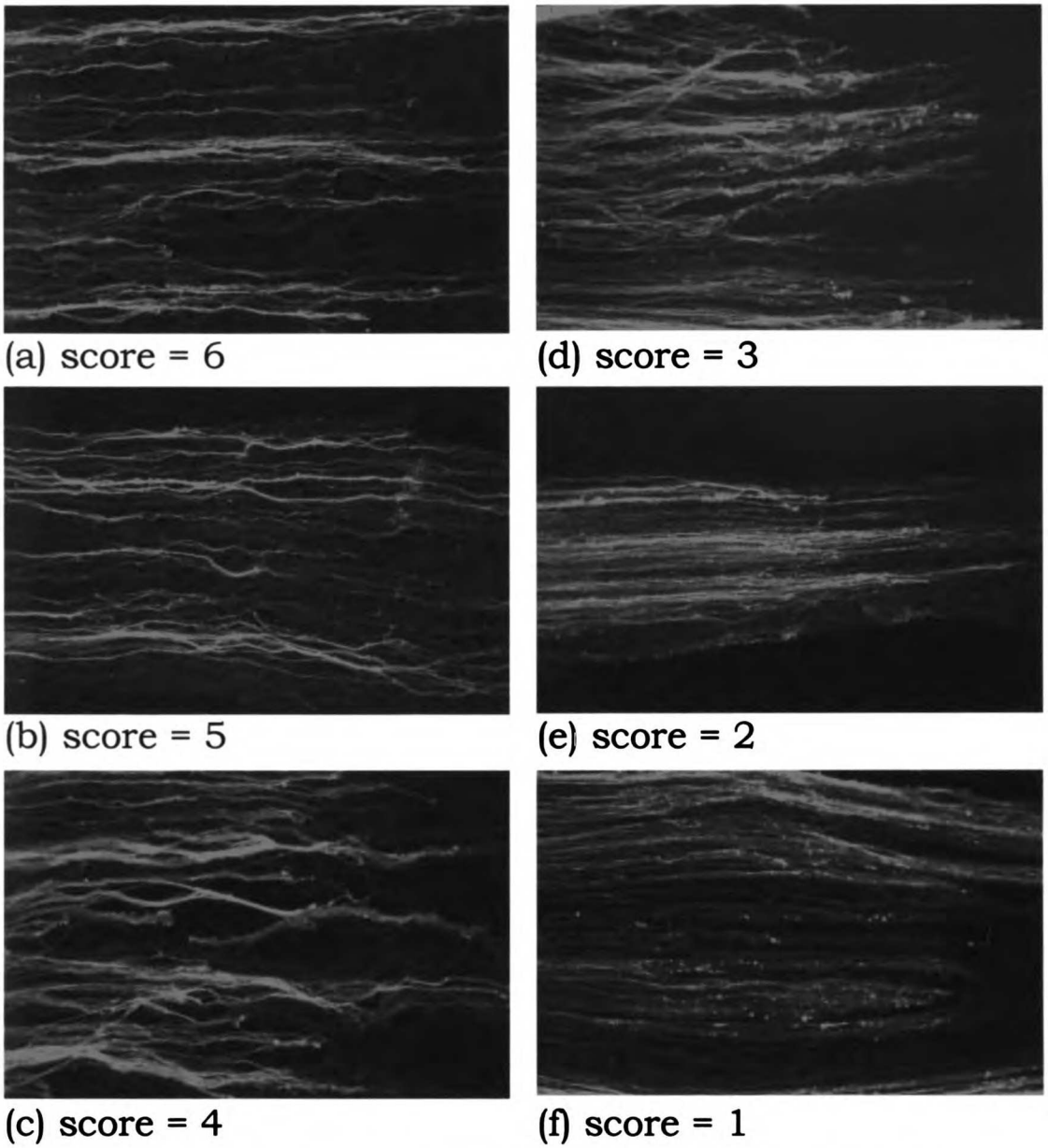


Fig. 2-7 Semi-quantitative scoring system for measuring the degree of neurite degeneration. GAP-43 immunostaining. Shown above are representative samples of different score values, from 6 (highly stable) to 1 (highly degenerative).

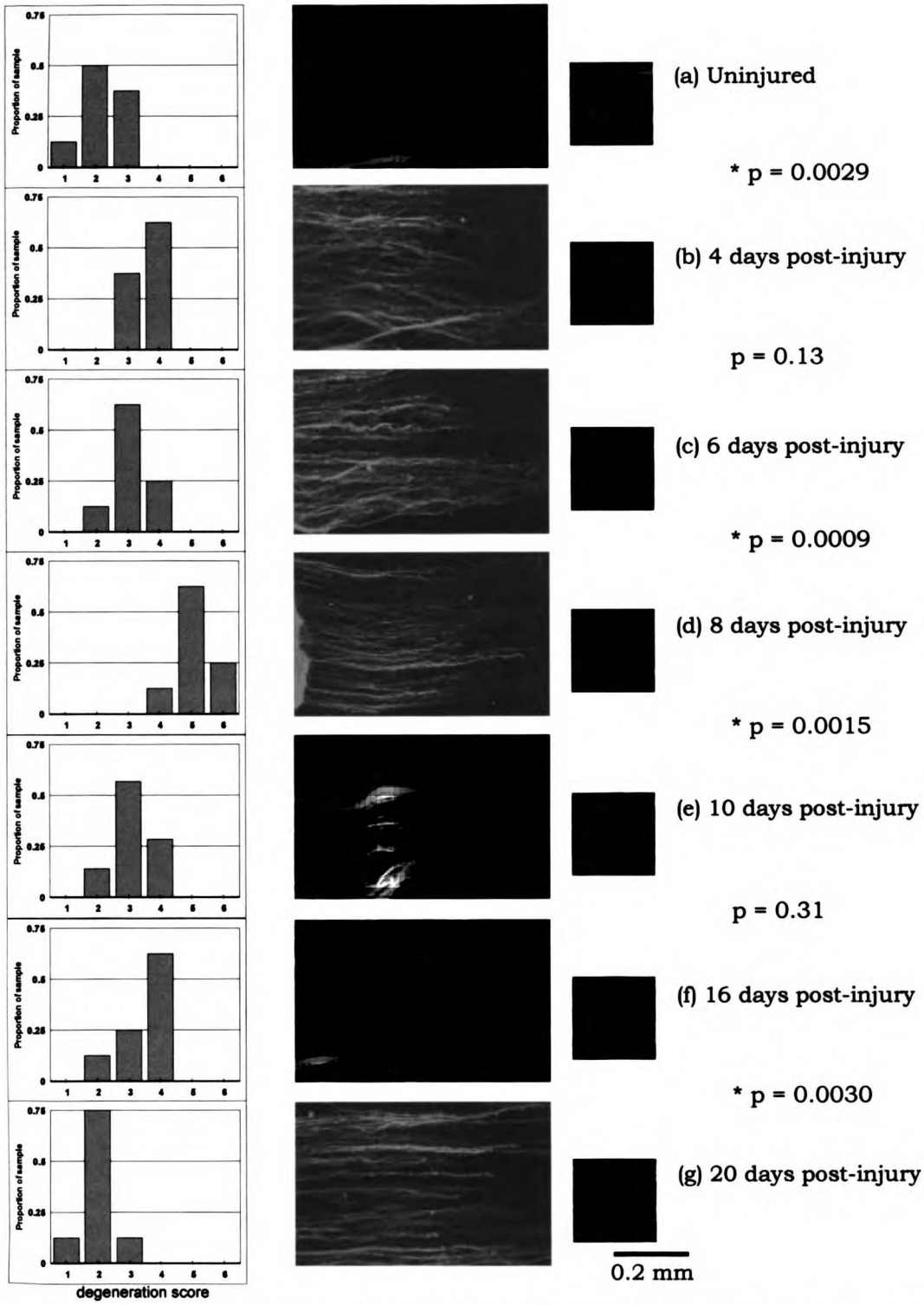


Fig. 2-8 Blind scoring of DRG neurite outgrowths on cryosections of adult sciatic nerves that had been pre-injured for different time durations. GAP-43 immunostaining. 8 samples were used for each time point. The right-shifting of the bars from (a) to (d) indicates an increasing trend of the degeneration score from 0 to 8 days post-injury while the left-shifting of the bars from (d) to (g) indicates a decreasing trend of the degeneration score from 8 to 20 days post-injury. The insert on the right side of each row is a magnified view of the neurites. The p-values of Wilcoxon's test between adjacent pairs of data are indicated (* denotes $p < 0.05$).

