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J.M. Grondona · M. Pérez-Martín · M. Cifuentes
J. Pérez · G. Estivill-Torrús · J.M. Pérez-Fígares
P. Fernández-Llebrez · E.M. Rodríguez

Neuraminidase injected into the cerebrospinal fluid impairs the assembly of the glycoproteins secreted by the subcommissural organ preventing the formation of Reissner's fiber

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Abstract Neuraminidase was injected into the cerebrospinal fluid of normal rats to investigate the assembly and fate of the desialylated Reissner's fiber glycoproteins. It was established that a single injection of neuraminidase cleaved the sialic acid residues of the Reissner's fiber glycoproteins that had been assembled before the injection, and of the molecules that were released over a period of at least 4 h after the injection. These desialylated glycoproteins underwent an abnormal assembly that led to the formation of spheres instead of a fiber. The number of these spheres increased during the 4-h period following the injection, indicating that neuraminidase did not prevent the secretion of the Reissner's fiber glycoproteins into the cerebrospinal fluid. The spheres remained attached to the surface of the subcommissural organ and became intermingled with infiltrating cells, many of which were immunocytochemically identified as macrophages. The latter were seen to contain immunoreactive Reissner's fiber material. It is concluded that the desialylated Reissner's fiber glycoproteins forming the spheres underwent an in situ degradation by macrophages, thus resembling the normal process undergone by the Reissner's fiber glycoproteins reaching the massa caudalis.

J.M. Grondona[1] (✉) · M. Pérez-Martín[1] · M. Cifuentes
J. Pérez · G. Estivill-Torrús · P. Fernández-Llebrez
Departamento de Biología Animal, Facultad de Ciencias,
Universidad de Málaga, E-29071 Málaga, Spain

J.M. Pérez-Fígares
Departamento de Biología Celular y Genética,
Facultad de Ciencias, Universidad de Málaga,
E-29071 Málaga, Spain

E.M. Rodríguez
Instituto de Histología y Patología, Facultad de Medicina,
Universidad Austral de Chile, Valdivia, Chile

Present address:
[1] These should be considered as equal first authors

Introduction

The subcommissural organ (SCO) is an ependymal brain gland located in the roof of the third ventricle, at the entrance of the cerebral aqueduct. The SCO secretes glycoproteins into the cerebrospinal fluid (CSF), most of which aggregate and form a thread-like structure named Reissner's fiber (RF, Reissner 1860). RF grows in a caudal polar direction by addition to its rostral end of newly secreted glycoproteins. RF extends along the cerebral aqueduct, the fourth ventricle, and the central canal of the spinal cord (Rodríguez et al. 1992). The SCO secretory proteins assembling into RF are high molecular weight, core glycosylated proteins displaying glucosamine-galactose-sialic acid as the terminal sugar chain (Herrera and Rodríguez 1990; Nualart et al. 1991; Meiniel et al. 1993; Grondona et al. 1994; López-Ávalos et al. 1996). A quantitative chemical analysis (Hadge and Sterba 1973) and the use of endoglycosidase F (Nualart and Rodríguez 1996) have shown that the protein backbone of RF glycoproteins represents about 80% of the molecular mass, with the remaining 20% corresponding to the complex-type carbohydrate chain, displaying sialic acid as the terminal residue.

Rodríguez et al. (1987b, 1992) have recognized three stages of RF glycoproteins after their release into the CSF. The pre-RF stage represents the newly released proteins that become aggregated and form a film lying on the cilia of the SCO cells. After about 1 h, the glycoproteins forming the pre-RF undergo a further degree of packaging to form the RF proper. The molecular events underlying the assembly of the RF glycoproteins are unknown. The molecules forming RF "travel" along the fiber (Sterba et al. 1967) to finally arrive at the terminal dilatation of the central canal located in the filum, known as the ampulla caudalis (Olsson 1958). When reaching the ampulla caudalis, the RF ends as an irregular mass, the so-called massa caudalis (Olsson 1955; Hofer 1964; Rodríguez et al. 1987a). The hydrolytic enzymes found in this structure by histochemical methods (Naumann 1968), the strong immunoreaction of the massa caudalis

with anti-RF sera, and the ultrastructure of the massa caudalis point to a restructuring or unpacking of the glycoproteins when passing from the RF stage to the massa caudalis stage (Rodríguez et al. 1992).

After reaching the massa caudalis stage, at least some of the RF glycoproteins lose their sialic acid residues, exposing galactose as the terminal residue (S. Rodríguez et al. 1987). The RF glycoproteins continuously arrive to the ampulla caudalis but the mass of the massa caudalis remains stable, thus indicating that the mechanisms leading to the discharge of this secretory material must operate continuously. In lower vertebrates this material escapes through the opening of the dorsal wall of the ampulla caudalis to finally reach the lumen of the local blood vessels (Hofer et al. 1984; Peruzzo et al. 1987). S. Rodríguez et al. (1987) postulated that the desialylated RF glycoproteins, with galactose exposed as the terminal residue, may become degradable by macrophages located in peripheral organs. The presence of macrophages within the ampulla (Olsson 1955; S. Rodríguez et al. 1987) was taken as an indication that a local degradation of desialylated RF glycoproteins may also occur.

