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IN VITRO EVIDENCE FOR A NEURITE GROWTH-PROMOTING ACTIVITY IN TREMBLER MOUSE SERUM

J. KOENIG,* Dj. HANTAZ-AMBROISE,† S. DE LA PORTE,* N. A. DO THI,‡ J. M. BOURRE,§ F. LA CHAPELLE‖ and H. L. KOENIG‡

*Lab. Neurobiologie Cellulaire, Université de Bordeaux II, Avenue des Facultés, 33405 Talence Cedex; †INSERM U. 153, Paris V; ‡Lab. Neurobiologie du Développement, Université Bordeaux I, Avenue des Facultés, 33405 Talence Cedex; §INSERM U. 26, Paris X; ‖INSERM U. 134, Paris XIII, France

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Abstract—Basal lamina components, such as heparan sulfate proteoglycan (HSPG) and laminin play an important role in neuritic outgrowth for CNS and PNS neurons in culture. The mutant mouse 'Trembler' is characterized by hypomyelinization and production of an excess of basal lamina layers around Schwann cells in peripheral nerves.

In order to analyse whether or not the serum of the mutant animals contains neurite outgrowth-promoting factors, we cultured rat spinal cord neurons in the presence of Trembler serum. Under these conditions, the outgrowth of neurites was increased approx. 2 times as compared to control serum. Trembler serum induces neuritic outgrowth characterized both by an increase in number of primary neurites emerging from the nerve cell body as well as by an increase in peripheral branching of neurites.

To characterize the factors implicated in this increase we added antibodies directed against HSPG or laminin to the mutant serum. As a result, the increase in neuritic outgrowth was reduced or abolished in both cases.

Trembler effect on neurite growth disappeared when the number of the non-neuronal cells was reduced, suggesting that the mutant serum did not act directly on neurons but by the intermediary action of non-neuronal cells.

Key words: Neuritic outgrowth, Schwann cell mutation, Serum, Extracellular matrix, Mitogenic factors.

At the neuromuscular junction, motor neuron terminals, Schwann cells and muscle cells are closely juxtaposed and interact by means of diffusible factors. Some of these factors are molecules, such as laminin and heparan sulfate proteoglycan which compose the basal lamina. In fact, it has been shown *in vitro* that Schwann cells[1,2] and muscle cells[6,10] synthesize and release both laminin and heparan sulfate proteoglycan (HSPG).

Schwann cell and muscle cell conditioned media increase neuritic outgrowth of a number of different types of neurons: chick ciliary ganglion cells,[13] sympathetic neurons[10] and rat spinal cord neurons.[6] In conditioned media, laminin and HSPG form a complex spontaneously.[11] Most authors have suggested that laminin is the only active factor for neuritic outgrowth in this complex. Nevertheless, the fact that anti-laminin antibodies added to different conditioned media do not totally block neuritic outgrowth induced by the heparan proteoglycan–laminin complex suggests that additional factors may contribute to some growth-promoting activity.[10]

Recently, we have shown that HSPG and laminin increase neurite growth of rat spinal cord neurons.[6] These two components of the basal lamina modulate neuritic outgrowth differently: heparan proteoglycan induces elongation of neurites while laminin increases neuritic branching. The extent of neurite growth was dependent on the concentrations of laminin and HSPG in the culture medium.[6] These results suggest that there is a direct relationship between the development of neuronal processes and the amount of basal lamina components secreted and/or synthesized by cells associated with neurons.

A multi-layered basal lamina around Schwann cells has been observed in Trembler mutant mice.[12] This mutation is characterized, in addition, by abnormal branching of nerve endings in certain muscles.[5,9] It is unclear whether or not excess secretion of basal lamina components in Trembler mice occurs into the blood, and the effects of Trembler mouse serum on neuritic outgrowth of rat spinal cord neurons are unknown.

The results presented in this paper show that Trembler serum added to neuron cultures increases neuritic outgrowth, resulting in a very dense network surrounding the perikaryon.

When antisera raised against HSPG and laminin were added to cell culture media, neuritic outgrowth induced by Trembler serum was reduced or even inhibited. These results suggest that

'neurite growth-promoting factors' in Trembler serum could be HSPG- and laminin-like molecules. However, it has not been possible to detect differences in the serum concentrations of these molecules in control and Trembler mice by ELISA. Furthermore, the fact that neuritic outgrowth induced by Trembler serum disappeared when non-neuronal cell populations were reduced by anti-mitotic agents suggests that mutant serum does not act directly on the neurons and that the increase in neurite growth is mediated by non-neuronal cells.

EXPERIMENTAL PROCEDURES

Culture techniques

Spinal cords from 14-day-old rat embryos were mechanically dissociated and cells were plated in standard medium at a concentration of 2×10^6 cells/35 mm dish precoated with 0.001% poly-L-lysine (Sigma). The standard medium consisted of 80% Eagle's minimal essential medium (MEM), 10% medium 199 with glutamine (Gibco), 10% horse serum (HS) (Gibco) with 0.5 ml of 20% streptomycin and 0.5% of 20,000 UT penicillin/ml. Cultures were incubated at 37°C in a humidified atmosphere of 8% CO_2 in air.

To allow for constant handling and survival of cells, all cultures were initially maintained for 24 hr in standard medium. After the first day, standard medium was replaced by different experimental media.

To reduce the number of non-neuronal cells, 5-fluodeoxyuridine (FUDR, Sigma) and uridine (Sigma) were added to the experimental media for 24 hr at concentrations of 10^{-5}M.

Preparation of sera from normal (control) and Trembler mice

Normal and Trembler mouse blood was collected from 4- to 6-month-old animals by the method of occular exsanguination. After 4–5 hr of storage at 4°C, the serum was carefully removed with a pipetman and centrifuged at 500 ***g*** for 10 min (Kontron ZT 365 centrifuge) to eliminate all blood cells. The sera were stored in 100 μl aliquots at −20°C. Immediately before the experiment, the serum was thawed and added to the culture medium, which was then filtered through a 0.22 μm millipore filter.

Media with normal (control) and Trembler sera

After 24 hr, varying amounts of sera from normal (C) and Trembler mice (TR) were added to the standard medium to give a final, total serum concentration of 10%. The effect of Trembler serum was observed at a concentration of 1% and increased to a maximum at 5% mutant serum. We used 5% HS+5% control serum for control medium and 5% HS+5% Trembler serum for Trembler medium.

Control and Trembler media supplemented with basal lamina components and antibodies

Heparan sulfate proteoglycan (HSPG purified low-density proteoglycan UL), laminin, anti-HSPG and anti-laminin antibodies were generous gifts from Dr M. Vigny (Inserm, U. 118, Paris). HSPG was purified from EHS sarcoma (Engelbreth–Holm–Swann) as recently described by Hassel *et al.*[7] and laminin was purified from EHS tumor.[8,18] They were added at a concentration of 4 μg/ml (HSPG) and 1 μg/ml (laminin) to control and Trembler media. Antibodies against HSPG and laminin were added to experimental media at a 1:100 dilution.

