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Short Communications

Laminin supports neurite outgrowth from explants of axotomized adult rat retinal neurons

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The influence of laminin on neurite outgrowth from explants of adult rat retina and its distribution in normal and lesioned rat optic nerves were examined. Neurite outgrowth required the presence of laminin in the substratum, and as with a goldfish retinal explant system, was markedly stimulated by prior axotomy. Except for blood vessels and the nerve sheath, normal rat optic nerve was devoid of laminin immunoreactivity. Unlike results seen in the goldfish optic nerve, injury to the rat optic nerve induced no observable increase in laminin content or change in its distribution. The differences in the in vivo regenerative capacities of these two species may in part be related to the differences in their abilities to provide a proper substratum for axon regrowth.

Injury to neurons of the vertebrate peripheral nervous system (PNS) usually results in an anabolic cell body response followed by regeneration of the cut axons through the nerve to the target tissue and ultimately in the reestablishment of functional connections[6,10]. This is not true for injuries incurred by the central nervous system (CNS) of higher vertebrates, which is characterized by the inability of neurons to regrow their axons. Transecting or crushing CNS axons of birds or mammals characteristically produces a weaker cell body response, transient and abortive sprouting of the injured axons and often the formation of a glial scar at the site of the injury, followed by neuronal loss[1,20,30]. Certain CNS tissues of lower vertebrates (e.g., the visual system of the goldfish) however, possess the ability to regenerate fully and it has long been hoped that some aspect of the biochemical response to CNS injury in these species will provide an insight that will eventually lead to successful regeneration in the mammalian CNS.

It was previously found that administering a conditioning lesion in the form of an optic nerve crush altered the physiology of the goldfish retina such that explants placed in primary culture one to two weeks following the crush extended neurites, while explants prepared from normal retinas did not[12,13]. This system has been exploited to examine the effects of drugs and trophic agents on neuritic outgrowth[11,24,28]. Most recently, we have used it to measure the influence of substratum-bound extracellular matrix (ECM) components on the rate and pattern of neurite outgrowth[7]. These studies were prompted by reports which showed that fibronectin, laminin and other ECM components support neurite growth from various PNS and embryonic CNS neurons grown in culture[4,17,21,25]. Although fibronectin had no effect in the goldfish retinal explant system, laminin, a basement membrane glycoprotein known to mediate cell–substratum adhesion[19,22,27], altered the pattern of the neurite field and stimulated the rate of outgrowth several-fold. This striking effect prompted us to ask whether laminin is present in the optic nerve in vivo. Using affinity-purified rabbit antibodies to murine laminin, we examined unoperated and axoto-

