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The Perineuronal Proteoglycan Surface Coat in the Adult Rat Brain, with Special Reference to its Reactions to Gömöri's Ammoniacal Silver

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The present study showed that many neurons in the adult rat brain possessed a perineuronal sulfated proteoglycan surface coat which reacted to cationic iron colloid and aldehyde fuchsin. This surface coat was stained supravitally with Ehrlich's methylene blue and doubly stained with Ehrlich's methylene blue and aldehyde fuchsin. The surface coat was also stained with Gömöri's ammoniacal silver and doubly stained with Gömöri's ammoniacal silver and cationic iron colloid. The surface coat was usually expressed together with a nerve cell surface glycoprotein net detectable with lectin *Wisteria floribunda* agglutinin. These findings indicate that the perineuronal proteoglycan surface coat is identical to Cajal's superficial reticulum and contains some collagenous elements. It was further demonstrated that collagenase digestion erased Gömöri's ammoniacal silver impregnation within the perineuronal proteoglycan surface coat.

Key words: brain, extracellular matrix, perineuronal proteoglycans, cell surface glycoproteins

Our previous histochemical and electron microscopic studies showed that many neurons in the central nervous systems of humans, dogs, cats, rats, mice and other animals possess a marked perineuronal sulfated proteoglycan surface coat which is stained with cationic iron colloid and aldehyde fuchsin (1-16). Additional experiments (8-10, 17) also showed that the surface coat reacts to Golgi's or Kopsch's silver nitrate and is identical to Golgi's reticular coating (18, 19). Recently, we also demonstrated that the perineuronal proteoglycan surface coat in the mouse brain is stained supravitally with Ehrlich's methylene blue, impregnated with Gömöri's ammoniacal silver and digested by collagenase (20, 21).

The present study reinvestigated the adult rat brain and confirmed our recent findings concerning the mouse brain. The retrosplenial cortex formed the material of the present study, as it contains many neurons with well developed perineuronal proteoglycan surface coats (7, 8, 12, 13, 20, 21).

Materials and Methods

Adult male Wistar rats weighing about 250 g were anesthetized with ethyl ether. After ligation of their thoracic aorta, they were treated as follows.

Staining with cationic iron colloid, ammoniacal silver, aldehyde fuchsin or lectin Wisteria floribunda agglutinin. The animals were perfused through the ascending aorta with 15 ml of Ringer's solution and with 15 ml of 4% paraformaldehyde in a 0.1 M cacodylate buffer (pH 7.2). Each brain was then isolated, and 1-2 mm-thick blocks traversing the retrosplenial cortex were prepared with a vibratome. These blocks were refixed in the 4% paraformaldehyde fixative, embedded in paraffin, and cut into 10-15 μm-thick sections.

The sections were deparaffinized with xylene and stained with our fine cationic iron colloid with pH values of 1.0-1.5 (22), Gömöri's ammoniacal silver (23, 24), Fujita's aldehyde fuchsin (25), or lectin *Wisteria floribunda* agglutinin (26). They were then embedded in balsam.

Cationic iron colloid stainability was demonstrated by a Prussian blue reaction. In the lectin labeling, peroxidase activity was demonstrated with diaminobenzidine. Counter-staining with nuclear fast red or Mayer's hematoxylin was sometimes done prior to the balsam-

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embedding. Controls for lectin-labeled sections consisted of adjacent sections treated with a phosphate buffer containing no agglutinin.

Double staining with ammoniacal silver and cationic iron colloid. Some sections stained with Gömöri's ammoniacal silver were successively stained with fine cationic iron colloid (pH 1.0–1.5) (20). They were then counter-stained with nuclear fast red and embedded in balsam.

Supravital staining with methylene blue. The animals were perfused through the ascending aorta with 5 ml of Ringer's solution, 15 ml of 0.25% Ehrlich's methylene blue in 0.9% NaCl, and 10 ml of saturated ammonium picrate (27). The brains were then isolated, and 1–2 mm-thick blocks traversing the retrosplenial cortex were prepared with a vibratome. These blocks were frozen and cut into 15–20 μm-thick sections and embedded in glycerol.

Double staining with methylene blue and aldehyde fuchsin, or methylene blue and lectin Wisteria floribunda agglutinin. Some sections from the supravitally methylene blue-stained (methylene blue/ammonium picrate-treated) samples were refixed with 4% paraformaldehyde in a 0.1 M cacodylate buffer (pH 7.2) for 10 min, stained with Fujita's aldehyde fuchsin or lectin *Wisteria floribunda* agglutinin, and embedded in balsam (20). Counter-staining with nuclear fast red was sometimes done prior to balsam-embedding.