Quantification of HSPG and laminin in Trembler serum

To determine levels of laminin and HSPG in Trembler serum we have used ELISA and ELISA sandwich techniques. For ELISA we used serial dilutions of undiluted serum, the same lots as for the cultures. HSPG and laminin levels were measured in control and Trembler serum as described by Rennard *et al.*[16] using specific immune sera directed against HSPG and laminin. Laminin and HSPG levels were estimated using a sandwich immunoassay by measuring the inhibition of binding of the first antibody to the antigen-coated well.

Characterization of non-neuronal cells

The relative proportion of non-neuronal cells by comparison with neuronal cells was determined by identifying cell types with specific markers: antibodies against glial fibrillary acid protein (GFAP) for astrocytes, antibodies against galactocerebrosides from oligodendrocytes,[15,17] and anti-Thy 1-1 antibodies (New England Nuclear, Boston, MA) coupled with fluorescein isothiocyanate for fibroblasts. Although we do not have a specific marker for Schwann cells, they were identified by their fusiform shape and their position along the length of the neurite following cresyl violet staining. Immune serum against GFAP was from Dako. The monoclonal antibody against galactocerebroside was a generous gift from Dr Pessac, Inserm U. 278, Paris and Dr Ranscht, La Jolla, U.S.A.

Neurite growth analysis

For a rough estimate of neuritic outgrowth we used direct observation of cultures by phase contrast microscopy. Neurons and neuritic extensions were detected by indirect immunofluorescence using tetanus toxin* and its immune serum.[15,17] Each set of experimental conditions was repeated at least three times until 50–70 neurons had been analysed. Neurons were photographed at a magnification of $\times 500$.

To quantify the amount of branching, each photo was covered with a transparent overlay with 11 concentric circles of increasing radii (3 mm increments) (Letraset 63233). The points of intersection between neurites and circles were counted. The distance in mm from the center of the perikaryon (abscissa) was plotted vs the number of neurite–circle intersections (ordinate) (Table 1). The sprouting index (SI) was defined for each of the 11 circles.

$$SI = \frac{\text{number of points of intersection/concentric circle exp. medium}}{\text{number of points of intersection/concentric circle control medium}}.$$

To quantify neuritic growth, the total length of the neurites and their branches was determined with the aid of a Kontron image analyser. This calculation allowed evaluation of neurite growth irrespective of the amount of sprouting.

Table 1. The effect of non-neuronal cells on neurite branching. In all experimental conditions, the spinal cord cells were cultured for 1 day in standard medium: MEM+199+10% HS, then cultured for 6 days in different experimental media. The number of points of intersection (± S.E.M.) between neurites and concentric circles is shown. These values indicate the degree of neurite branching with high (A) or low (B) proportion of non-neuronal cells in the control medium after 6 days of culture (C 6d) and in Trembler medium, 6 days (Tr 6d) of culture

	Number of points of intersection in different media						
	A = high proportion of non-neuronal cells			B = low proportion of non-neuronal cells			
Distance from perikarya (mm)	C 6d	Tr 6d	$SI = \frac{Tr\ 6d}{C\ 6d}$	C 6d	$SI = \frac{CB}{CA}$	Tr 6d	$SI = \frac{Tr\ B}{Tr\ A}$
3	6 ± 0.3	15 ± 0.7	2.50	5 ± 0.6	0.8	3 ± 0.2	0.2
6	6 ± 0.2	20 ± 0.7	3.33	9 ± 0.7	1.5	3 ± 0.1	0.15
9	7 ± 0.6	22 ± 0.9	3.14	12 ± 1.7	1.71	4 ± 0.2	0.18
12	8 ± 0.5	24 ± 1.0	3.0	13 ± 1.0	1.62	5 ± 0.1	0.20
15	8 ± 0.7	28 ± 1.3	3.5	13 ± 1.8	1.62	5 ± 0.5	0.17
18	8 ± 0.5	32 ± 2.3	4	14 ± 0.8	1.75	4 ± 0.3	0.12
21	7 ± 0.4	33 ± 1.9	4.71	14 ± 1.9	2	4 ± 0.7	0.12
24	6 ± 0.3	35 ± 2.1	5.83	15 ± 1.5	2.5	3 ± 0.5	0.08
27	5 ± 0.5	37 ± 1.7	7.4	15 ± 0.9	3	3 ± 0.2	0.08
30	5 ± 0.4	38 ± 1.9	7.6	15 ± 1.1	3	3 ± 0.1	0.08
33	5 ± 0.4	43 ± 2.1	8.6	15 ± 1.3	3	3 ± 0.2	0.07

* Tetanus toxin and its immune serum were a generous gift from Prof. B. Bizzini, Institut Pasteur, Paris.

RESULTS

Definition of neurite growth types

Neurite growth is of two types: elongation or sprouting as we have recently reported.[6] The elongation type of neurite growth is characterized by increases in the length of neurites growing out of the nerve cell body, with little branching along their length. In contrast, the sprouting type of neurite growth is characterized by an increase in the number of neurites: either of primary neurites budding from the cell body (proximal sprouting), or of branches raising from primary neurites (distal sprouting).

Neuritic outgrowth in control medium. During the first 24 hr cells were grown in standard medium plated at 2×10^6 cells/35 mm dish; 50% of plated cells were attached to the substrate after 1 day of culture. Dissociated embryonic spinal cord cells were heterogeneous. There were numerous non-neuronal cells of ill-defined shape adhering to the substrate during the first 2 hr of culture, whereas isolated neurons characterized by large nuclei adhered later, 6–10 hr after plating.

The isolated neurons exhibited short, thin neurites at the end of the first day of culture. The standard medium was then replaced by control medium. During the second and third day of culture, large neurites emerged from isolated nerve cell bodies. During the fifth and sixth day of culture short, thin branches emerged from these large neurites.