In the present investigation, neuraminidase was injected into the CSF of normal rats with the aim of cleaving the sialic acid residues of the glycoproteins secreted by the SCO in order to establish: (1) whether the desialylation of the newly released glycoproteins interferes with their assembly into pre-RF or RF; and (2) whether or not the newly released but desialylated RF glycoproteins become degraded, in situ, by macrophages.

Material and methods

Animals

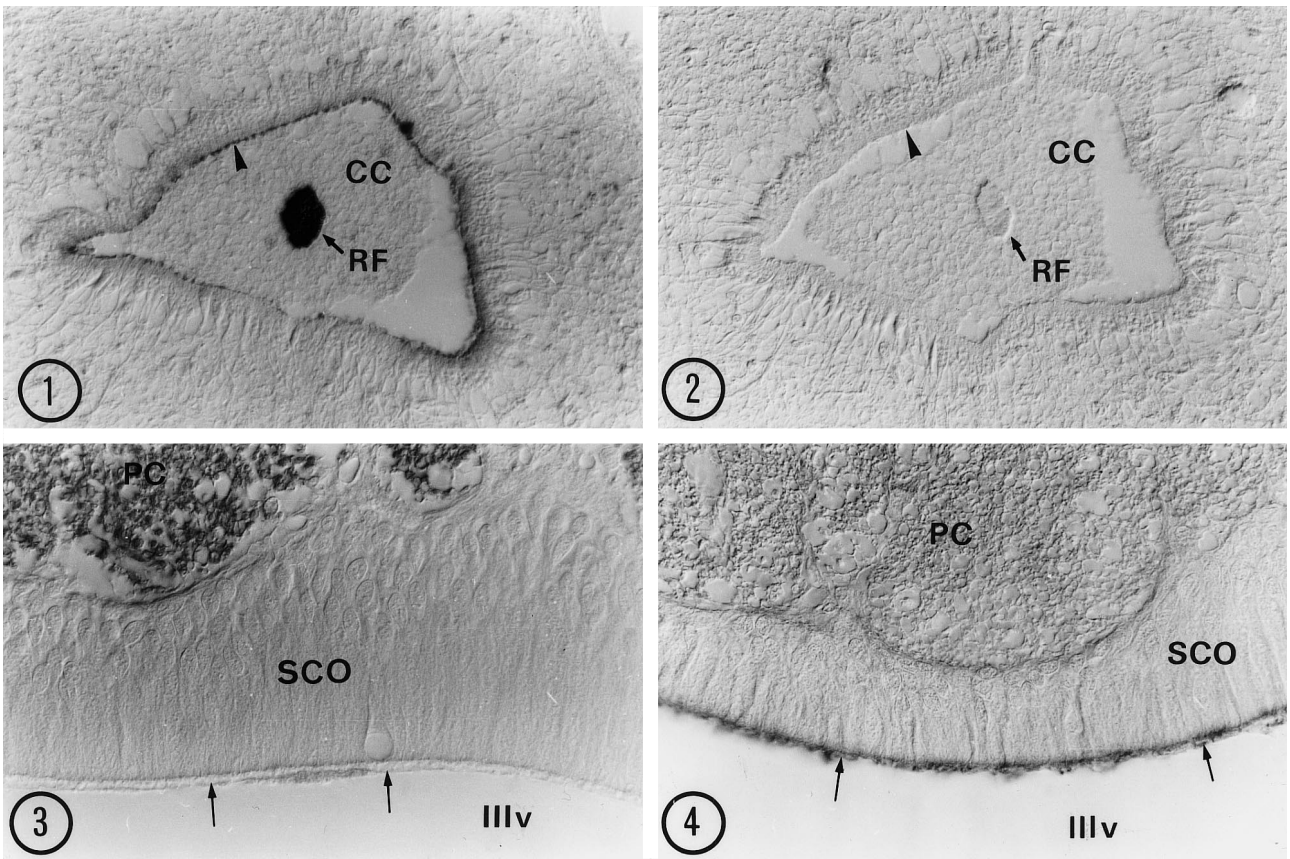
Forty-six adult Sprague Dawley rats (body weight 250–300 g) of both sexes were used. The animals were kept under a photoperiod of 12 h light: 12 h dark and a room temperature of 25°C. They were fed ad libitum with rodent food. Handling, care and processing of the control and experimental animals were carried out according to principles approved by the council of the American Physiological Society and national laws (B.O.E. 67, 1988, Spain). All animals were anesthetized with ether.