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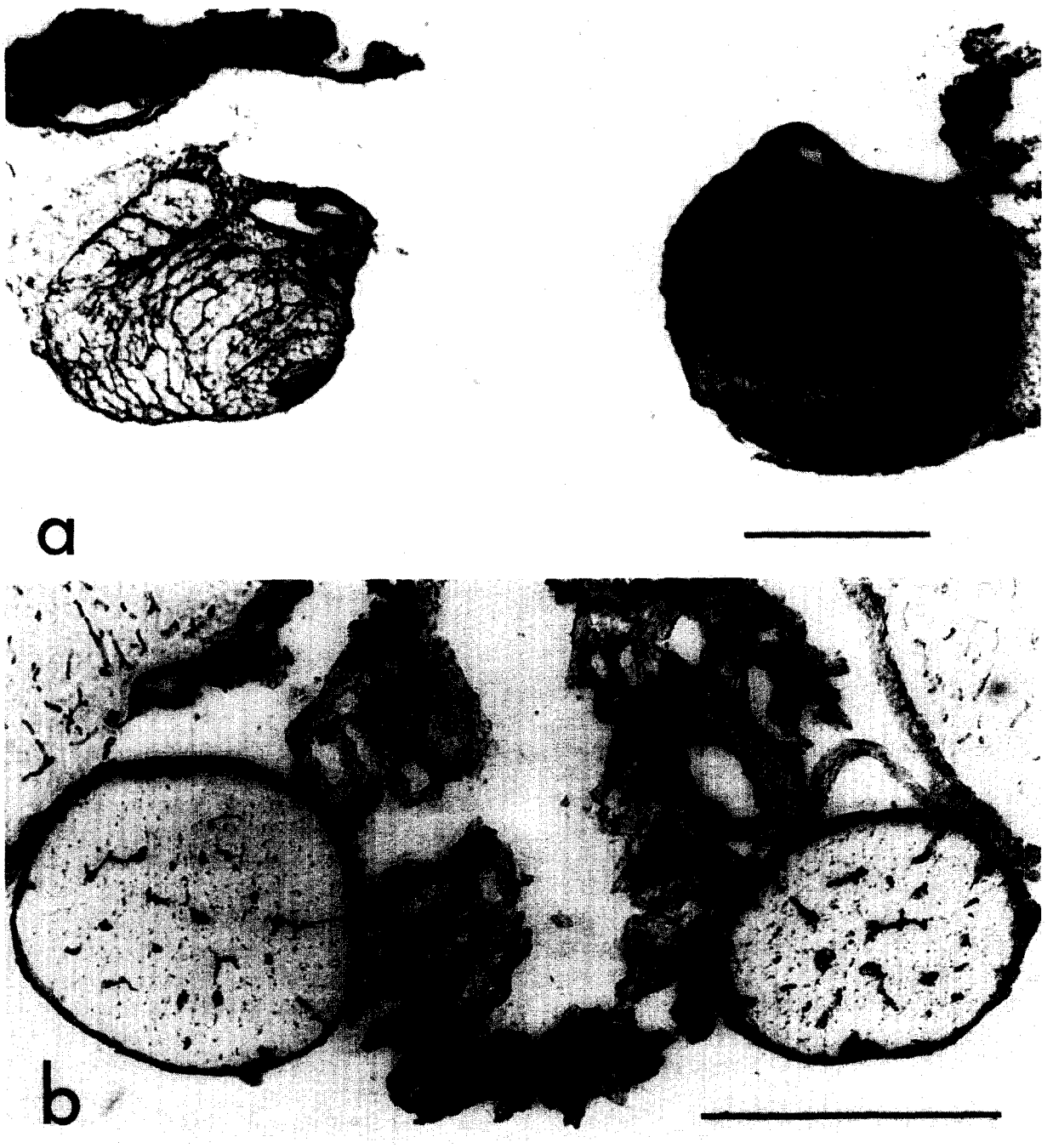

Fig. 1. Anti-laminin staining in normal and damaged optic nerves of the adult goldfish and rat. a: the optic nerve and tract of a goldfish was removed and frozen in embedding medium 56 days after intraorbital crush of the nerve (shown on the right). The tissue was cut into 8-μm sections, mounted, reacted with affinity-purified rabbit anti-laminin and peroxidase-stained with an avidin–biotin conjugate reaction[7]. Note marked increase in anti-laminin staining in contrast to normal nerve (at left). Similar results were obtained at various intervals between 7 and 85 days PC. b: brains and optic nerves of adult rats removed 29 days after lesioning the nerve shown on the right[31] and prepared as in (a). Note absence of anti-laminin staining as in normal nerve (at left). Similar results were obtained at 15 days PC. The sections shown are central to the site of crush. Bars, 500 μm.