Collagenase digestion. Some sections were rinsed with distilled water and incubated with 40 U/ml collagenase (*Cl. histolyticum*) (Elastin Products Co., MO, USA) in distilled water for 60 min at 37 °C (28). These sections were impregnated with Gömöri's ammoniacal silver (21). Control sections were incubated in distilled water containing no enzyme and stained with ammoniacal silver.

Light microscopy. All sections prepared as described above were observed with a transmission light microscope (Olympus BX 50 or BH2).

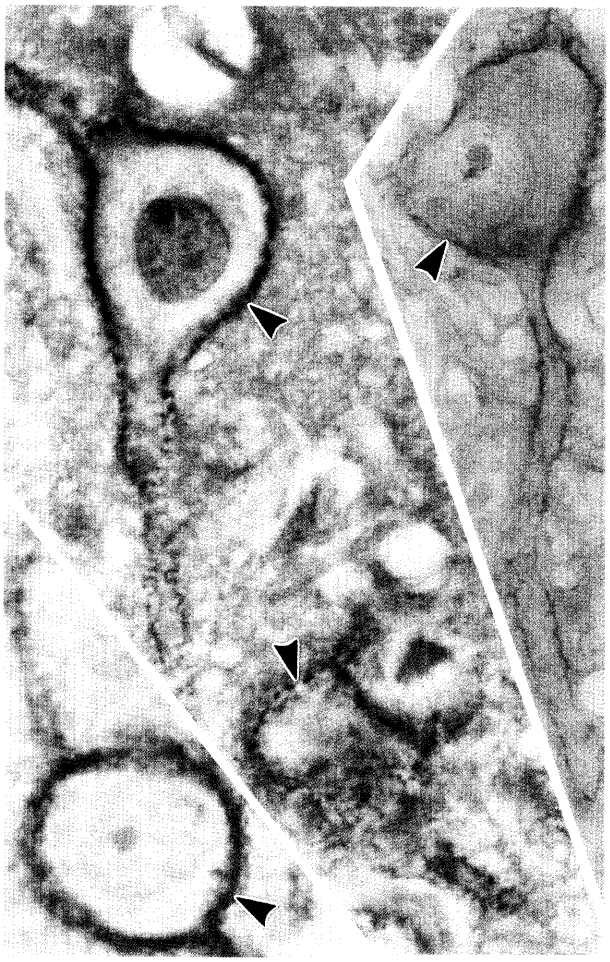

Fig. 1 Retrosplenial cortex of an adult rat brain stained with cationic iron colloid (pH 1.5), treated for a Prussian blue reaction, and counter-stained with nuclear fast red. A perineuronal proteoglycan surface coat is demonstrated by a Prussian blue reaction (single arrowhead). **Upper Inset** shows a retrosplenial section stained with aldehyde fuchsin and nuclear fast red. The core protein of the perineuronal proteoglycan surface coat reacts to aldehyde fuchsin (single arrowhead). **Lower Inset** shows a retrosplenial section of an adult rat brain treated with lectin *Wisteria floribunda* agglutinin. Some neurons express cell surface glycoproteins with terminal *N*-acetylgalactosamine reactive to lectin *Wisteria floribunda* agglutinin (single arrowhead). × 1,200; Insets, × 1,200.

Results

Staining with cationic iron colloid, ammoniacal silver, aldehyde fuchsin or lectin Wisteria floribunda agglutinin. Many neurons (15–20% of the neurons) in the retrosplenial cortex possessed a surface coat which reacted to the cationic iron colloid (Fig. 1), Gömöri's ammoniacal silver (Fig. 2), Fujita's aldehyde fuchsin (Fig. 1 Upper Inset), or lectin *Wisteria floribunda* agglutinin (Fig. 1 Lower Inset). In each case, the surface coat appeared as a fine meshwork surrounding the nerve cell bodies and their main processes (Figs. 1, 2). In the control sections for lectin labeling, no neurons were labeled with lectin.