Experimental groups

Neuraminidase was injected in the right lateral ventricle using a pump, via a canula stereotaxically positioned (0.5 posterior from

Figs. 1, 2 Transverse sections through the bovine central canal (*CC*) of the spinal cord, stained with the lectin *Limas flavus* agglutinin (LFA) before (**Fig. 1**) and after (**Fig. 2**) in vitro neuraminidase treatment. Both Reissner's fiber (*RF*) and the ependymal surface (*arrowhead*) bind LFA before but not after the enzymatic treatment. ×120

Figs. 3, 4 Saggital sections through the subcommissural organ (SCO) of a control (**Fig. 3**) and a neuraminidase (10 μg) injected (**Fig. 4**) rat (t=1 h.) stained with the lectin peanut agglutinin (PNA). In the control rat, pre-RF (*arrows*) has no affinity for PNA. In the neuraminidase injected rat the disorganized pre-RF (*arrows*) strongly binds PNA. (*PC* Posterior commissure, *IIIv* third ventricle) ×180



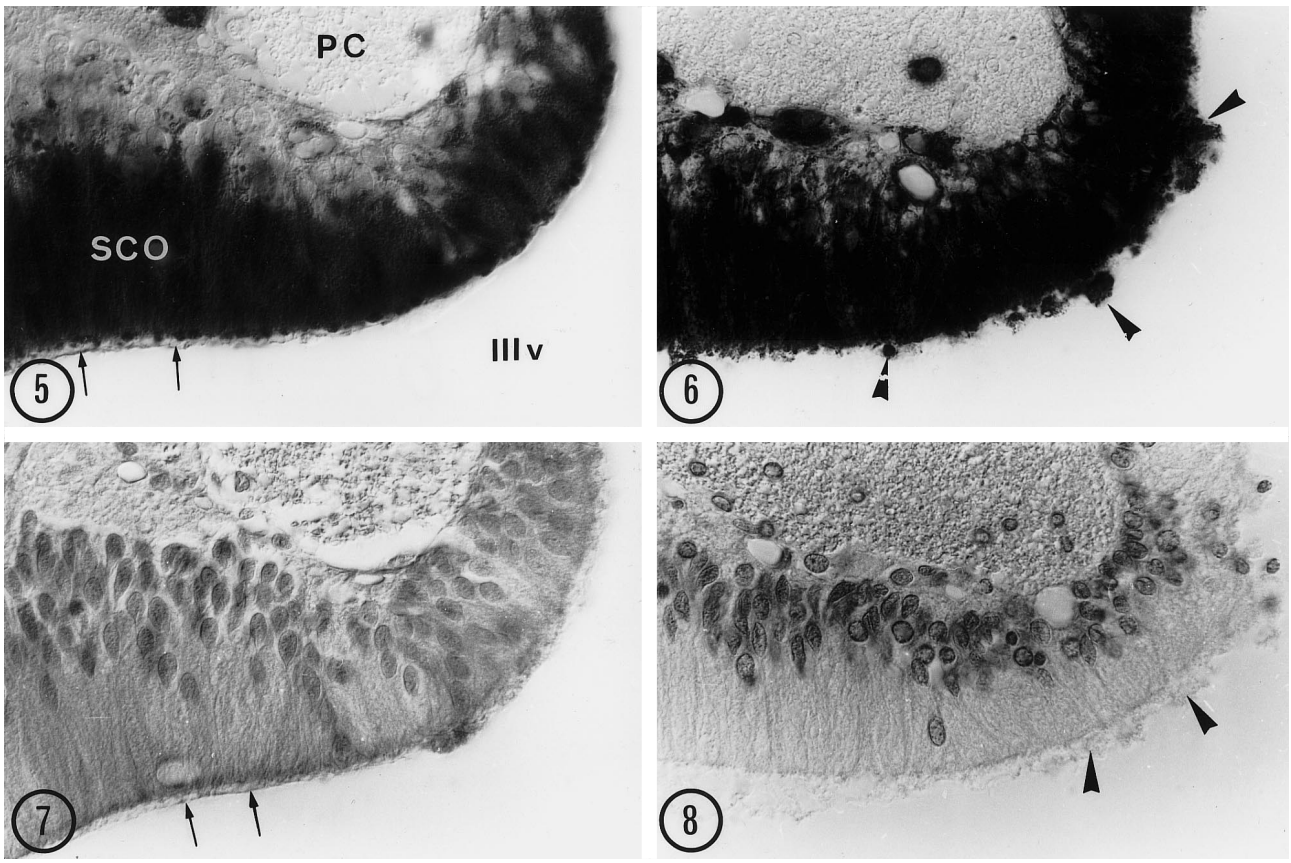
Bregma, 1.5 lateral from sagittal suture, and 3.5 mm ventral from dura). A single dose of 10 μg of neuraminidase from *Clostridium perfringens* (catalogue number 107590, 1995; Boehringer, Mannheim, Biochemica, Germany), dissolved in 20 μl distilled water, was administered to 12 rats at a rate of 2 μl/min for 10 min. These rats were killed at different time intervals after the administration of neuraminidase: immediately after the injection (t=0), 1 h, 2 h and 4 h. Three other rats were injected with 1 μg of neuraminidase and killed 30 min. later. Control rats were injected with 20 μl distilled water and killed at various postinjection intervals. Untreated normal rats were also used. The animals were transcardially perfused with 0.9% NaCl, and then with Bouin's fixative. The brains were dissected out and immersed in the same fixative for 2 days. They were dehydrated, embedded in paraffin, and cut either transversally or sagittally (10 μm thick) sections.