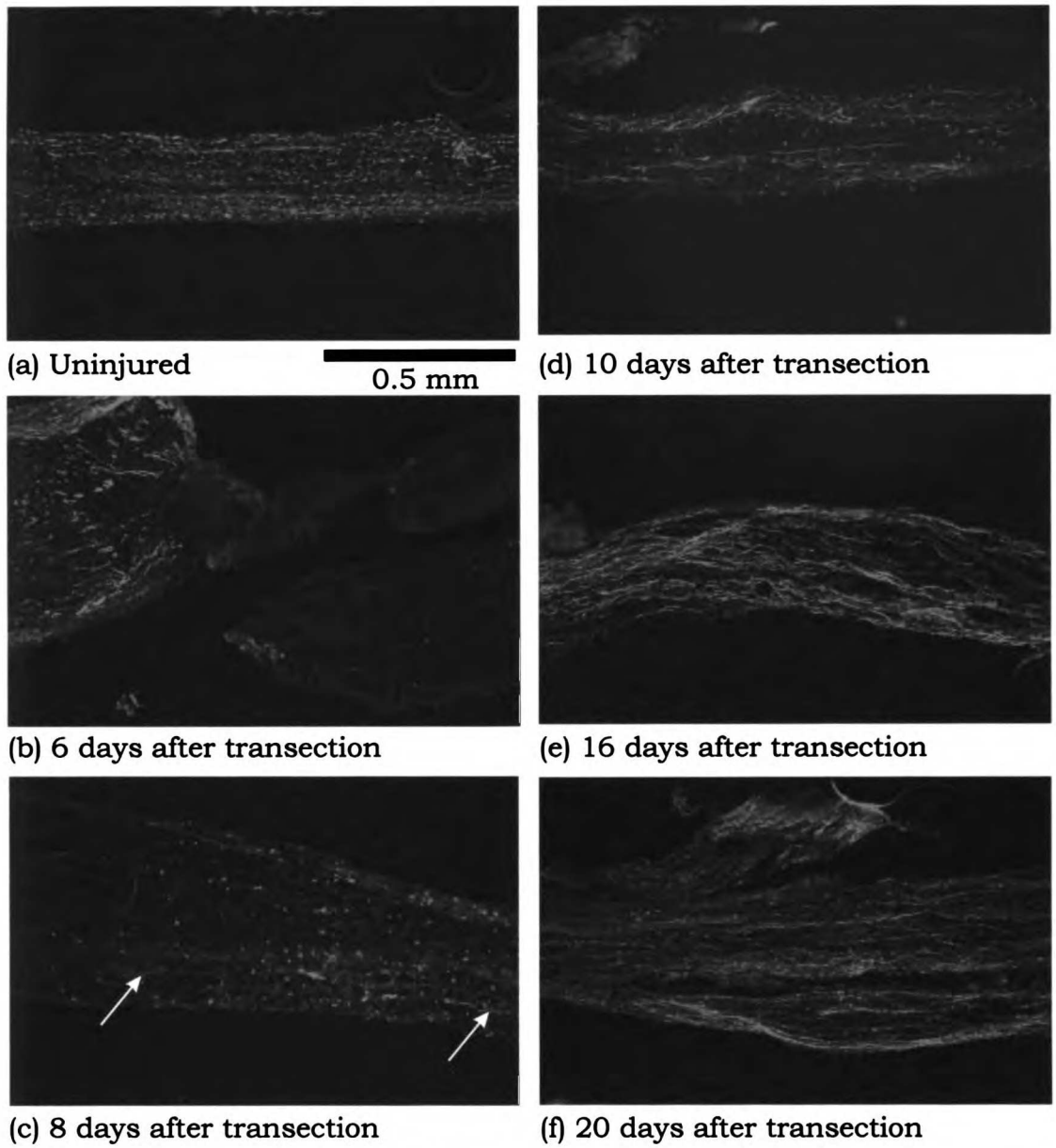
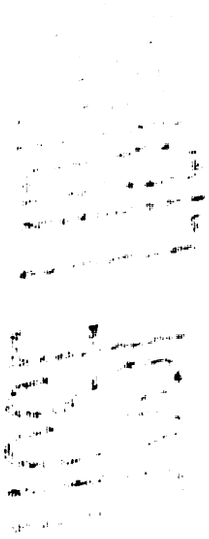
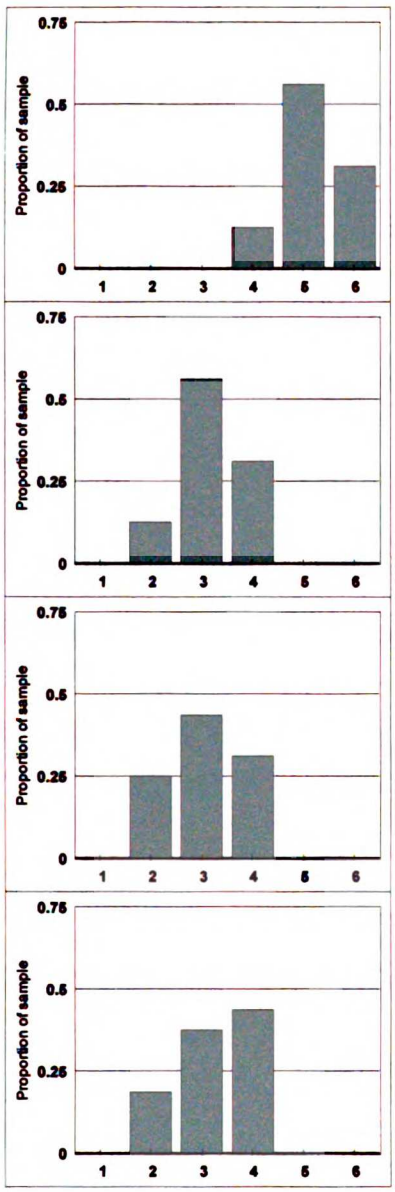


Fig. 2-9 Neurofilament (NF-200) immunostaining of sciatic nerves at different time points following transection injury. The photographs are oriented proximal-to-distal from left to right. (a) An uninjured nerve exhibited abundant NF-200 staining in the form of short, stubby fragments. This type of staining disappeared from the distal segment by 2 days after transection. (b) By 6 days after transaction, many regenerating neurites (seen here as long, thread-like NF-200 staining) had amassed at the proximal stump but penetration into the distal stump had not yet occurred. (c) At 8 days after transection, regenerating neurites (arrows) appeared in the distal segment for the first time. (d) to (e) From 10 to 20 days after transection, additional neurites continued to arrive at the distal segment.





(a) 8 days after transection

* p = 0.0001

(b) 11 days after transection

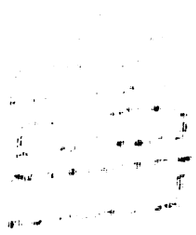
p = 0.67

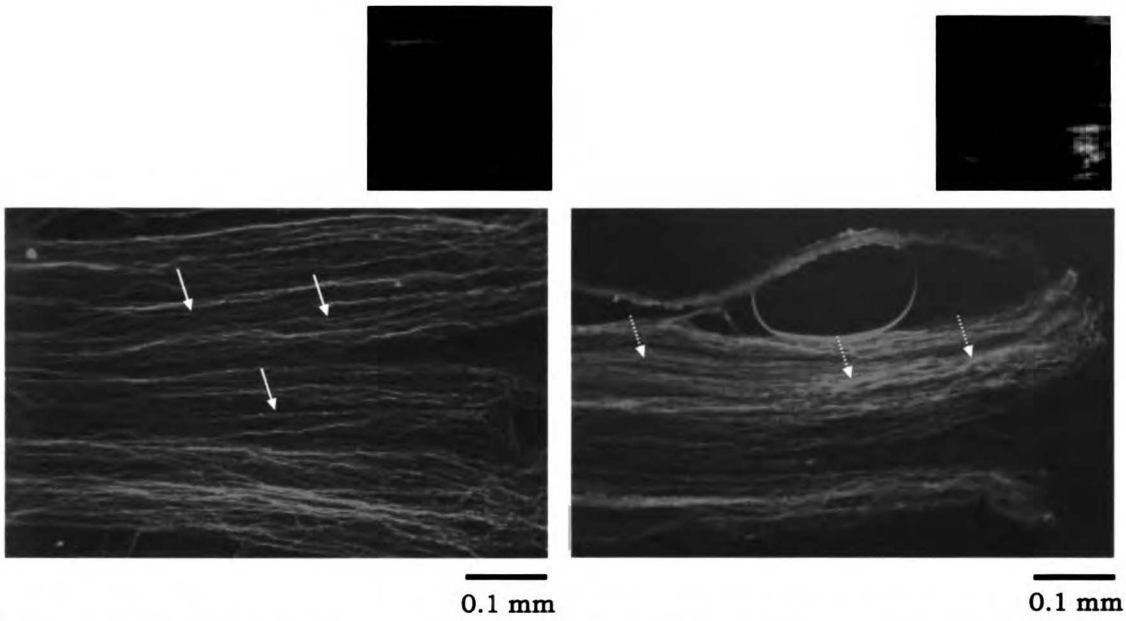
(c) 15 days after transection

p = 0.49

(d) 18 days after transection

Fig. 2-10 Blind scoring of DRG neurite outgrowths on cryosections of adult sciatic nerves that had been pre-transected and the regenerating neurites disallowed from entering the distal segment. 16 samples were used for each time point. The shifting of the bars to the left from (a) to (d) indicates a decreasing trend of the degeneration score from 8 to 18 days post-transection. The p-values from Wilcoxon's test between adjacent pairs of data are indicated (* denotes $p < 0.05$).