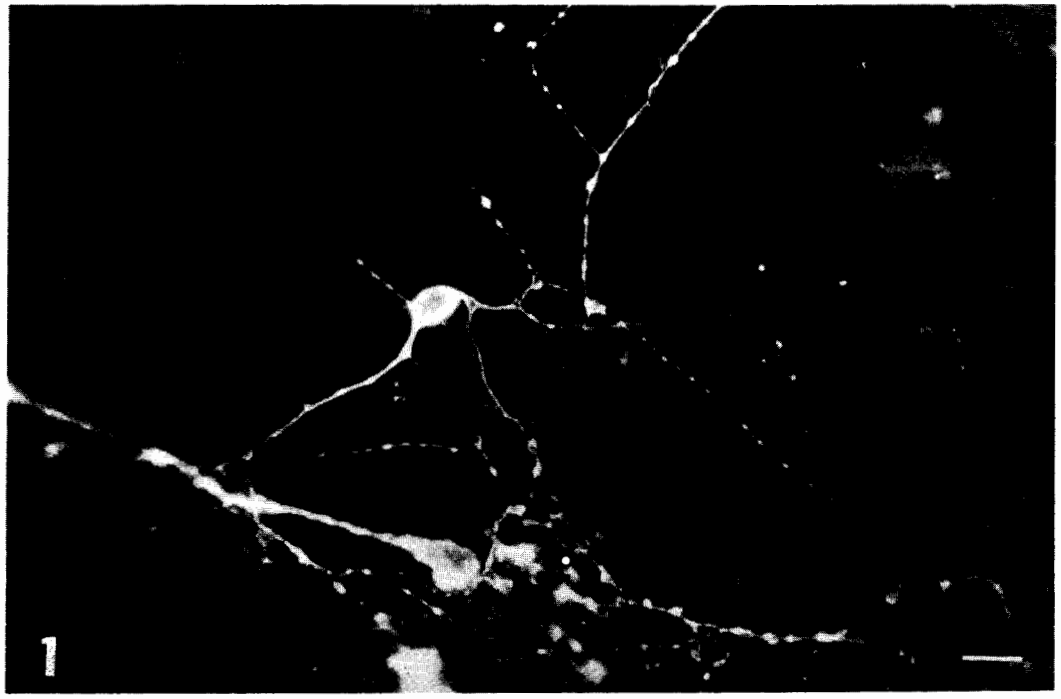
From the fourth day of culture, neurons and astrocytes were visualized by immunofluorescence using tetanus toxin and by immune serum raised against GFAP, respectively. Oligodendrocytes could not be labeled by antibodies against galactocerebrosides until day 6 or 7 of culture. We chose the seventh day of culture to analyse differentiation of spinal cord cell types. At this time, in control medium, the non-neuronal cells formed a layer under the neurons representing approximately 25% of the total cell population: astrocytes (17%), oligodendrocytes (2–3%), and fibroblasts and Schwann cells (approximately 3–4%).

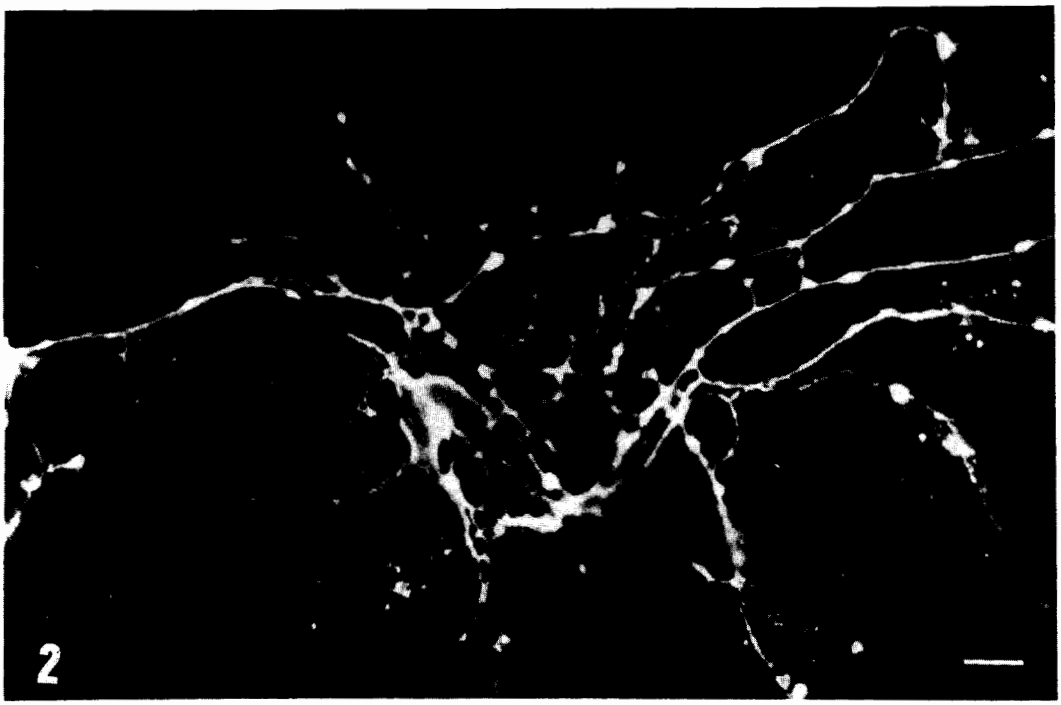
Neuritic growth in the presence of FUDR. When cultures were treated with FUDR between days 2 and 3, distal sprouting was increased or induced (Fig. 2) compared with that noted in control cultures (Fig. 1). Table 1B shows that the sprouting index (SI) increased from a distance of 6 mm from the cell body, at all points along the growing neurites, but was particularly pronounced in the terminal portion of the neurites. At 27 mm or more from the perikaryon, the SI was 3, as compared to 0.8 at 3 mm, thus demonstrating a proximo-distal branching gradient (Table 1B). This sprouting involved the distal part of neurites only and therefore corresponded to distal sprouting (Fig. 5c). This sprouting appeared only when non-neuronal cells were scarce and may have been repressed when they were more numerous. We have called this type of sprouting 'non-neuronal cell repressed sprouting' (Fig. 5c).

Trembler serum. Twenty-four hours following cell plating, standard medium was replaced by Trembler medium. Forth-eight hours later, increases were noted in the number of primary neurites emerging from the nerve cell bodies and of neurite branches (Fig. 10A). The number of branches increased significantly from day 4 to day 6 of culture. By day 6, an average of 15 neurites had emerged directly from the nerve cell body (proximal sprouting). There was also an increase in the number of branches along the length of the neurites (distal sprouting). At 6 mm (on the photo $\times 500$) from the perikaryon the average number of neurite–circle intersections was 20, whereas at 33 mm from the cell body this value was 43 (Table 1A). The SI was 2.5 for the circle 3 mm from the perikaryon and 8.6 for the 33 mm circle (Table 1A, Figs 3, 5 and 10A). The two types of sprouting occurring when neurons were cultured in the presence of Trembler serum resulted in a significant increase in the total length of the neuritic arbor (86% as compared to control values after 6 days in culture) (Fig. 13).