Histochemical and immunocytochemical procedures

Adjacent serial sections were processed by the following methods:

1. Hematoxylin-eosin.
2. The lectin *Limax flavus* agglutinin (LFA) has affinity exclusively for sialic acid (Roth et al. 1984). For LFA binding, paraffin sections were hydrated, treated with hydrogen peroxide to block endogenous peroxidase, and sequentially incubated in: (1) 7 μg/ml unlabeled LFA (Calbiochem, San Diego Calif., USA) in 0.1 M phosphate-buffered saline (PBS) pH 7.3, for 1 h at 22°C; (2) an antiserum raised in rabbits against LFA (from E.M. Rodríguez, Valdivia, Chile), diluted 1:5000 in 0.1 M TRIS buffer, pH 7.8, containing 0.7% lambda carrageenan (Sigma, Madrid, Spain) and 0.3% Triton X-100 (Sigma) (TCT), for 18 h, at 22°C; (3) anti-rabbit IgG developed in goat (from our laboratory), diluted 1:50 in TCT for 30 min at 22°C; (4) rabbit PAP (Sigma), diluted 1:200 in TCT, for 30 min at 22°C. To reveal peroxidase, 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) was used as electron donor.
3. The lectin peanut (*Arachis hypogaea*) agglutinin (PNA) has affinity for terminal residues of galactose. Galactose is the subterminal sugar residue in complex-type glycoproteins (Sharon and Lis 1982). The removal of sialic acid residues from SCO glycoproteins leaves galactose as the terminal residue, which, in turn, results in the binding of PNA (Herrera and Rodríguez 1990). The sections were incubated in 4 μg/ml peroxidase-labeled PNA (Sigma) in 0.1 M PBS pH 7.3 for 1 h at 22°C. Peroxidase activity was demonstrated as described above.
4. The lectin concanavalin A (Con A) has affinity for terminal residues of mannose and glucose. If the sialic acid residue is removed from complex-type glycoproteins of the SCO, then Con A expresses its affinity for internal mannosyl residues (Herrera and Rodríguez 1990). Binding of peroxidase-labeled Con A (Sigma) was done as described for PNA. The working concentration was 5 μg/ml.
5. The secretory material of the SCO and RF was revealed by immunocytochemistry, using the immunoperoxidase method of Sternberger et al. (1970). An antiserum raised in rabbits against the constitutive glycoproteins of the bovine RF (AFRU; Rodríguez et al. 1984) was used at a 1:1000 dilution as the primary antibody. The immunocytochemistry procedure was the same as that used for anti-LFA (see above).
6. Immunocompetent cells producing IgG were revealed in paraffin sections by the immunoperoxidase method using anti-rat IgG as primary antibody.

Figs. 5–8 Sagittal sections of control rats (**Figs. 5, 7**), and of neuraminidase (10 μg) injected rats at t=0 (**Figs. 6, 8**), stained with hematoxylin-eosin (**Figs. 7, 8**) and immunostained with an antiserum against RF glycoproteins (AFRU) (**Figs. 5, 6**). Pre-RF (*arrows*) was present in the control rat, and missing in the neuraminidase injected rat, which instead showed disorganized fibrous material and irregular immunoreactive spheres (*arrowheads*). (*PC* Posterior commissure, *SCO* subcommissural organ, *IIIv* third ventricle) ×180



7. Intraventricular macrophages were identified by using three monoclonal antibodies: OX42 (Sera-Lab (Loughborough, England (U.K.); MAS370), OX18 (Sera-Lab; 101b), and OX6 (Sera-Lab; MAS043b). These antibodies, respectively, recognize the complement type 3 receptor and major histocompatibility complex class I and class II antigens, present in intraventricular macrophages and in suprachoroidal macrophages (Lu et al. 1994, 1996). Two neuraminidase injected rats (10 μg, t=4 h) were used to test the presence of macrophages. Frozen sections (10-μm-thick) were subsequently fixed either with acetone or periodate-lysine-paraformaldehyde. Working dilution (OX42, OX18, and OX6) was 1:20. The secondary antibody was an anti-mouse IgG (raised in our laboratory; dilution 1:50). Immunostaining procedures were performed as described above.