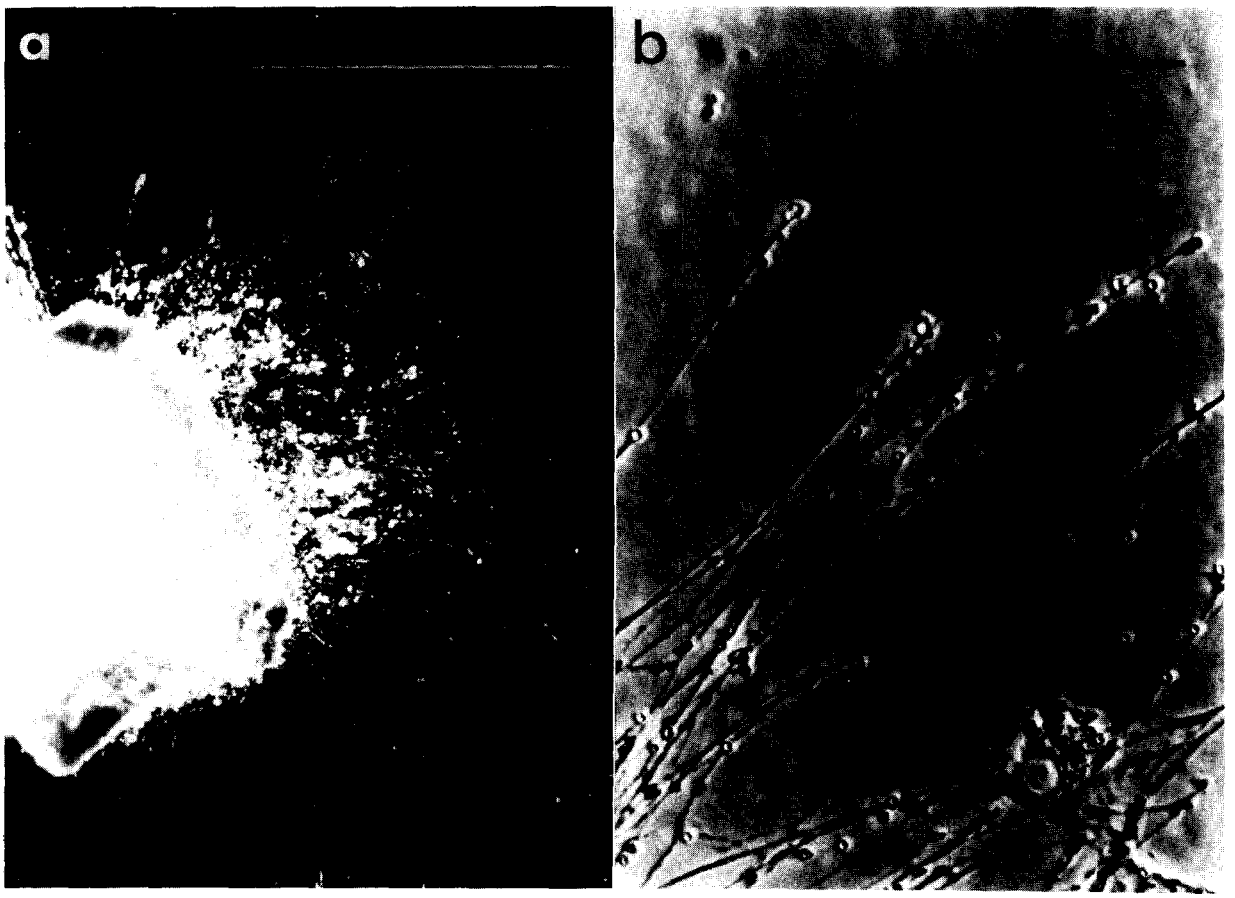

Fig. 2. Neuritic outgrowth from explants of adult rat retina. Retinas were removed 10 days after optic nerve crush[31], and explanted into culture dishes. a: explant from PC retina growing on the combined substratum as photographed using pseudo-darkfield illumination at 7 days in vitro. Bar, 500 μm. b: phase-contrast photomicrograph of neurites shown in (a). Bar, 100 μm.

mized goldfish optic nerves. In the normal nerve, laminin immunoreactivity was found only in vascular endothelium and the invaginated pial membrane which surrounds the nerve fascicles (Fig. 1a). Shortly after nerve crush and continuing for several weeks, the anti-laminin staining was greatly increased and more diffuse, with reactivity within nerve fascicles and in the surrounding basement membrane, but not outside of the fascicles (Fig. 1a). Enhanced antilaminin staining of the optic nerve also occurred following enucleation of the eye, a result that eliminated the possibility of direct or indirect contribution to the laminin content by the regrowing axons of the retinal ganglion cells, and led to the conclusion that laminin is produced by non-neuronal cells of the fish optic nerve in response to axonal degeneration. Given the demonstrated association of axonal growth cones with basal lamina in vivo[5] and the enhancement of neurite growth rates on laminin substrata in vitro[7,21], its role in regenerating nerves is surmised to be either to guide axons or simply to increase the rate of axonal elongation, thus promoting regrowth within a time critical for successful reconnection.