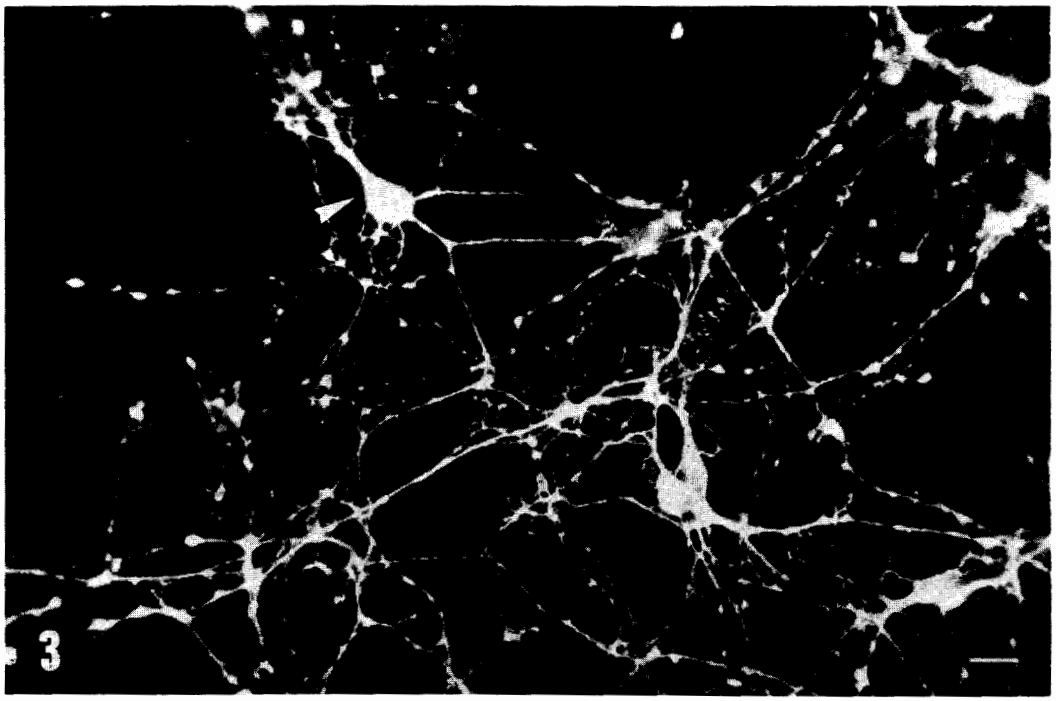
Trembler serum plus FUDR. When non-neuronal cells were scarce (about 5%) after inhibition of their replication by FUDR, the number of neurites emerging from the cell bodies and of neuritic branching was equal to or less than that found in control cultures (Table 1). We have called this type of sprouting observed only in the presence of a large number of non-neuronal cells, 'non-neuronal cell-induced sprouting' (Table 1, Figs 5b, d and 10A, B).

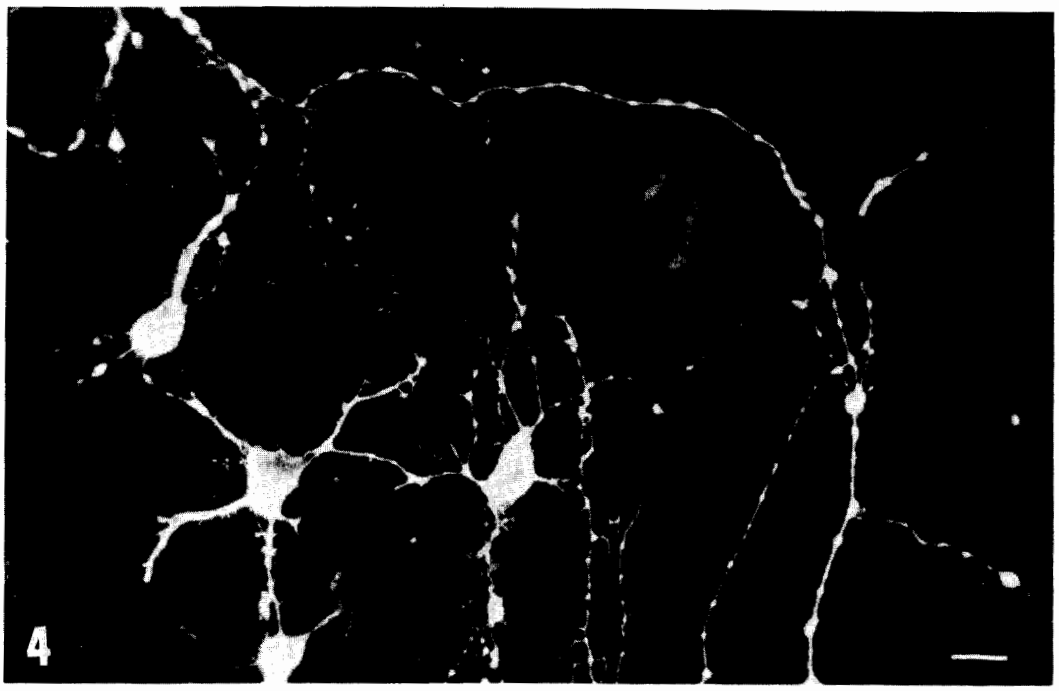
Effect of anti-HSPG antibodies on proximal and distal sprouting induced by Trembler serum. When the standard culture medium was replaced after 24 hr by Trembler medium containing