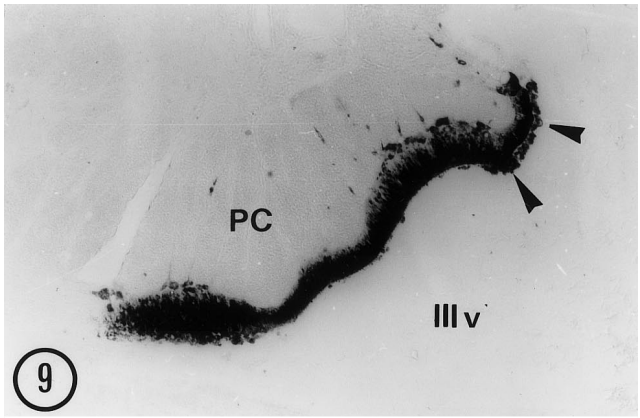
In vitro and in vivo control of neuraminidase activity

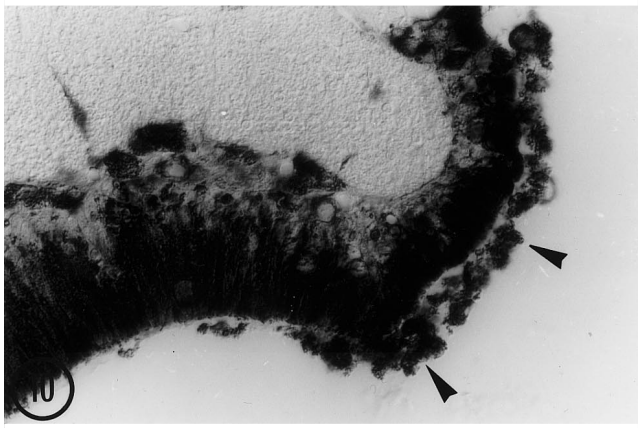
To test the in vitro enzymatic activity of the batch of neuraminidase used to inject the rats, paraffin sections of bovine spinal cord containing RF were hydrated and incubated with the enzyme, 0.3 mg/ml in 0.1 M sodium acetate buffer, pH 5.6, containing 0.004 M calcium chloride, for 18 h, at 37°C. Digested and undigested sections were processed for LFA and PNA binding. The enzymatic activity of neuraminidase was tested in vivo by PNA binding to complex-type glycoproteins present in SCO sections from neuraminidase injected rats (see above). Moreover, we have evaluated in vivo neuraminidase activity by using explants from bovine lateral ventricle walls. These explants contain ependymal cells which have a glycocalix rich in sialic acid. Explants were incubated both in cultured medium (RPMI 1640 medium; Sigma) and in bovine CSF. After incubation, explants were processed for lectin histochemistry. In both cases, treatment of the explants with neuraminidase cleaved terminal sialic acid, leaving galactose as the terminal sugar residue. For negative controls, we perfused rats with inactivated neuraminidase (both by heating and by incubating with trypan blue) with no effects on RF integrity.

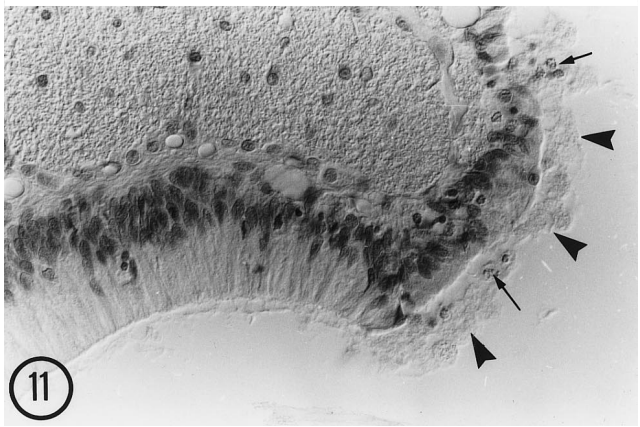
Results

In vitro and in vivo analysis of the enzymatic activity of neuraminidase

In order to test neuraminidase activity on RF glycoproteins, sections of the bovine spinal cord containing RF were incubated with neuraminidase and subsequently processed for LFA (sialic acid affinity) and PNA (galactose affinity) binding. In untreated sections, RF strongly bound LFA (Fig. 1) while PNA binding was very weak (data not shown). After neuraminidase treatment, RF lost its affinity for LFA (Fig. 2) and increased its affinity for PNA (not shown), indicating that neuraminidase had cleaved sialic acid from the RF glycoproteins, exposing galactose. Neuraminidase activity was studied in vivo by LFA and PNA binding on SCO sections from control and neuraminidase injected rats. The pre-RF of control rats, seen on the surface of the SCO (see below), did not bind PNA (Fig. 3) but it bound LFA (not shown). By contrast, the disorganized pre-RF of neuraminidase-injected rats was PNA positive (Fig. 4), while LFA affinity was greatly decreased (not shown). Rat RF showed the same behavior as the pre-RF (data not shown).