When unfixed, frozen sections of brain and optic nerves from adult rats were examined, we found that anti-laminin also bound to blood vessels and the epineurial sheath, which was devoid of invaginations (Fig. 1b). More significantly, we found that unlike the goldfish, the amount and distribution of laminin was not altered following retro-orbital crush or retinectomy[31] (Fig. 1b). Thus, the adult rat optic nerve does not appear to provide a laminin-enriched environment in response to axotomy. Since it has been shown that glial cells of the embryonic rat brain can

TABLE I

Neurite growth from adult rat retinas explanted 6–9 days after crush

The presence of neurites was judged by pseudo-darkfield and phase-contrast microscopy of the living cultures at 2–4 days in culture. Results are the sum of 4 separate experiments. The difference between neurite outgrowth from unoperated and axotomized explants on the poly-L-lysine (PLYS) + laminin (LAM) substratum is significant at $P < 0.01$ (χ^2 test, df = 1). Values are means ± S.E.M.

Treatment group	*Substratum*	*Number plated*	*Percent attached*	*% Growing of attached*
Unoperated	PLYS	216	55.1	0.9 ± 0.8*
	PLYS + LAM	198	37.4	12.0 ± 4.2
Axotomized	PLYS	207	52.7	0.9 ± 1.2
	PLYS + LAM	216	55.6	37.0 ± 11.0

express laminin[14,18] one could suppose that the support cells of the adult optic nerve have lost the ability to produce laminin in response to a signal from the degenerating axons, or that the effective neuronal signal is absent. It is also possible that the observed species differences are related to differences in response of various glial cell types to traumatic denervation. Astrocyte-like cells, which have been associated with laminin production[16,18] are known to proliferate in the axotomized goldfish optic nerve[32] while they do not in the optic nerve of the rat, in which the bulk of glial proliferation can be accounted for by phagocytic multipotential glia[29]. Liesi has recently shown that laminin immunoreactivity colocalizes with astrocytic glial fibrillary acid protein (GFAP) immunoreactivity in sections of normal goldfish optic nerve and that sections of normal and degenerating rat optic nerve are GFAP-positive but laminin-negative[15]. Thus, while optic nerves of both goldfish and rat contain GFAP-reactive glia, only the goldfish glia express laminin.

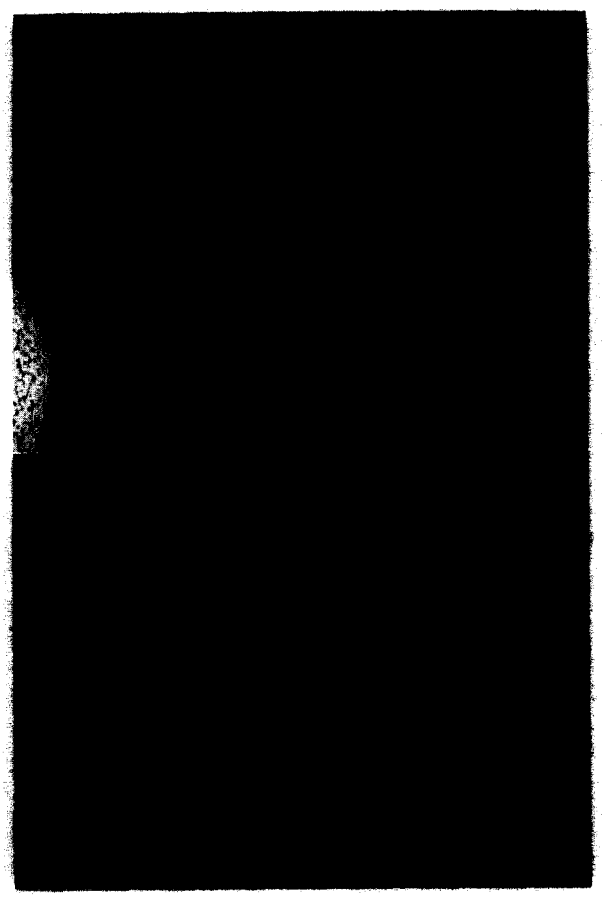

Fig. 3. Scanning electron micrographs of a rat retinal explant removed 10 days after optic nerve crush and cultured for 48 h on a PLYS + laminin-coated glass coverslip. The specimen was fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical-point dried and coated with gold–palladium. a: low-power photomicrograph. Note that the path taken by the neurite is free of non-neuronal cells. Bar, 100 μm. b: higher magnification of the growth cone shown in (a). Bar, 10 μm.