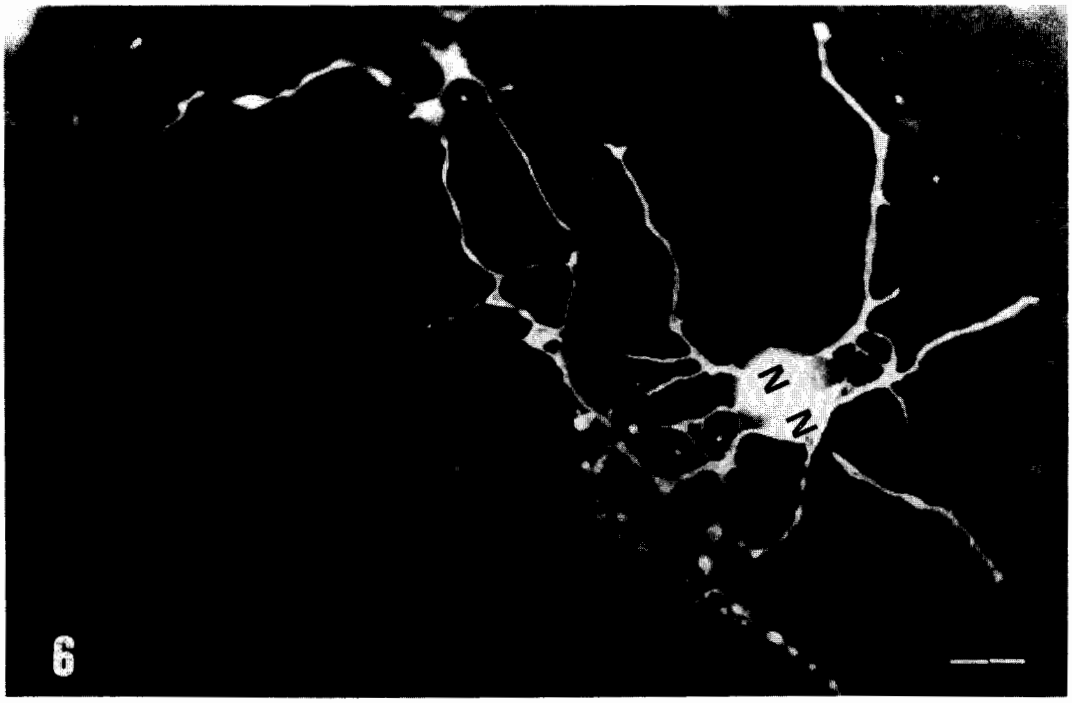


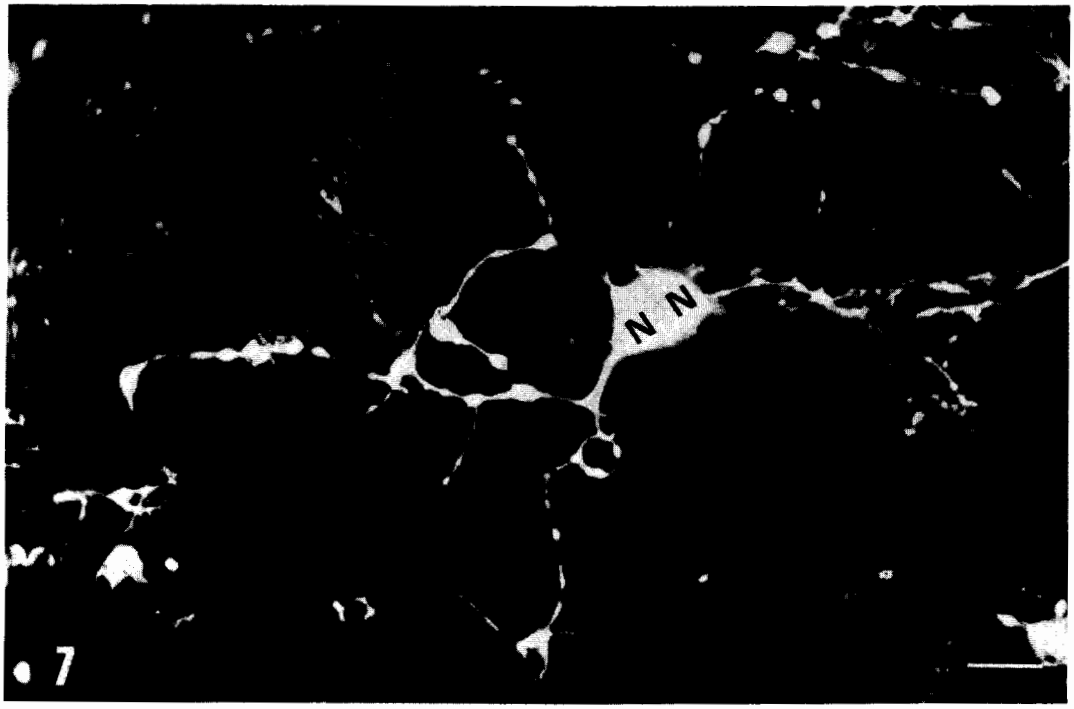
Figs 1 and 2. Neurons cultured in the presence of control serum for 6 days. In the absence (Fig. 1) and in the presence of anti-mitotic drugs (Fig. 2). Note that the branches of the neurites are more numerous when the non-neuronal cells are less numerous (Fig. 2). Indirect immunofluorescence using tetanus toxin. Bars = 20 μm.



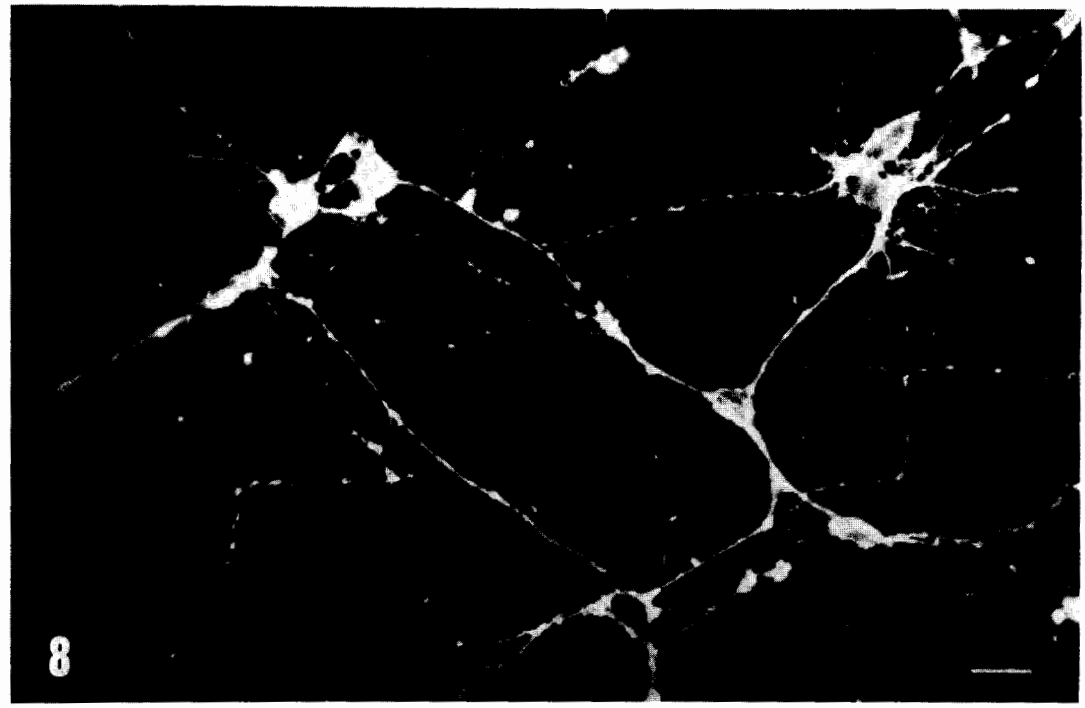


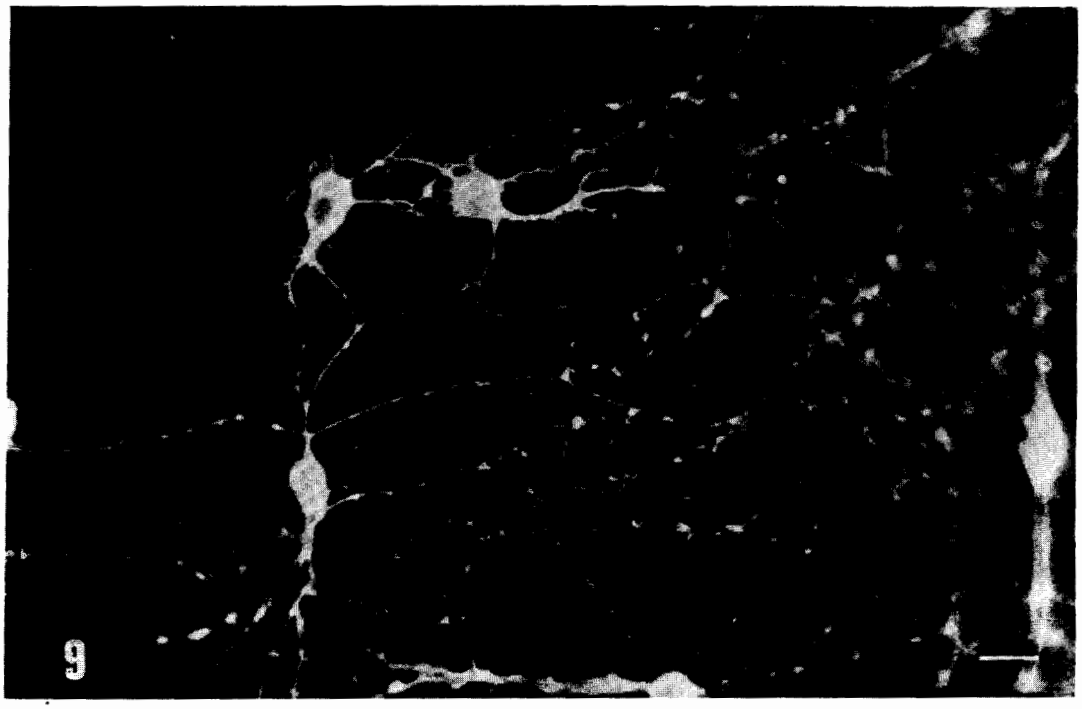
Figs 3 and 4. Neurons cultured for 6 days in medium containing Trembler serum. Note that many neurites (about eight per cell) emerge from the perikaryon (arrow) and that a network formed of numerous branches encloses the nerve cell body in the presence of a large number of non-neuronal cells (Fig. 3). When the number of non-neuronal cells is considerably reduced by an anti-mitotic drug (Fig. 4) the proximal sprouting is less affected than the distal sprouting which is totally inhibited. Indirect immunofluorescence using tetanus toxin. Bars = 20 μm.