Figs. 9–11 Sagittal sections through the SCO of a rat injected with neuraminidase (10 μg) and killed 1 h after injection. Immunostaining with AFRU revealed extracellular secretory material, mostly in the form of irregular spheres (**Figs. 9, 10**) (*arrowheads*). Hematoxylin-eosin staining of an adjacent section showed very few cellular components **Fig. 11** (*arrows*) among the eosin-positive material (*arrowheads*). (*PC* Posterior commissure, *IIIv* third ventricle) **Fig. 9** ×30, **Figs. 10, 11** ×120

Effects of neuraminidase on pre-RF and RF

In the SCO of normal control rats (Figs. 5, 7) the secretory material released into the CSF first condenses, forming a thin film on top of the ependymal microvilli and cilia of the SCO (Figs. 5, 7). This film has been regarded as pre-RF (Rodríguez et al. 1986, 1987b). RF

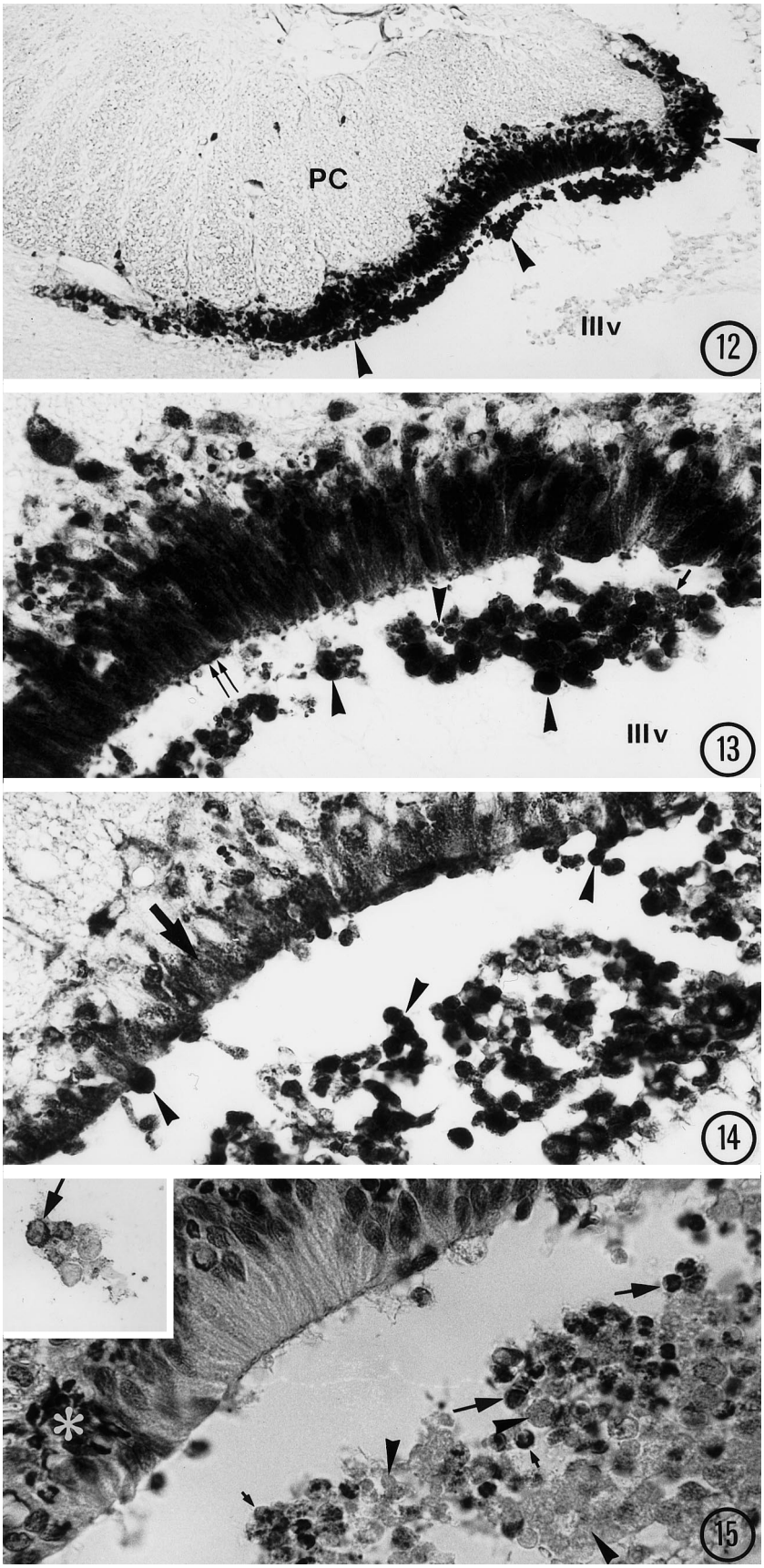


Fig. 12–15 Sagittal sections through the SCO of a rat killed 4 h after neuraminidase (10 µg) injection. AFRU immunostaining (**Figs. 12, 13**). Abundant immunoreactive spheres of different sizes appear near the surface of the SCO (*arrowheads*). *Two arrows* point to an incipient immunoreactive sphere on the surface of the SCO. The *small arrow* points to immunoreactive material in the cytoplasm of an infiltrating cell. Concanavalin A (Con-A) binding (**Fig. 14**). All the spheres of secretory material (*arrowheads*) display Con A affinity. *Arrow* points to a weakly labeled SCO cell. Hematoxylin-eosin staining (**Fig. 15**). Numerous cellular elements, most of which are macrophages (*large arrows*) and neutrophils (*small arrows*) appear intermingled with the secretory spheres (*arrowheads*) that are weakly stained with eosin. *Inset* shows immunoreactive cells to OX 42; note that adjacent cells are immunonegative. The *asterisk* indicates a focus of infiltrating cells between SCO ependymal cells. (*PC* Posterior commissure, *IIIv* third ventricle) **Fig. 12** ×145, **Figs. 13, 14** ×525, **Fig. 15** ×575

forms by the confluence of pre-RF filaments. Both pre-RF and RF react with AFRU (Fig. 5).

All rats injected with 1 and 10 μg neuraminidase survived; the latter suffered damage of the ciliated ependyma (see Grondona et al. 1996). In the rats injected with 1 μg neuraminidase, pre-RF and RF were present. However, the RF present in the cerebral aqueduct had lost the appearance of a highly condensed structure; it also presented variations in its thickness (data not shown). In the fourth ventricle, the RF material appeared as irregular masses lying on the ventricular floor (data not shown).