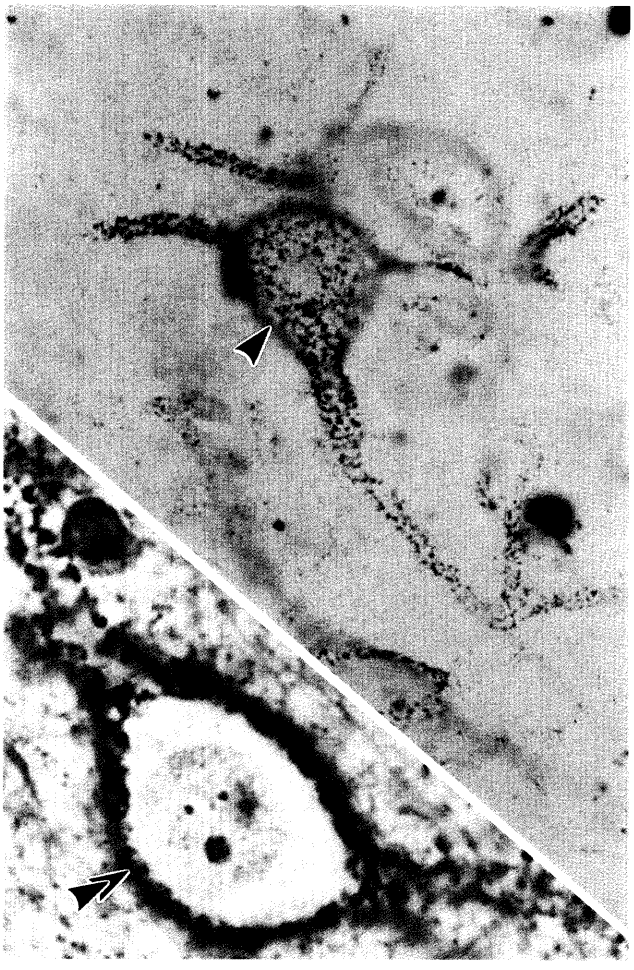

Fig. 2 Retrosplenial cortex of an adult rat brain stained with Gömöri's ammoniacal silver. The perineuronal proteolgycan surface coat contains some collagenous elements reactive to ammoniacal silver (single arrowhead). **Inset** shows a retrosplenial section treated with Gömöri's ammoniacal silver and cationic iron colloid. The perineuronal proteoglycan surface coat is doubly stained with ammoniacal silver and cationic iron colloid (double arrowheads). × 1,200; Inset, × 900.

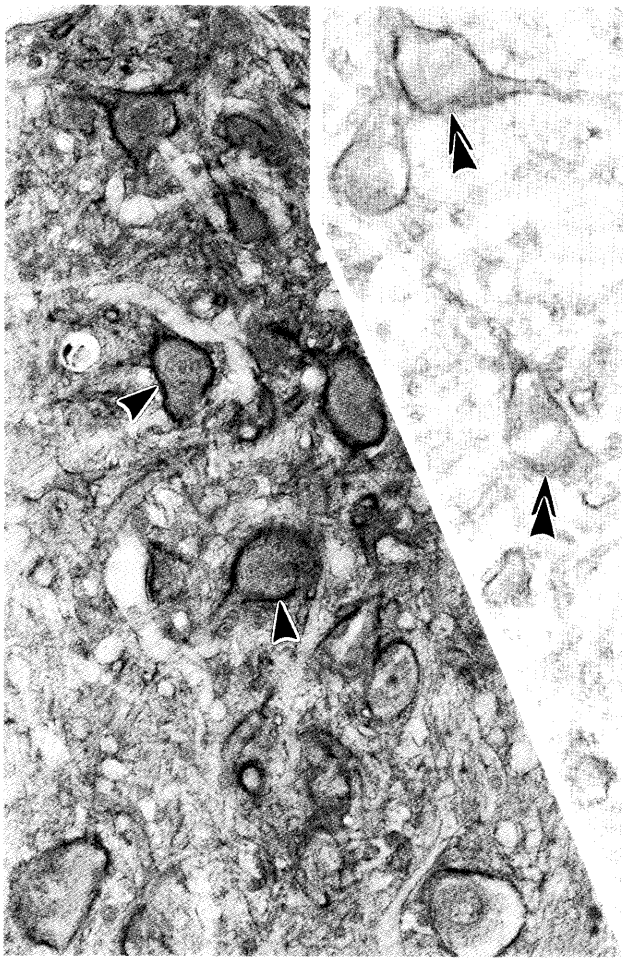

Fig. 3 Retrosplenial cortex supravitally stained with Ehrlich's methylene blue. The surface coat is stained supravitally with methylene blue (single arrowhead). **Inset** shows the retrosplenial cortex successively stained with Ehrlich's methylene blue and aldehyde fuchsin. The surface coat is stained doubly with methylene blue and aldehyde fuchsin (double arrowheads). × 500; Inset, × 500.

Double staining with ammoniacal silver and cationic iron colloid. In the retrosplenial cortex, neurons stained with ammoniacal silver always reacted to the cationic iron colloid. More precisely, the surface coat was constantly stained with ammoniacal silver and cationic iron colloid (Fig. 2 Inset).

Supravital staining with methylene blue. Supravital methylene blue staining by vascular perfusion preferentially stained the nerve cell surfaces (Fig. 3). Thus, 10–15% of the neurons in the retrosplenial cortex possessed a surface coat which reacted to methylene blue. The surface coat thus stained with methylene blue showed a meshwork structure surrounding the cell bodies and main processes of the nerve cells (Fig. 3).