Figs 6 and 7. Two neurons (N) cultured in medium containing Trembler serum for 6 days in the presence of anti-HSPB (Fig. 6) or in the presence of anti-laminin (Fig. 7). Note that proximal sprouting is less affected in the presence of anti-HSPG (Fig. 6) than in the presence of anti-laminin (Fig. 7). In the latter case neuritic growth is less than that observed in control medium (Fig. 1). Indirect immunofluorescence after binding with tetanus toxin and its immune serum. Bars = 20 μm.





Figs 8 and 9. Neurons cultured in medium containing Trembler serum for 6 days in the presence of HSPG and laminin. In the presence of HSPG, the proximal and distal sprouting are much reduced (Fig. 8). The neurites are also very long. In contrast, in the presence of laminin both types of sprouting are more numerous (Fig. 9) than cells grown in Trembler medium alone. Indirect immunofluorescence using tetanus toxin and its immune serum. Bars = 20 μm.

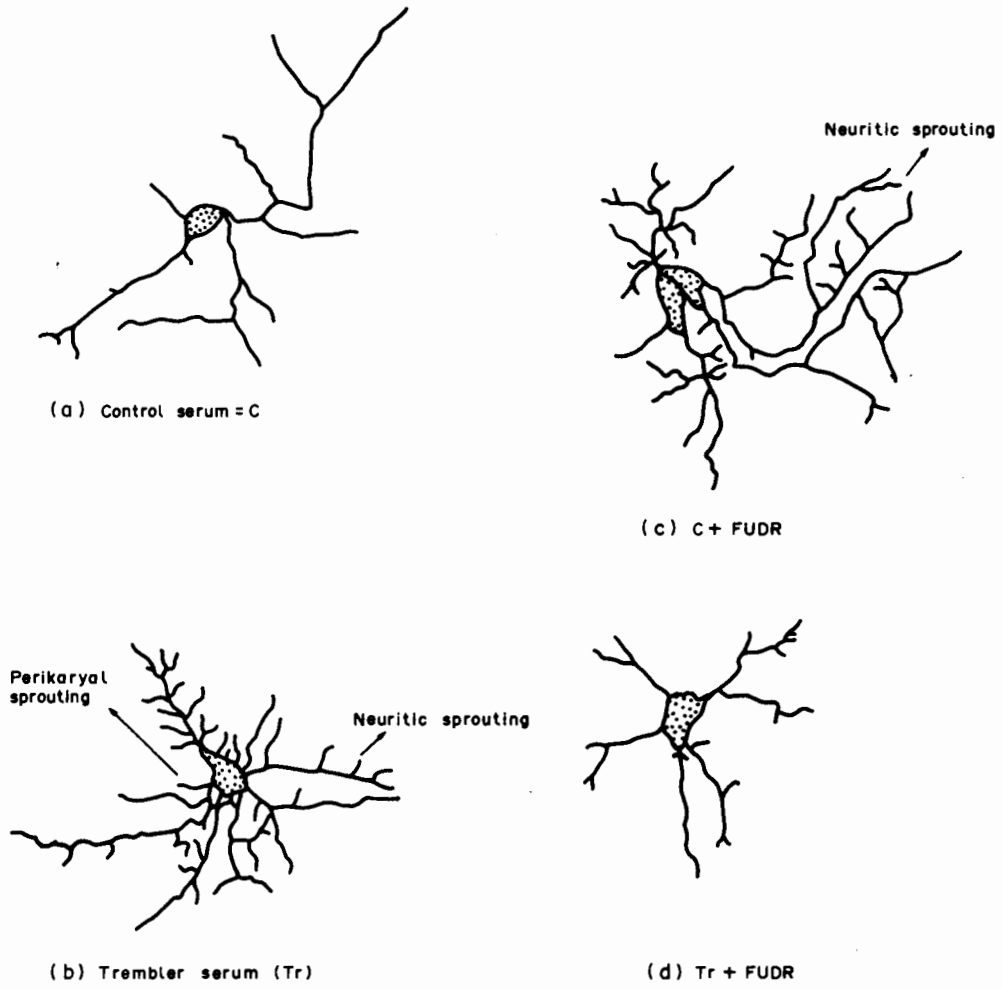


Fig. 5. The transformation of neurons into neurons exhibiting proximal (number of neurites emerging from perikaryon) and distal sprouting (b) in Trembler serum. This sprouting was apparent when the non-neuronal cells were abundant (b) and absent when these cells were scarce (d).

immune serum raised against HSPG, reductions were noted in total neurite length and in the frequency of neuritic sprouting, by comparison with neurons grown in Trembler medium. In the presence of anti-HSPG antibodies, the total neurite length was reduced by 38% relative to neurons grown in Trembler medium without antibody (Fig. 13). Anti-HSPG antibodies particularly affected distal branching of neurites, whereas they modified only slightly proximal sprouting (Figs 6 and 11).