The pre-RF and the RF of the rats injected with 10 μg neuraminidase underwent important changes. At t=0 the film of pre-RF material had disappeared; in its place, masses of an irregular shape and size appeared. These masses were weakly stained with eosin (Fig. 8) and were strongly reactive with AFRU (Fig. 6). The RF detached from the SCO and appeared in the form of irregular masses along the fourth ventricle (data not shown). The SCO ependymal cells proper were not affected at t=0.

One hour after the injection of 10 μg neuraminidase, the amount of the immunoreactive masses increased, especially in the vicinity of the cephalic end of the SCO (Figs. 9, 10). These masses bound PNA and Con A (not shown). No or few cellular elements, similar to those described in the 4-h group (see below), were observed in the third ventricle, close to the immunoreactive masses (Fig. 11). The SCO ependymal cells showed a normal appearance. The detached RF continued to appear as irregular masses in the fourth ventricle (not shown).

Four hours after the injection of 10 μg neuraminidase, the immunoreactive masses became spherical, with a diameter ranging between 2 and 8 μm (Fig. 13). They were numerous and distributed throughout the surface of the SCO (Fig. 12). These spheres bound PNA (not shown) and Con A (Fig. 14). No RF material was seen in the aqueduct and fourth ventricle (not shown). Numerous cells infiltrated the meninges, the brain ventricles, the subependymal neuropil, and some circumscribed areas of the ependyma of the SCO. The infiltrating cells were especially abundant in the vicinity of the luminal surface of the SCO (Fig. 15), where they intermingled with the AFRU immunoreactive spheres (Fig. 15). Some of the infiltrated cells contained AFRU immunoreactive material in their cytoplasm (barely seen in Fig. 13). Most of these cells were identified as neutrophils by their nuclear morphology. Some appeared to be macrophages since they labeled with the three monoclonal antibodies, OX42 (see inset in Fig. 15), OX18, and OX6 (data not shown), used as macrophage markers (Lu et al. 1994, 1996). There were a few cells resembling lymphocytes; they might correspond to T lymphocytes. B lymphocytes or plasma cells were absent since all cells were negative for the anti-rat IgG serum.

Discussion

According to the experience of the authors, there are variations between different batches of a neuraminidase with the same label, or between enzyme preparations of different sources, with respect to their specificity and efficiency in cleaving sialic acid residues. The in vivo and in vitro analyses of the enzymatic activity of the neuraminidase used demonstrated that this preparation did cleave sialic acid residues without affecting the subterminal galactose residue. Both properties were essential for the aims of the present investigation.