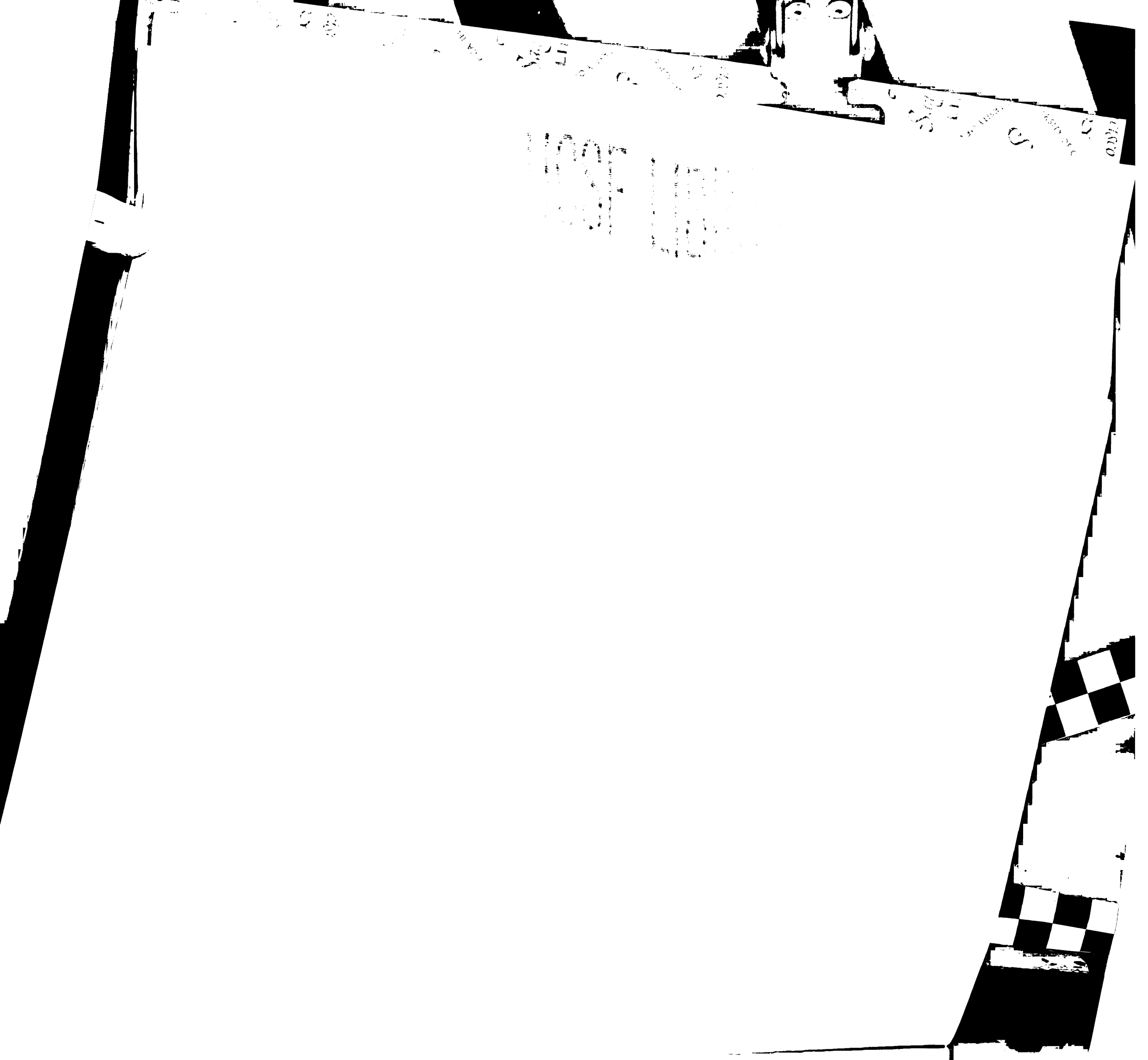


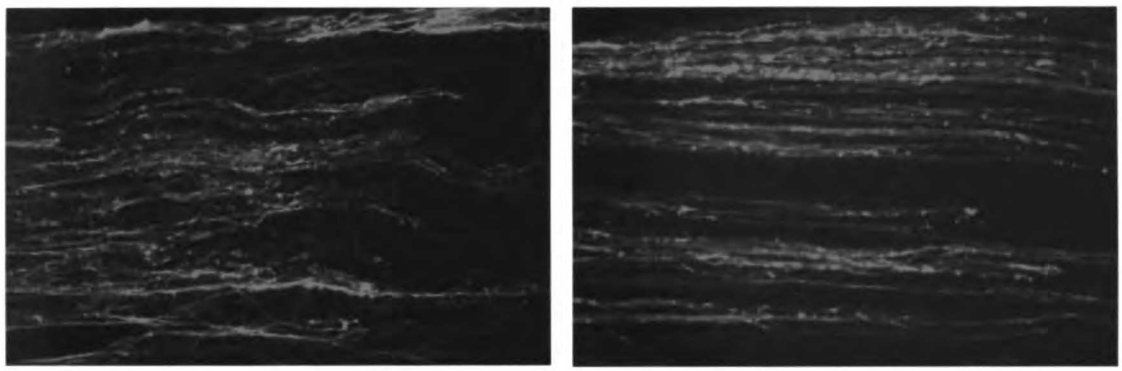


(a) Adult DRG neurite outgrowth on neonatal sciatic nerve substrate

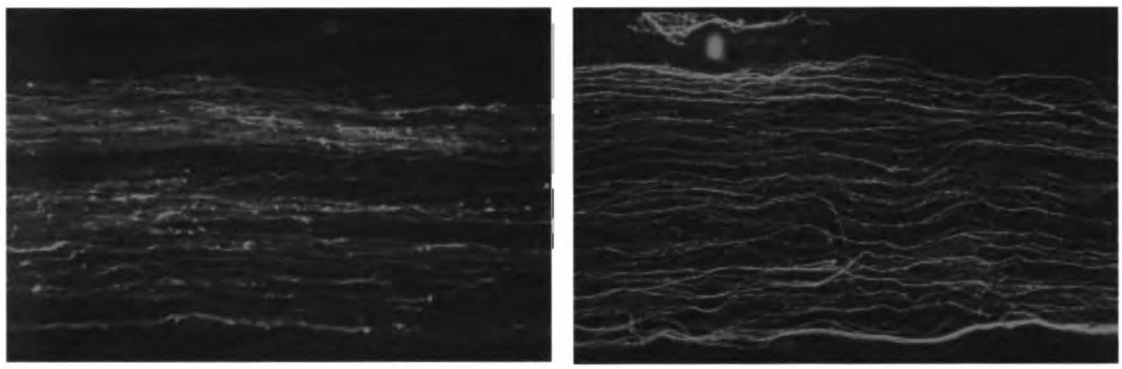
(b) Adult DRG neurite outgrowth on adult sciatic nerve substrate

Fig. 2-11 GAP-43 immunostaining. (a) Adult DRG consistently extend large amount of highly stable neurites (solid arrows) on substrate of neonatal sciatic nerve. (b) Negative control showing degenerative neurites (dotted arrows) growing on substrate of adult sciatic nerve. The inserts show the neurites in magnified view.





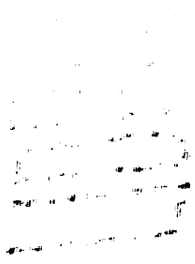
(a) Schwann cell-conditioned medium (b) Fibroblast-conditioned medium

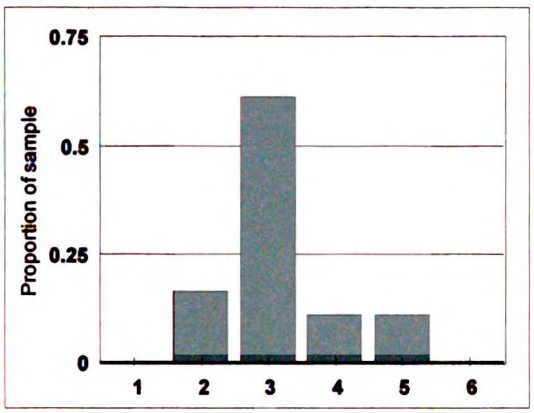


(c) DME / LH negative control (d)Macrophage-conditioned medium

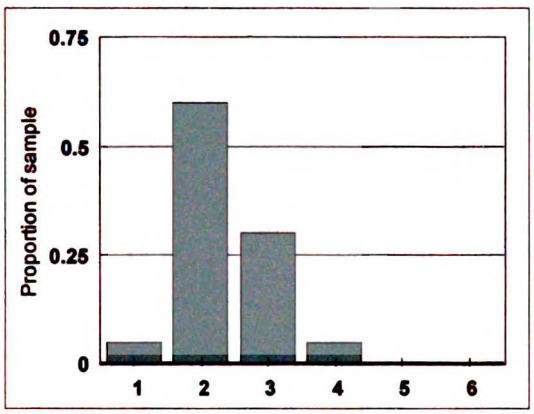
0.1 mm

Fig. 3-1 DRG neurite outgrowths on cryosections of normal adult sciatic nerves pretreated with different conditioned media or plain culture medium. GAP-43 immunostaining. DRG neurites degenerated on cryosections pretreated with Schwann cell-conditioned medium, fibroblast-conditioned medium and non-conditioned medium. However, DRG neurites remained healthy on cryosections pretreated with macrophage-conditioned medium.





(a) Macrophage-conditioned medium



(b) DME/LH (control)

Degeneration score

Fig. 3-2 Blind scoring of DRG neurite outgrowths on cryosections of adult sciatic nerves that had been pre-treated with (a) macrophage-conditioned medium (18 samples) or (b) DME/LH culture medium (20 samples). The neurite outgrowths in (a) are significantly healthier than those in (b) (Wilcoxon's $p = 0.0024 < 0.05$).