The recent finding that rat retinal ganglion cells will extend neurites into a peripheral nerve graft sutured to the sclera demonstrates that these mammalian CNS neurons possess an intrinsic ability to regrow their axons[26]. We reasoned, on the basis of this and our own goldfish studies, that explants of adult rat retina might be able to extend neurites in vitro provided they are given a conditioning lesion and a proper substratum for neurite growth. Right optic nerves of adult Sprague–Dawley rats were crushed (under ether anesthesia) by inserting forceps behind the eye and crushing the retro-orbital tissue mass including the optic nerve[2]. Loss of pupillary constriction in the unoperated eye in response to light stimulus delivered to the operated eye was considered evidence of a complete crush. Normal and 6–10 day postcrush retinas were removed[31], cut into 500-μm squares and cultured (9 per 35-mm dish) as described previously for goldfish retinal explants[13] with the fol-

lowing modifications: rat retinas and explants were prepared at room temperature in L-15 medium with 5% fetal calf serum, 0.1 mg/ml gentamycin sulfate and buffered with HEPES to pH 7.4. Explants were plated onto plastic dishes precoated with either poly-L-lysine (PLYS; 1 mg/ml) or PLYS followed by laminin (20 μg/ml)[7]. The medium was then replaced with Dulbecco's minimum essential medium containing 20% fetal calf serum, 0.1 mM 5-fluorodeoxyuridine, 0.2 mM uridine, 0.1 mg/ml gentamycin sulfate and was buffered with bicarbonate/CO_2 to pH 7.4. Cultures were maintained at 37 °C and examined for outgrowth 2–7 days later.

Culture substrata consisting of PLYS alone did not support outgrowth from explants prepared from either normal (N) or postcrush (PC) retinas (Table I). When the substratum also contained laminin, however, explants prepared from PC retinas usually extended neurites for distances of 0.1–1 mm (Fig. 2). Explants of N retinas also grew neurites onto substrata containing laminin, but significantly less often than did the PC explants (Table I). Both phase contrast and scanning electron micrographs (Fig. 3) show that the rat retinal explant fibers are typical neurites, presumably originating from ganglion cells, as has been demonstrated in goldfish retinal explants[9]. The magnitude of neurite outgrowth in these experiments is less than that seen in cultures of goldfish retinal explants or cultures of embryonic mouse retinal explants[25], possibly because the adult rat explant culture system is not yet optimized. Other ECM components such as fibronectin and collagen may have selective effects on regrowth. It is nevertheless clear that prior injury to the nerve enhances the neuritogenic potential of adult rat retina and that laminin is an effective substratum for growth of neurites from this adult mammalian CNS tissue.

Recent studies on peripheral nerve regeneration indicate that growth cones of regenerating axons select the laminin-rich inner lamina lucida of the Schwann tube[3,8,23]. Since we have shown that neurite outgrowth from adult rat retinal explants is supported by laminin in the culture substratum, we propose that the presence of laminin in peripheral nerve tissue may explain in part the ability of PNS grafts to support regeneration of CNS axons, including neurites grown out from mammalian retinal ganglion cells[26]. The correlation we have presented should not be taken to mean that the presence of laminin in the extracellular matrix suffices to bring about CNS regeneration: other extraneuronal factors, perhaps originating from supporting cells, may be important as well. The demonstration in the present study of the stimulating effect of a prior lesion on neurite outgrowth also indicates a role for neuronal factors in neurite outgrowth in the mammalian CNS. The lack of laminin in the parenchyma of the rat optic nerve may nevertheless account significantly for its failure to regenerate. The provision of a laminin-rich extracellular environment may thus prove a useful approach for promoting successful in vivo CNS regeneration.

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