The ventricular system of the rat contains about 150 μl of CSF; this CSF volume is completely renewed 4–5 times a day (Davson and Segal 1996). Therefore, a single injection of a compound into the rat ventricular CSF would result in a maximal CSF concentration of such a compound at t=0; as the CSF is progressively renewed, the concentration of the injected compound should progressively decrease, fully disappearing 4–5 h after the injection. This dynamic phenomenon must be kept in mind when interpreting the results of the present investigation. Similarly relevant is the dynamic of the secretory material of the SCO after its release into the CSF. Upon release, the glycoproteins aggregate into fibrils that form a network on top of the microvilli and cilia of the SCO, which at the light microscopic level appears as a film covering the surface of the SCO, designated pre-RF (Rodríguez et al. 1987a, 1992). The newly released glycoproteins stay at the pre-RF "station" for between one and several hours (Herrera 1988; S. Rodríguez et al. 1990; Rodríguez et al. 1992). Then, the pre-RF fibrils undergo a further degree of packaging to form the RF proper (Sterba et al. 1967; Rodríguez et al. 1992). Once in the RF, the glycoproteins move along the fiber at a fixed rate, which varies with the species. In the mouse, a molecule of glycoprotein would take about 10 days to move from the rostral to the caudal end of the RF (Ermisch 1973); in the rat, this molecular trip would take about 12 days (Herrera 1988). This means that in the experimental studies used in the present investigation (up to 4 h), the glycoproteins released by the SCO after the injection of neuraminidase would be in the pre-RF and in a very short portion of the rostral RF.

The changes observed 30 min after the injection of 1 μg neuraminidase indicate that: (1) the enzyme can cleave sialic acid residues from RF glycoproteins that have already achieved their highest degree of assembly, that is, the RF stage; and (2) the presence of a pre-RF suggests that the dose of 1 μg neuraminidase is not enough to cleave the sialic acid residues from all the released RF glycoproteins. This could explain why this low dose, in contrast to the 10 μg dose, results in the partial unpacking of the RF glycoproteins already assembled, but is not followed either by the dissolution of the RF or by its detachment from the SCO.

The effects produced by the dose of 10 μg neuraminidase observed at postinjection intervals between t=0 and 4 h lead to the following suggestions:

1. The disappearance of pre-RF at t=0 indicates that this high dose of neuraminidase results in the loss of sialic acid residues of most or all RF glycoproteins that had been secreted before the injection; this in turn would

lead to the disarrangement of the network of fibrils forming pre-RF, and to the detachment of the RF.
2. The appearance at t=0 of AFRU immunoreactive masses on the surface of the SCO must be the result of an abnormal packaging of the desialylated RF glycoproteins that were forming the pre-RF before the injection.
3. The fact that the AFRU immunoreactive masses increased in number 1 and 4 h after the injection indicates, firstly the neuraminidase present in the CSF does not prevent the secretion of RF glycoproteins by the SCO and, secondly, that the newly released glycoproteins would also become desialylated and abnormally packed into masses. A general conclusion would be that the desialylated RF glycoproteins continue to assemble, but in such a way that the assembled molecules form individual masses or spheres instead of a single RF.
4. It is tempting to correlate the transformation of the irregular masses into distinct spheres, observed 4 h after the injection, with a higher degree of aggregation of the desialylated RF glycoproteins, resembling the "transformation" of pre-RF into RF described for the normal rat.

Which mechanism underlies the formation of spheres of different diameters and what is causing their retention in the vicinity of the ventricular surface of the SCO, remain open questions.

The injection of neuraminidase into the ventricular CSF leads to several changes in the CNS, including the invasion of the ventricular cavities by white blood cells (Grondona et al. 1996). These cells, especially macrophages and neutrophils, become numerous in the vicinity of the SCO and intermingle with the AFRU immunoreactive spheres. It is known that sialoglycoproteins become degradable after the loss of their sialic acid residues (Durocher et al. 1975; Lefort et al. 1984). They, thus, become available to macrophages as galactose remains as the terminal sugar residue (Kawasaki et al. 1986; Li et al. 1988, 1990). The fact that the RF glycoproteins forming the spheres lack sialic acid residues and display galactose as the terminal residue, and the occasional presence within the neighboring macrophages of AFRU immunoreactive material, strongly suggests that the desialylated RF glycoproteins forming the spheres undergo an in situ degradation by macrophages. This would resemble the normal process undergone by the RF glycoproteins reaching the massa caudalis. Indeed, lectin histochemical and ultrastructural immunocytochemical studies have shown that, when reaching the massa caudalis stage, the RF glycoproteins lose their sialic acid residues exposing galactose as the terminal residue, before reaching the local capillaries (Peruzzo et al. 1987; S. Rodríguez et al. 1987). Thus, in the ampulla caudalis, sialidases should occur naturally. S. Rodríguez et al. (1987) postulated that the desialylated RF glycoproteins reaching the bloodstream, with galactose exposed as the terminal residue, would become degradable by macrophages located in peripheral organs. The presence of macrophages within the ampulla (Olsson 1955; S. Rodríguez et al. 1987) was taken as an indication that a local degradation of desialylated RF glycoproteins may also take place. The present findings strongly support the view that the desialylated RF glycoproteins, wherever they are, become degradable by macrophages, a cell type known to display receptors for terminal galactose residues. Experimental RF degradation after neuraminidase injection and natural massa caudalis decomposition may not be identical events since unknown additional enzymes might be implicated in the degradation of the massa caudalis, while only neuraminidase is considered to be acting in this experimental work. This could explain the absence of spheres in the naturally desialylated massa caudalis.

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