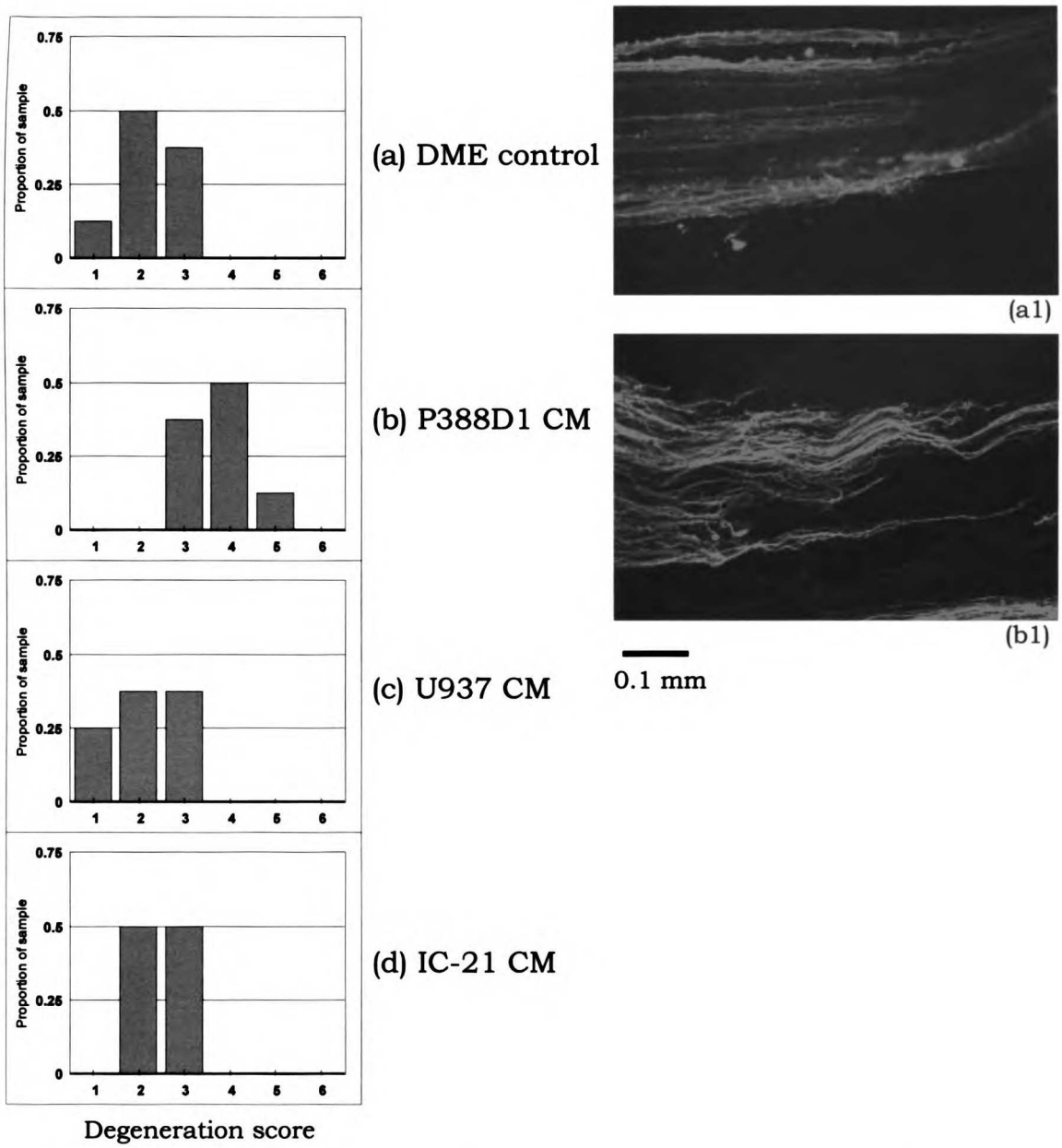
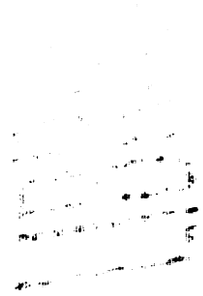


Fig. 3-3 The effect of pre-incubation of adult sciatic nerve cryosections with conditioned-media of several macrophage-like cell lines. Nerve cryosections that are pre-treated with P388D1-conditioned medium (b and b1) support more stable neurite outgrowths compared to DME control (a and a1) ($p = 0.003 < 0.05$) while nerve cryosections pretreated with U937 (c) and IC-21 (d) conditioned-media do not support stable outgrowth above the level of DME control (a) ($p = 0.82$ and 0.52, respectively). (a1) and (b1) show GAP-43 immunostaining.



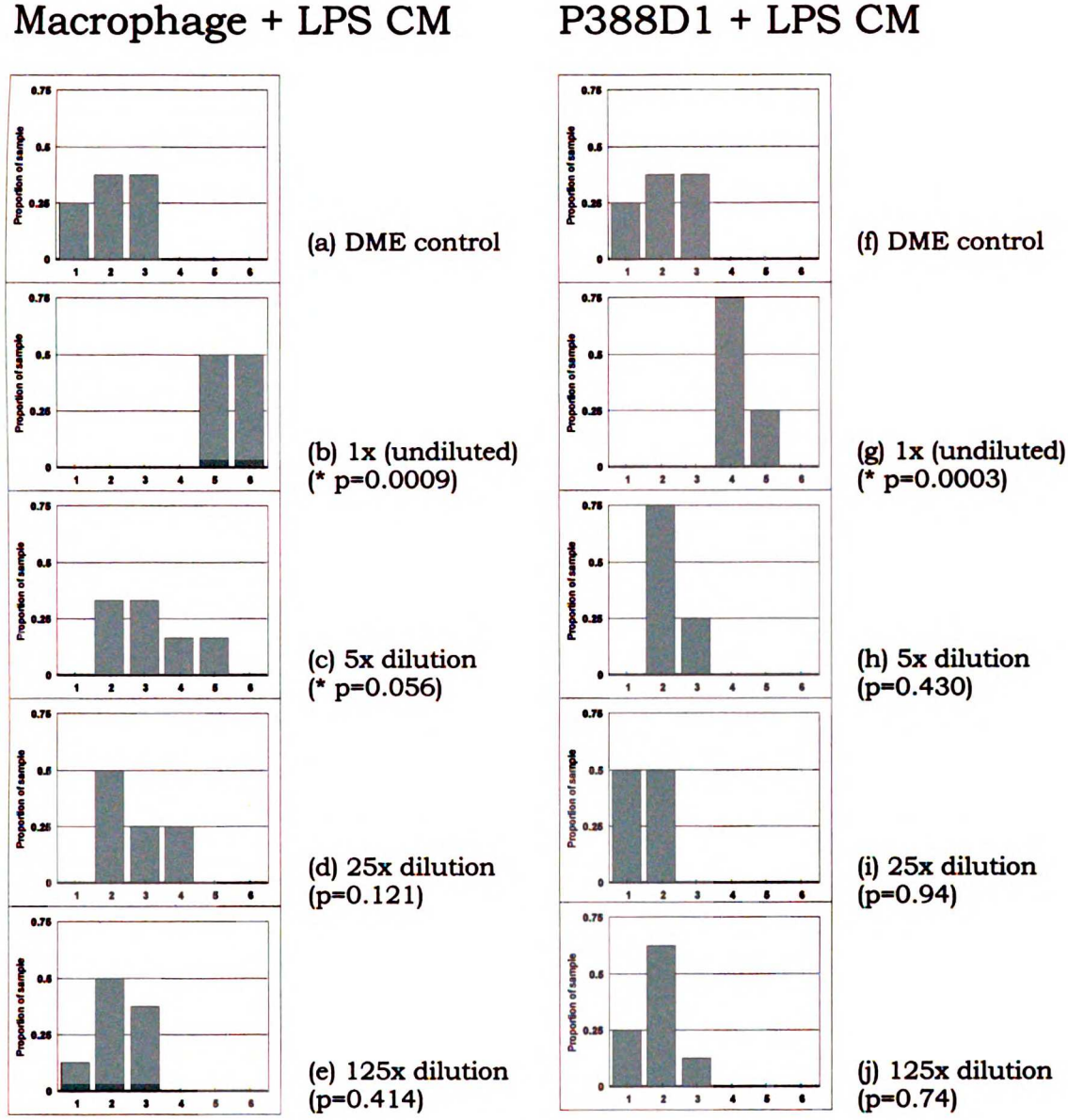
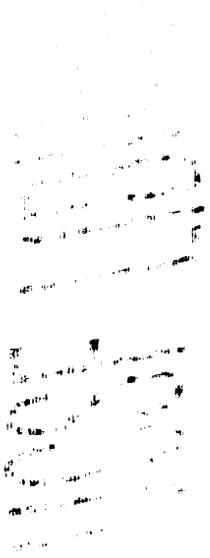


Fig. 3-4 Testing serial dilutions of conditioned media from LPS-activated macrophages and LPS-activated P388D1 for transforming activity above the level of DME control. Five-fold serial dilutions were used to pre-treat normal nerve cryosections before they were used as substrate for DRG neurite outgrowths. Wilcoxon's two-sample test (one-sided) was performed on the degeneration scores obtained from each CM dilution against those from control (DME). The p-values are indicated beside the charts.