Effect of anti-laminin antibodies on proximal and distal sprouting induced by Trembler serum. In the presence of Trembler medium containing anti-laminin antibodies, the emergence of neurites from the cell body, and their subsequent branching, were similar to those observed when neurons were cultured in the presence of control medium (Figs 7, 11 and 13). Total neurite growth was reduced by 55% as compared to that of neurons grown in Trembler medium without anti-laminin

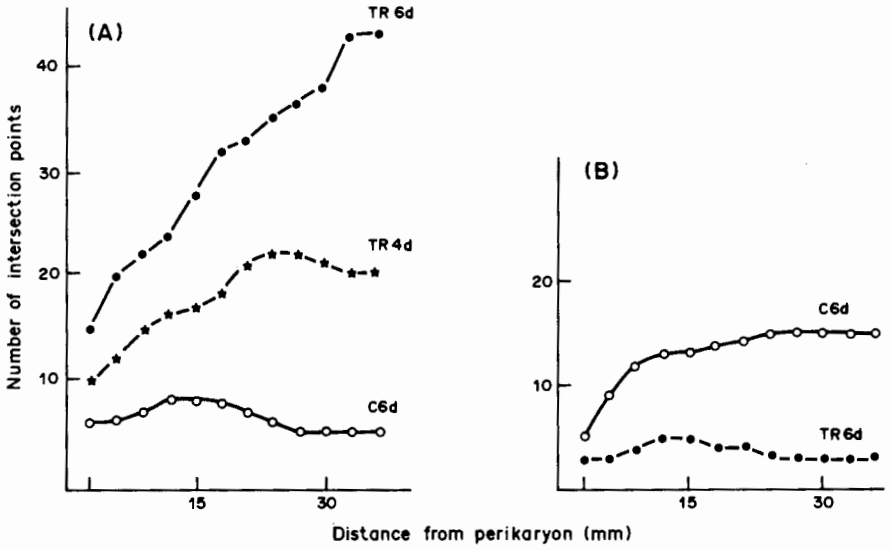


Fig. 10. Curves showing the number of points of intersection between neurites and concentric circles as a function of distance from the perikaryon for neurons cultured for 6 days in control plating medium (C 6d), for 4 days in Trembler medium (Tr 4d) and for 6 days (Tr 6d) in the presence of high (A) or low (B) proportions of non-neuronal cells (see Table 1).

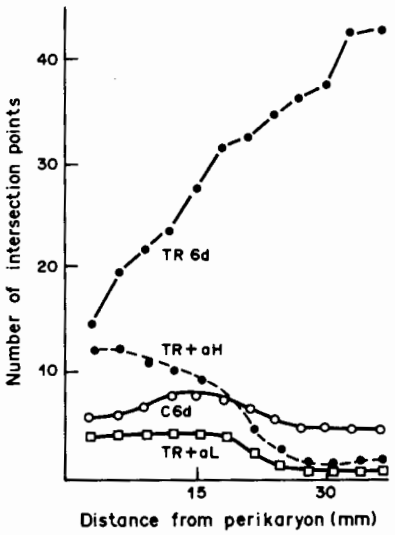


Fig. 11. Effect of antibodies on neuritic branching of neurons cultured in the presence of Trembler serum. Both anti-HSPG (aH) and anti-laminin (aL) antibodies affected neuritic branching when added to Trembler medium.

antibodies. Note that the neurite growth seen in Trembler medium supplemented with anti-laminin antibodies was 17% less than that in control cultures (Fig. 13). Thus, anti-laminin antibodies completely inhibited the proximal and distal sprouting induced by Trembler serum.

Determination of HSPG and laminin antigen in Trembler serum. The later results suggest that Trembler serum contains HSPG- and laminin-like molecules which could act as neurite-sprouting factors. We were not able to detect any laminin or HSPG in either Trembler or control sera by ELISA methods. This suggests that the two sera contain less than 10 ng of antigen per ml.

We have recently shown that two components of the basal lamina, HSPG and laminin, added to a medium containing 10% horse serum, increased neuritic growth and induced, respectively, elongation and branching of neurites.[6] We have investigated whether HSPG and laminin antigens added to Trembler medium influence neuritic growth in the same way.

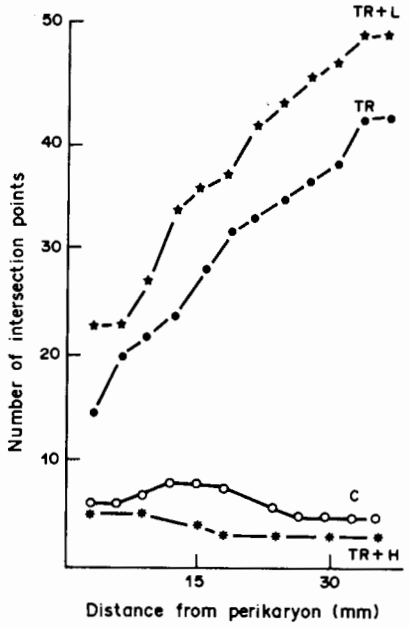


Fig. 12. Curves showing the number of points of intersection between neurites and concentric circles as a function of the distance from the perikaryon for neurons cultured for 6 days in Trembler plating medium (Tr), in laminin (L) and in HSPG (H) supplemented media. The concentration of laminin and HSPG were 1 and 4 μg/ml, respectively. The maximum number of points of intersection was obtained with laminin-supplemented medium. Means ± S.E.M. were calculated for 50 neurons.

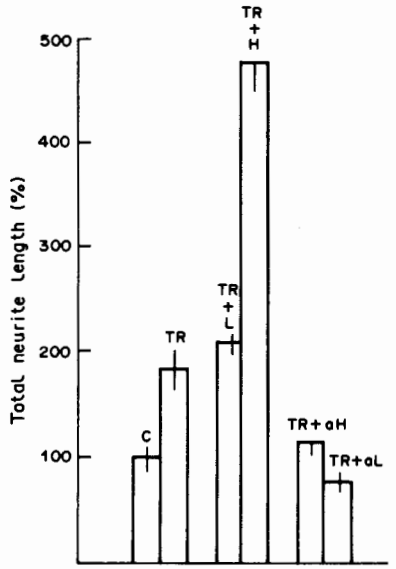


Fig. 13. Total neurite length (± S.E.M.) after 6 days of culture in control (C) and Trembler (TR) media supplemented with laminin (TR+L), HSPG (TR+H), anti-laminin antibodies (TR+aL) or anti-HSPG (TR+aH) antibodies. Measurements were performed in triplicate with 50 neurons for each treatment in three dishes.

Effect of HSPG on spinal cord neurons cultured in Trembler serum. Addition of HSPG to Trembler medium caused general neuritic elongation. The number of neurites emerging from the perikarya was greatly reduced (at most five per cell), and neurites were very long, and had fewer branches along their length (Fig. 8), as compared to those observed in medium containing Trembler serum alone. The total length of neuritic branching increased 2.5 fold as compared with the Trembler value (Fig. 13). The SI was less than that of neurons cultured in control medium. SI values were smaller at points removed from the perikaryon, showing that HSPG-supplemented Trembler medium reduced distal sprouting in particular (Fig. 12).

Effect of laminin on spinal cord neurons cultured in Trembler serum. Laminin addition to Trembler medium increased only the branching of neurites. The SI increased particularly in the region close to the neuronal cell body. The number of neurites emerging from the perikarya increased by a factor of 1.5, and that of branches of the main neurites by a factor 1.2. Laminin increased the total neuritic length by 115% as compared to the Trembler value and 215% as compared to control values (Fig. 13).

DISCUSSION

From the results described above, it appears that Trembler serum affects neurite growth by increasing both the number of neurites emerging from the nerve cell bodies and the number of peripheral neuritic branches. Neurons cultured in the presence of Trembler serum for 6 days had three times as many neurites emerging from the perikaryon as neurons cultured in control medium. The number of branches budding from the neurites increased by a factor (SI) of 3.3 at a distance of 6 mm (on the micrograph) from the perikaryon and by a factor of 8.6 at 33 mm from the perikaryon (Table 1, Fig. 10). We have called this type of sprouting, which corresponds to neurites budding both from the nerve cell body and from the neurites 'proximal and distal sprouting'. The fact that the sprouting index (SI) increased at greater distances from the cell body indicates a proximo-distal gradient of sprouting.