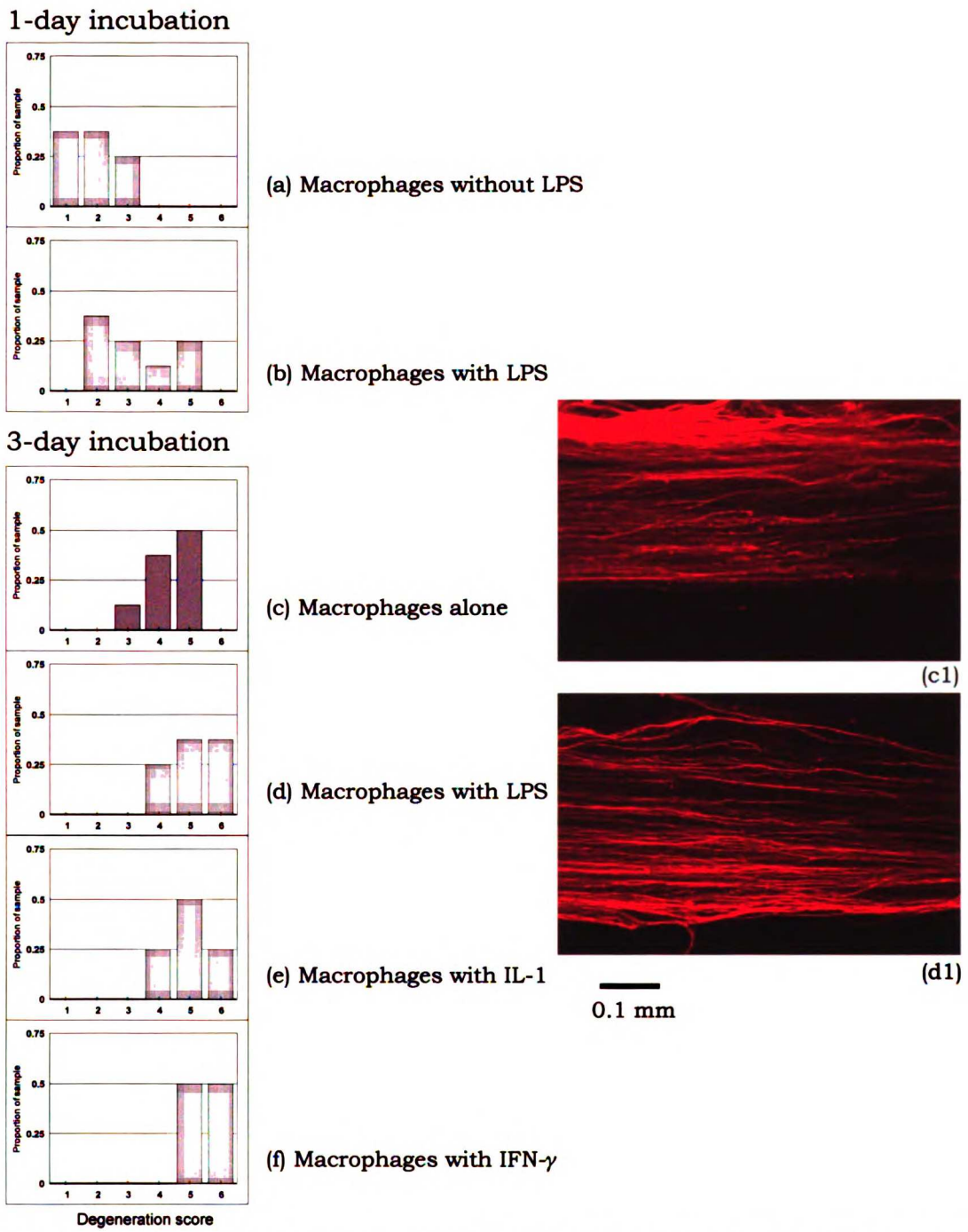
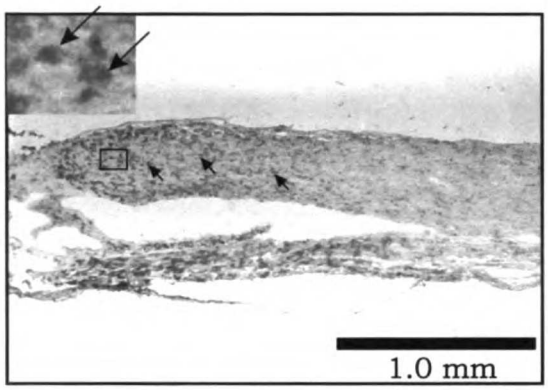
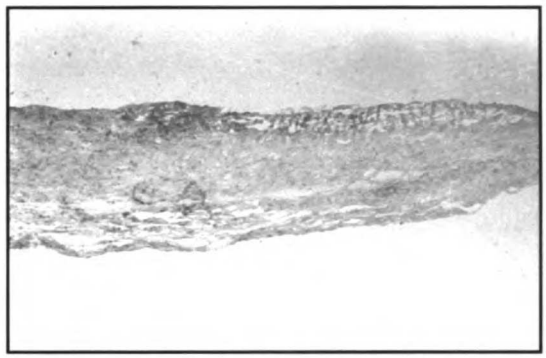


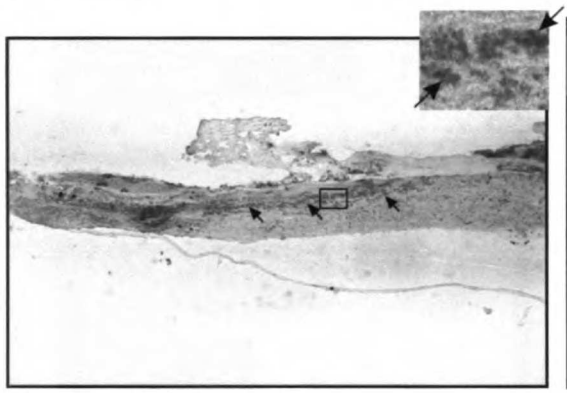
Fig. 3-5. Macrophages secrete more transforming activity upon activation (n=8 per treatment). In the first day of incubation, unactivated macrophages (a) clearly secrete a much lower level of transforming activity than LPS-activated macrophages (b) ($p = 0.019 < 0.05$). After three days of incubation, the transforming activity in the CM of unactivated macrophages (c) has accumulated to a level approaching that in CM of macrophages activated with LPS (d), IL-1 (e) or IFN-γ (f) ($p= 0.052$, 0.077 and 0.004 respectively). The photographs show slightly more stable DRG neurites growing on cryosections pretreated with LPS-activated macrophage CM (d1) than on cryosections prtreated with unactivated macrophage CM (c1) (GAP-43 immunostatining).



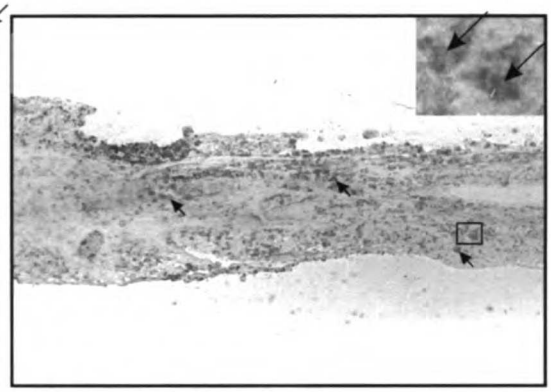
(a) 4 days after transection (just distal to wound site)



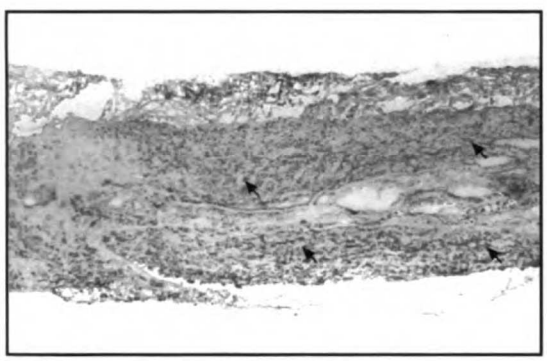
(b) 4 days after transection (further distal from wound site)



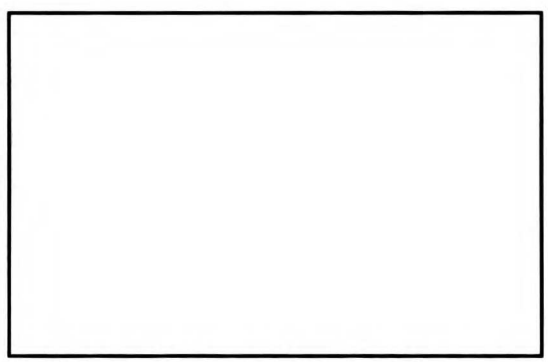
(c) 6 days after transection



(d) 8 days after transection

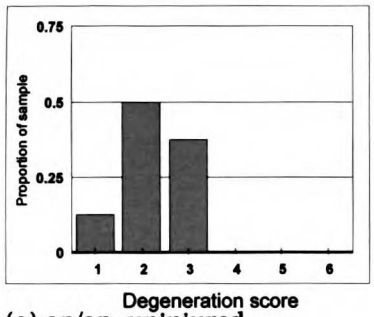


(e) 10 days after transection

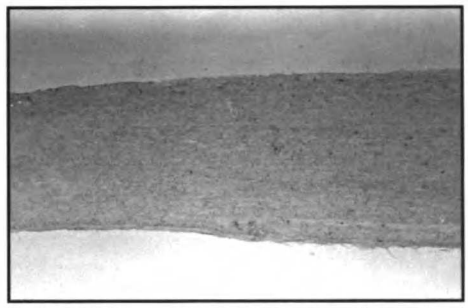


(f) 10 days after transection (negative control)

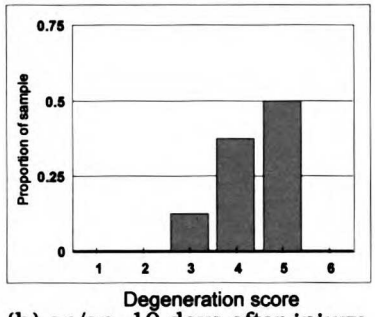
Fig. 3-6 F4/80 immunostaining of macrophages in the distal segment of sciatic nerves at different time points (4 to 10 days) after transection. (a) At 4 days after injury, macrophages (arrows; also in magnified insert) began to infiltrate the sciatic nerve at the distal stump but (b) they were not present further distal from the wound site. (c) More macrophages (arrows) appeared in the distal segment by 6 days after injury and (d) by 8 days and (e) 10 days after injury, macrophages (arrows) were present throughout the distal segment.



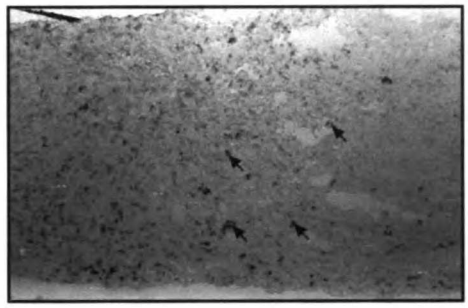
(a) op/op, uninjured



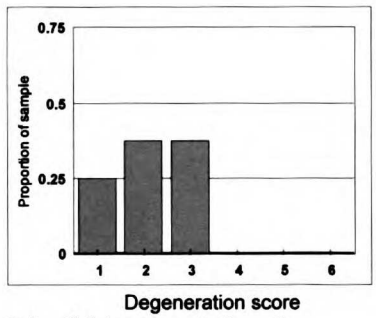
(a1) F4/80 immunostaining of macrophages



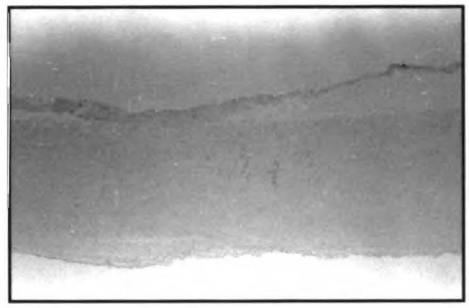
(b) op/op, 10 days after injury



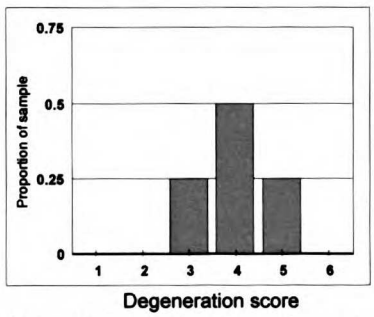
(b1)



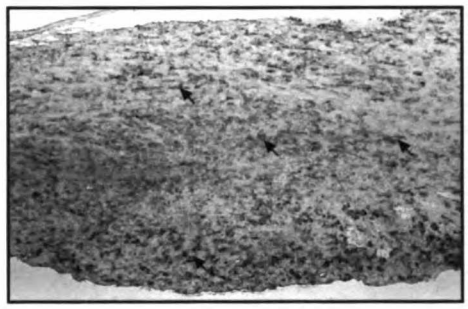
(c) wild type, uninjured



(c1)



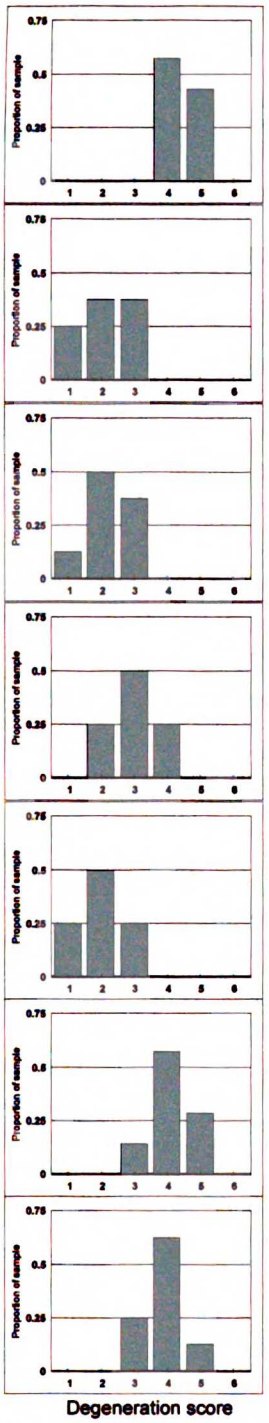
(d) wild type, 10 days after injury



(d1)

0.1 mm

Fig. 3-7 Testing of post-injury sciatic nerve cryosections from op/op (osteopetrotic) mice and wild type mice for the ability to support stable neurite outgrowth. (c) & (d) While wild type mice display an increase in the ability to support stable outgrowth after injury ($p = 0.0021 < 0.05$), (a) & (b) op/op mice also unexpectedly display a similar increase ($p = 0.0012 < 0.05$). The photographs on the right column show F4/80 immunostaining of macrophages (arrows) in the corresponding nerve cryosections. Notice that (b1) the post-injury op/op mice sciatic nerve has been infiltrated by macrophages.



(a) macrophage CM control

(b) DME control

(c) boiled CM

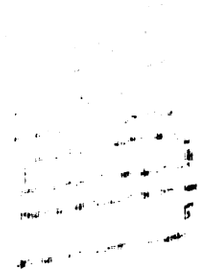
(d) CM + trypsin + trypsin inhibitor

(e) DME + trypsin + trypsin inhibitor (control)

(f) dialyzed CM

(g) PEG concentrated and dialyzed CM

Fig. 3-8 Testing the activity of LPS-activated macrophage-conditioned medium (CM) as protein. Macrophage CM was pre-treated as indicated before it was used to pre-incubate uninjured nerve cryosections, which were then tested for the ability to support stable neurite outgrowth (8 samples per test). (c) Boiling of macrophage CM reduced the activity ($p=0.001$, (a) vs. (c)) to a level not significantly different than that of DME control ($p=0.82$, (b) vs. (c)). (d) Trypsinization of the macrophage CM also significantly decreased the activity ($p=0.0011$, (a) vs. (d)). (f) The activity was retained after dialysis of macrophage CM ($p=0.0020$, (b) vs. (f)). See text for more information.



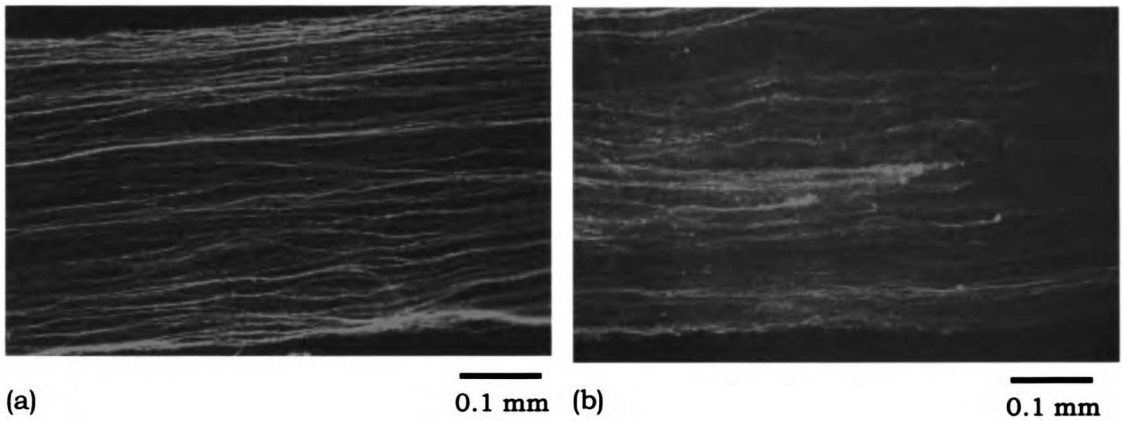
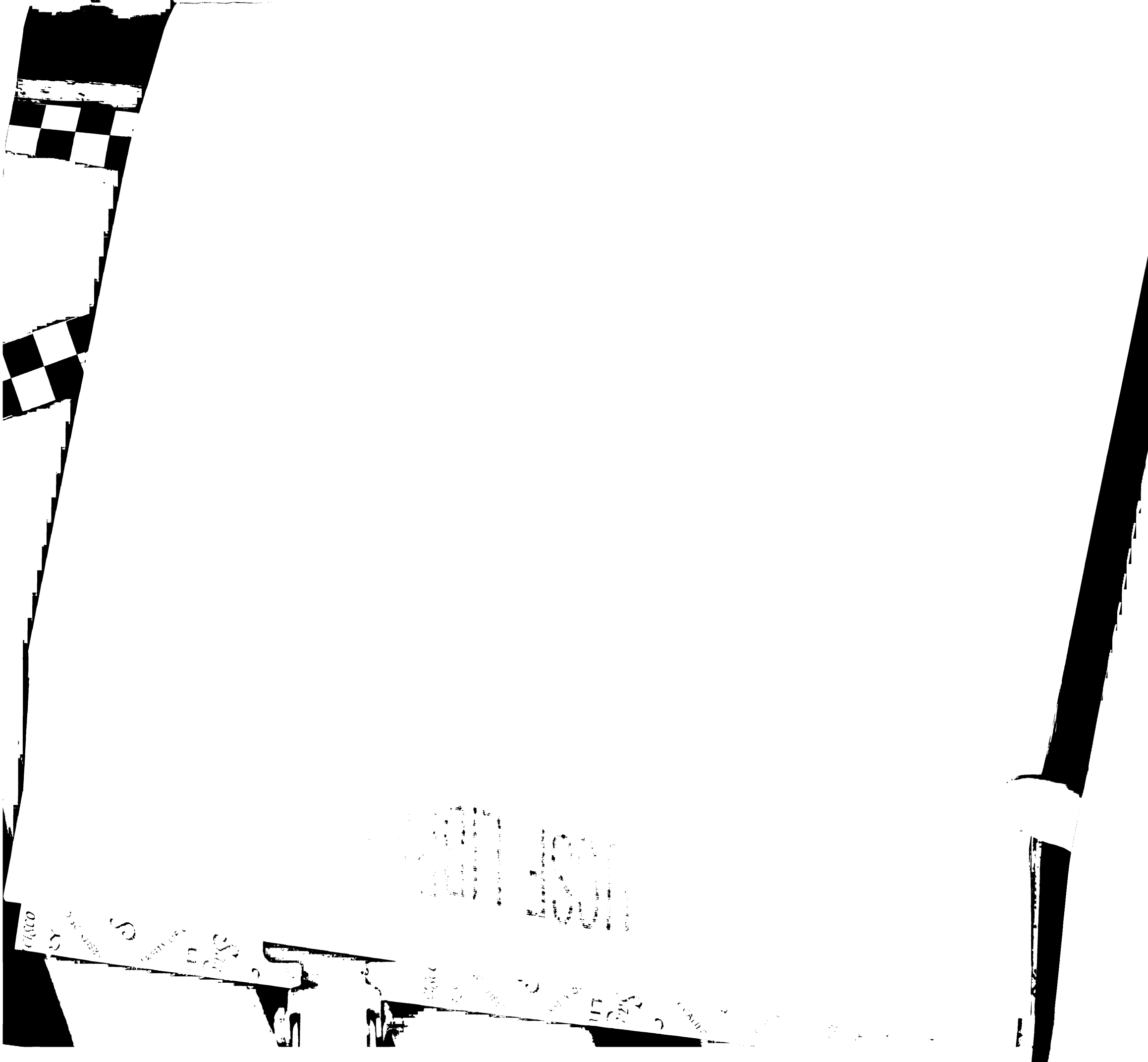
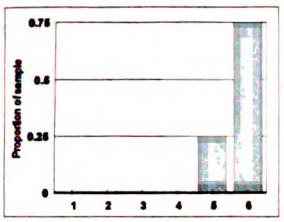
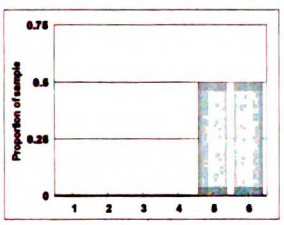


Fig. 3-9 DRG neurite outgrowths on adult sciatic nerve cryosections briefly pre-treated with 0.01% trypsin (a) or with DME medium (b). GAP-43 immunostaining. Notice highly stable neurites in (a).