We have shown that the increase in neurite growth of neurons cultured in Trembler medium was reduced greatly when immune sera directed against HSPG and laminin were added to the Trembler serum. This inhibition of the Trembler effect has led us to postulate that Trembler serum contains HSPG- and laminin-like molecules. However, we have now shown using ELISA that the concentration of these two molecules in both Trembler and control sera is less than 10 ng/ml. The very low concentrations of these two molecules in Trembler serum cannot account for the Trembler effect. Thus, Trembler serum probably does not act directly on neurons but may act by way of non-neuronal cells, by stimulating proliferation of non-neuronal cells, which in turn might synthesize and release HSPG- and laminin-like molecules into the medium. In Trembler medium, the number of non-neuronal cells is high and the concentration of HSPG- and laminin-like molecules secreted by these cells would be sufficient to induce neuritic outgrowth characteristic of the Trembler effect. In standard medium, Schwann cells of the rat dorsal root ganglion synthesize components of the basal lamina such as fibronectin, laminin and HSPG.[14] Rat RN22 Schwannoma cells also secrete a neurite growth-promoting factor composed of a laminin–HSPG complex.[2] The laminin secreted by rat Schwannoma cells, as well as the HSPG synthesized by rat dorsal root Schwann cells, have structures similar but not identical to those antigens isolated from the EHS tumor of the mouse. Nevertheless, antibodies raised against tumoral HSPG and laminin recognize HSPG and laminin secreted by rat Schwannoma cells.[2] These results allow us to understand why anti-HSPG and anti-laminin antibodies, raised against antigens extracted from the EHS tumor, inhibit the biological effect of factors secreted by rat non-neuronal cells in our cultures.

The Trembler serum could induce neuritic extension not only by increasing the number of non-neuronal cells which synthesize neurite-promoting factors but also by stimulating significantly the synthesis of these molecules.

We intend to test this hypothesis by studying the synthesis and release of HSPG- and laminin-like molecules by normal mouse Schwann cells cultured in the presence of Trembler serum and the influence of these molecules on neurite growth.

We cannot exclude the possibility that Trembler serum contains other growth-promoting factors that act directly on neuritic growth in addition to those molecules acting upon the non-neuronal cells. These putative factors present in serum would not be detected by antibodies raised against the two principal components of the basal lamina, HSPG and laminin. The Trembler serum could contain one or more factors with different physiological roles, including mitogenic and neurite growth-promoting activities. It has been shown recently that a retina-derived growth factor (RDGF), also induces neuritic extension in PC_{12} cells.[21]

The two types of neuritic growth observed in spinal cord cells cultured in Trembler serum, proximal sprouting and distal sprouting, were modulated differently by anti-laminin and anti-HSPG antibodies. The two antibodies modified Trembler serum-induced neuritic branching in

two different ways. Anti-laminin antibodies decreased both proximal and distal sprouting whereas anti-HSPG antibodies primarily decreased distal sprouting. The effect of anti-HSPG antibodies on neuritic branching was pronounced particularly in the peripheral portion of the neurites where the number of branches was lower than that observed in cultures with control medium (normal mouse serum). Addition of anti-laminin antibodies to Trembler serum reduced both proximal and distal sprouting to values below control values, showing that anti-laminin totally inhibited the Trembler effect (Fig. 11). We suggest that laminin is 'the neurite-inducing factor' that acts both on neuritic emergence from the perikaryon (proximal sprouting) and distal sprouting, while HSPG could be the factor acting only on growth of neuron processes.

Davis *et al.*[3] have reported that laminin may control the number of neurites emerging from ciliary ganglion neurons. The results obtained after addition of laminin to Trembler medium are consistent with this observation. In Trembler medium containing laminin, the number of neurites emerging from the perikaryon, as well as the branching of the elongated neurites, increased by 30–50%. In contrast, when HSPG was added to Trembler medium, neurite growth was modified by elongation of the neurites and inhibition of distal sprouting (Fig. 12). The antigens HSPG and laminin had the same effect on neuritic growth in standard medium containing 10% horse serum.[6]

Laminin and HSPG could modulate cellular and neuritic sprouting mediated by specific membrane receptors distributed differently around the periphery of the perikaryon and along the length of the neuritic outgrowths. It has been shown recently that the receptor protein for laminin is an 'integrin-related glycoprotein' and may be responsible for neuronal interactions with laminin.[19]

Two receptor systems on astrocytes surfaces that function in neuronal process outgrowth have been identified. One of the receptors, B1 integrin, binds with several ECM proteins including laminin, fibronectin and type IV collagen. The second receptor is a 'ligand' for N-cadherin, an adhesive protein. These results suggest that the adhesive interactions with glial cells may be important for neuronal process extension.[20]

The fact that the Trembler effect is inhibited when the number of non-neuronal cells is reduced by the addition of an antimitotic agent (Table 1) suggests that factor(s) present in serum could act directly on non-neuronal cells. These factors might induce replication of non-neuronal cells which could in turn synthesize and release HSPG- and laminin-like molecules. We have observed that Trembler serum promotes 2-fold proliferation of normal Schwann cells '*in vitro*', 24 hr after addition of serum (N. A. Do Thi, unpublished results). We have now performed experiments to investigate whether conditioned medium of normal Schwann cells cultured in Trembler medium contains basal lamina component-like molecules which act as neurite growth-promoting factors for spinal cord neurons.

In vivo laminin and HSPG synthesized and deposited by Trembler Schwann cells could promote terminal budding of the motor axon in the mutant muscle. The very high concentrations of laminin and HSPG detected by immunofluorescence in the basal lamina of mutant Schwann cells compared to control values (F. Mellouk, unpublished results) could explain the extensive growth (terminal branching) of motor axon endings.[9] Moreover, it is possible that in the Trembler muscle, Schwann cell extensions accompany terminal sprouting as Duchen has described in muscles treated with Botulinum toxin.[4]

Under these conditions, the basal lamina of the Schwann cell would be tightly juxtaposed to the growing axon, thus allowing intimate interactions which could modulate neurite growth. The extensive growth of nerve terminals is observed only in the soleus muscle of the mutant in which supernumerary synapses have been described.[9]

In conclusion, the results obtained from cultures of spinal cord neurons grown in Trembler medium allow us to hypothesize that '*in vivo*' dysfunction of Schwann cells results in increased synthesis and accumulations of certain components of the basal lamina, which may cause or participate in the pronounced expansion of nerve terminals found at Trembler muscle synapses. We can also suggest that in adult Trembler mouse the mitogenic factor(s) (unpublished results) present in Trembler serum may stimulate proliferation of Schwann cells, which in Trembler mice are 10 times more numerous than in normal mice.

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