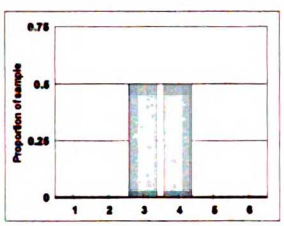




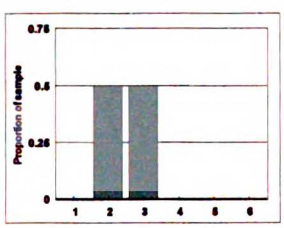
(a) 0.05% trypsin, 37 °C, 3 min.



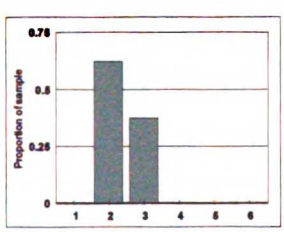
(b) 0.01% trypsin, 37 °C, 3 min.



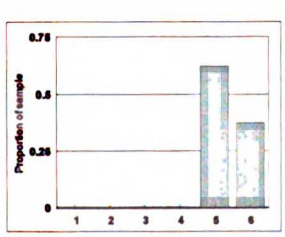
(c) 0.002% trypsin, 37 °C, 3 min.



(d) 0% trypsin, 37 °C, 3 min. (control)

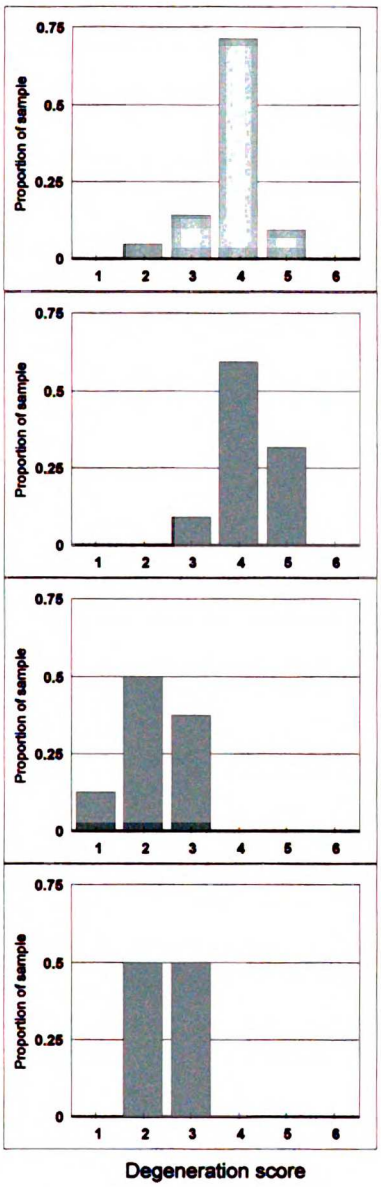


(e) 0.01% trypsin, room temp., 3 min.



(f) crude preparation of bacterial collagenase, 37 °C, 3 min.

Fig. 3-10 Normal adult sciatic nerve cryosections were pre-treated with various concentrations of trypsin under various conditions as indicated (n=8 per treatment). The pretreated cryosections were then tested for the ability to support stable DRG neurite outgrowths. Compared to control (0% trypsin, (d)), trypsin shows significant transforming activity at concentrations as low as 0.002% (37 °C, 3 min., (c)) ($p=0.007<0.05$). (e) Activity of 0.01% trypsin at room temperature (3 min. incubation) is not sufficient to transform nerve cryosections ($p=0.67>0.05$, (e) vs. (d)) while (f) crude preparation of bacterial collagenase (37 °C, 3 min.) displays a similar transforming activity as trypsin ($p=0.0006<0.05$, (f) vs. (d)).



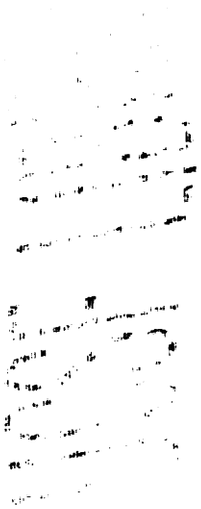
(a) SL-KO 10-day post-transection
(n = 21)

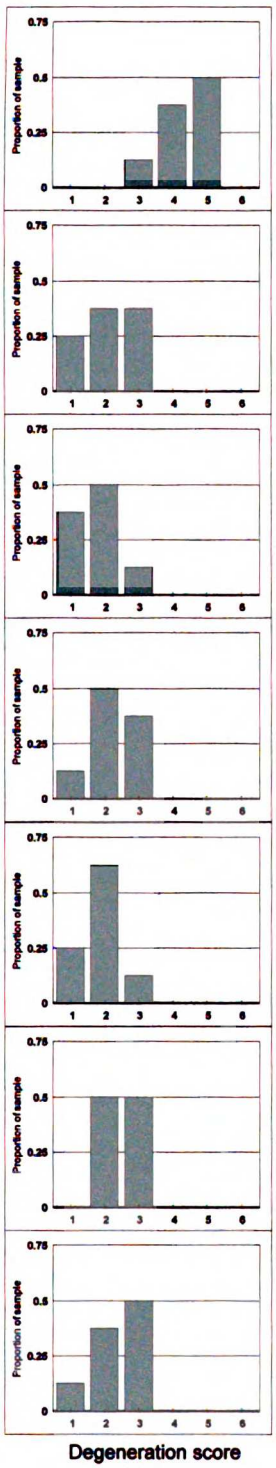
(b) wild type 10-day post-transection
(n = 22)

(c) SL-KO uninjured
(n = 8)

(d) wild type uninjured
(n = 8)

Fig. 3-11 Testing pre-injured and uninjured nerve cryosections from stromelysin knock-out (SLKO) mice for the ability to support stable neurite outgrowth. By 10 days after nerve transection, both SLKO mice (a) and wild type mice (b) sciatic nerves displayed significant increase in the ability to support stable outgrowth compared to their uninjured counterpart ($p=0.0001<0.05$, (a) vs. (c); $p=0.0001<0.05$, (b) vs. (d)). The level of stable outgrowth on SLKO nerve cryosections is not significantly different from that on wild type nerve cryosections at the 0.05 level ($p=0.07$, (a) vs. (b)).





(a) macrophage CM
(p = 0.0013)

(b) DME control

(c) transglutaminase
(p = 0.37)

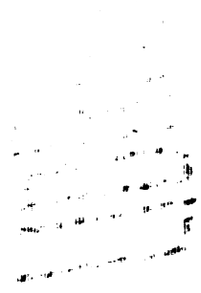
(d) chondroitinase ABC
(p = 0.82)

(e) phosphatidylinositol phospholipase c
(p = 0.53)

(f) hyaluronidase
(p = 0.39)

(g) heparinase
(p = 0.53)

Fig. 3-12 Testing non-proteolytic matrix-modifying enzymes for the ability to transform normal sciatic nerve cryosections to support stable neurite outgrowth (n=8 per treatment). The p-values indicated are derived from Wilcoxon's two-sample test of each treatment vs. DME control (b). Besides macrophage-conditioned medium (positive control, (a)), none of the tested enzymes show transforming activity above the level of DME control ($p >> 0.05$).



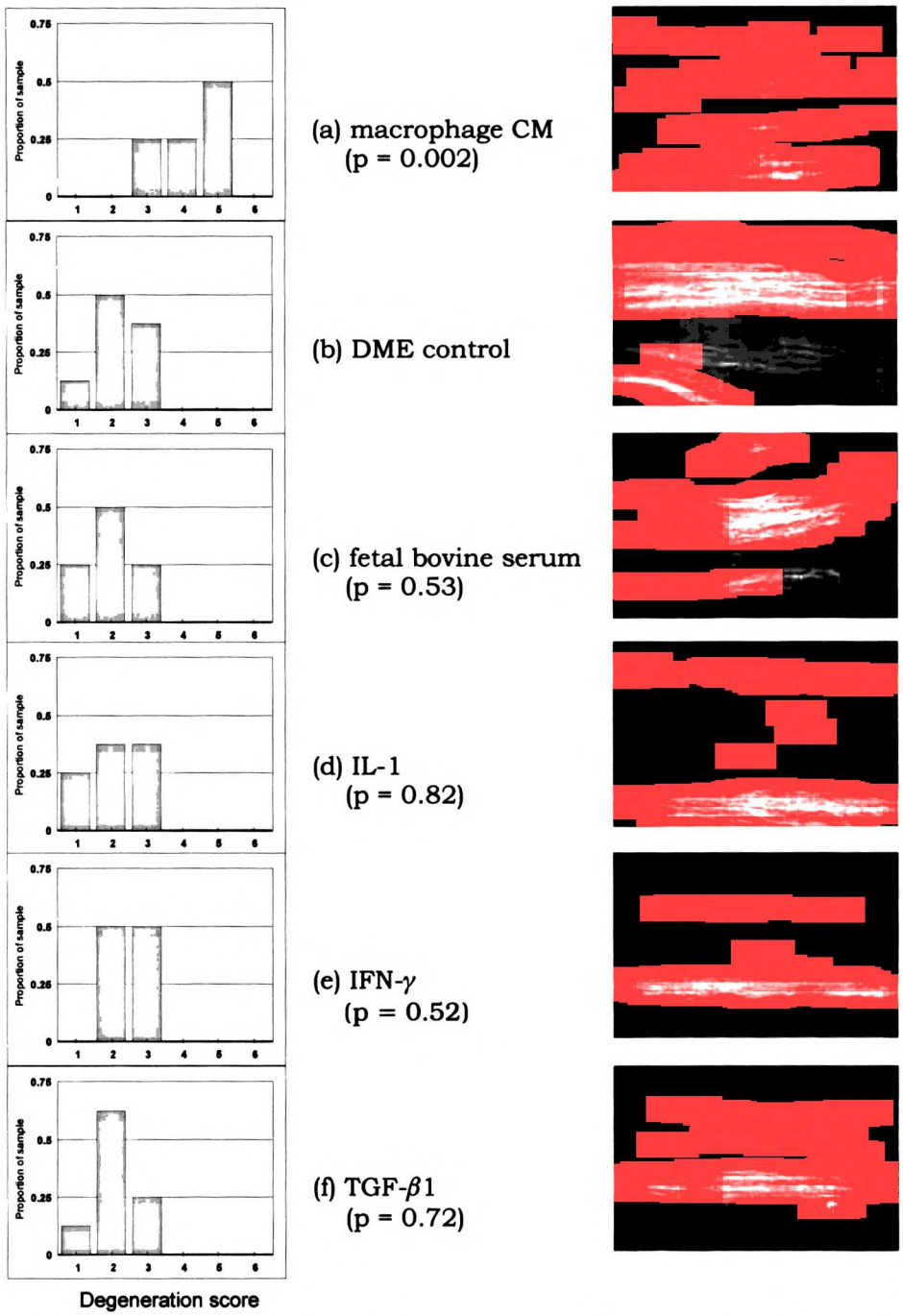
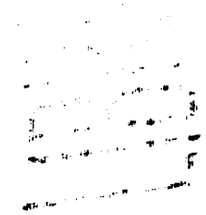
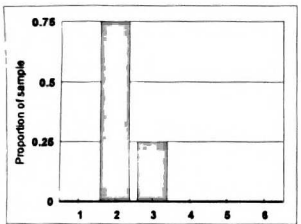
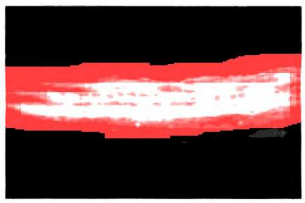


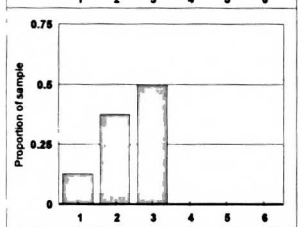
Fig. 3-13 Testing various growth factors for the ability to mimic the activity of macrophage-conditioned medium (n=8 per treatment). The photographs on the right column (GAP-43 immunostaining) show the condition of neurite outgrowths on cryosections that were pre-treated with the corresponding growth factors for 24 hours. The p-values indicated are derived from Wilcoxon's two-sample test of each treatment vs. DME control (b). Besides macrophage-conditioned medium (positive control, (a)), none of the tested substances show transforming activity above the level of DME control ($p >> 0.05$).



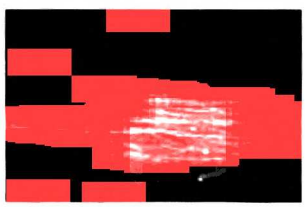


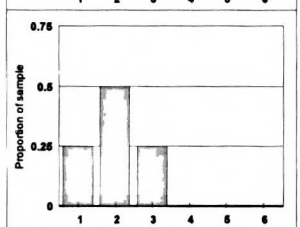
(g) LIF
(p = 0.95)



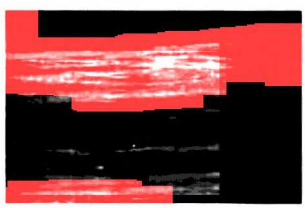


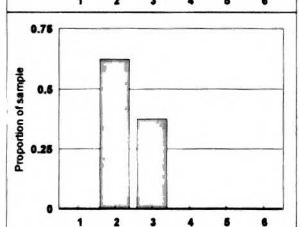
(h) bFGF
(p = 0.73)



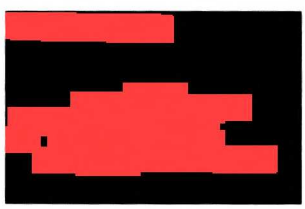


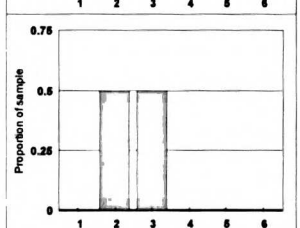
(i) EGF
(p = 0.53)



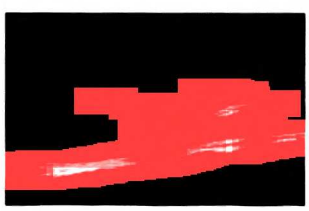


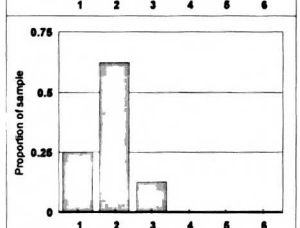
(j) insulin
(p = 0.81)



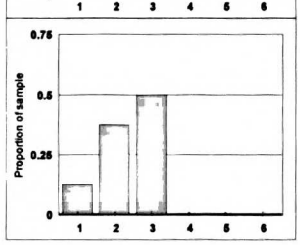


(k) HGF
(p = 0.52)





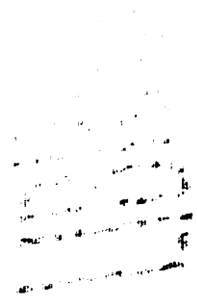
(l) PDGF
(p = 0.29)



(m) IGF-1
(p = 0.73)

Degeneration score

Fig. 3-13 continued from previous page.



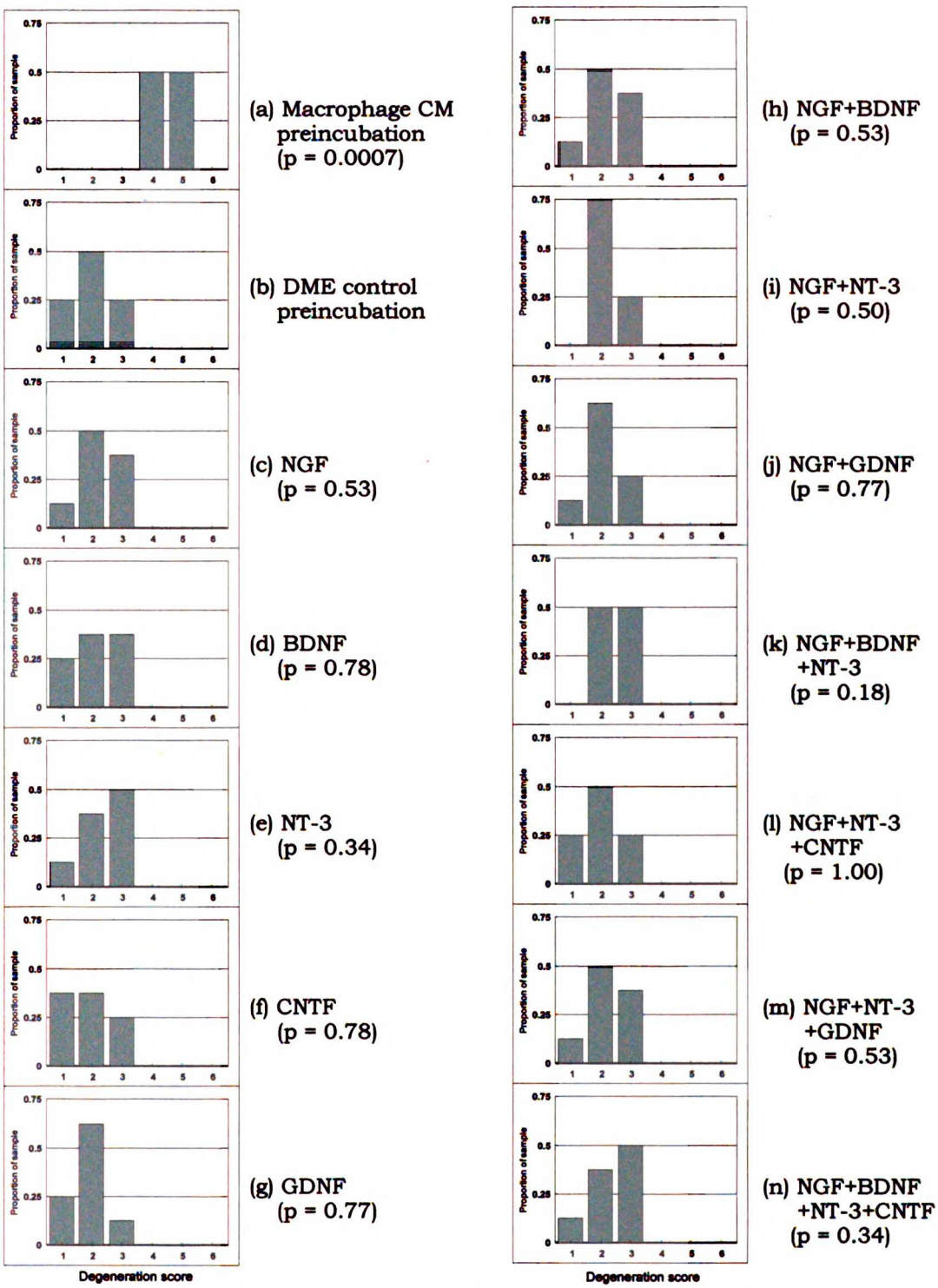


Fig. 3-14 Testing neurotrophic factors for the ability to induce stable neurite outgrowth on normal sciatic nerve cryosections (n=8 per treatment). The neurotrophic factors were present during the 24-hour pre-incubation period and during the 6-day culturing of DRG explants. The p-values indicated are derived from Wilcoxon's two-sample test of each treatment vs. DME control (b). None of the tested neurotrophins were able to induce stable neurite outgrowth above the level of DME control ($p >> 0.05$).