Neither perivascular reticular fibers nor glial cell nuclei reacted to methylene blue. Elastic fibers around the arterial vessels also showed no reaction to methylene blue.

Double staining with methylene blue and

aldehyde fuchsin. In the retrosplenial cortex, the neurons supravitally stained with methylene blue always reacted to aldehyde fuchsin (Fig. 3 Inset). More precisely, the surface coat was constantly doubly stained with methylene blue and aldehyde fuchsin.

Double staining with methylene blue and lectin Wisteria floribunda agglutinin. The neurons supravitally stained with methylene blue were not always labeled with lectin *Wisteria floribunda* agglutinin (Fig. 4); 5% of the neurons stained with methylene blue were not labeled with lectin *Wisteria floribunda* agglutinin, and 5% of the neurons labeled with this lectin were not stained with methylene blue.

Collagenase digestion. In the collagenase-treated sections, the perineuronal surface coat was not stained with Gömöri's ammoniacal silver (Fig. 5A). In the control sections, the perineuronal surface was well stained with ammoniacal silver (Fig. 5B).

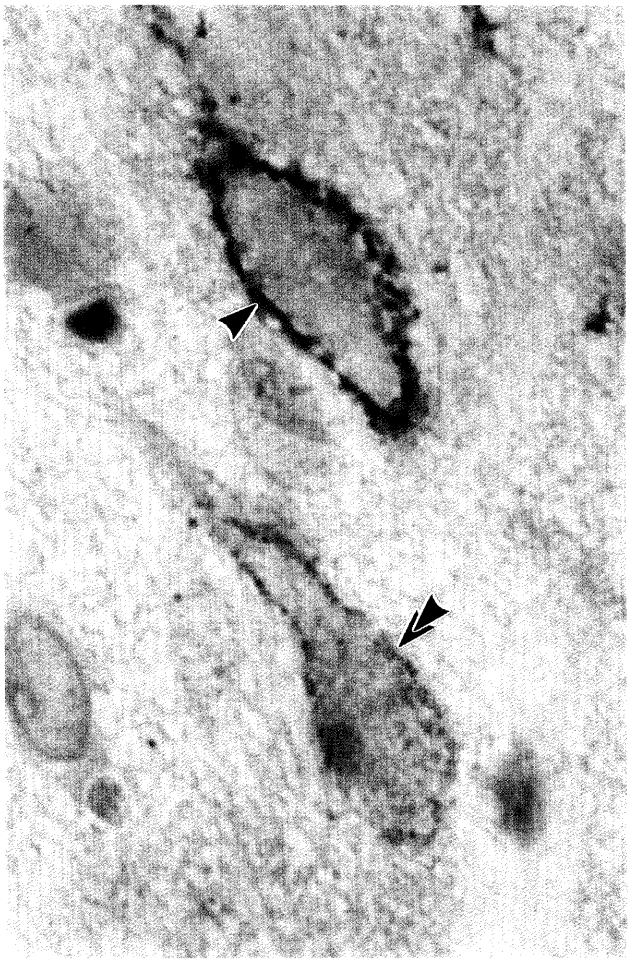

Fig. 4 Retrosplenial cortex successively stained with methylene blue, lectin *Wisteria floribunda* agglutinin and nuclear fast red. The surface coat is usually stained doubly with methylene blue and lectin agglutinin (double arrowheads). In some nerve cells, the surface coat is stained only with methylene blue (single arrowhead) or lectin agglutinin. × 1,000.

Discussion

Recent histochemical, immunohistochemical and biochemical studies of humans and various animals, including mice, have shown that mature as well as immature central nervous tissues express many proteoglycans and that some of these proteoglycans form an extracellular matrix to cover certain neuron subsets (26, 29–43).

The present study, together with our previous studies of humans, dogs, cats, mice and other animals, including lower vertebrates such as reptiles and fish (5–7, 9, 12, 14–16, 20), confirmed that such perineuronal proteoglycan surface coats are stained with cationic iron colloid and aldehyde fuchsin and that cationic iron colloid and aldehyde fuchsin stain the sulfate groups and core proteins of the proteoglycans, respectively. Distribution of the neurons with this proteoglycan surface coat in the mouse brain and spinal cord was compared with that of the neurons whose cell surfaces were labeled with lectin *Wisteria floribunda* agglutinin (5–8, 12, 14–16).

Our previous studies of human, rat and mouse brain samples (8–10, 17) showed that the perineuronal proteoglycan surface coat is usually stained with Golgi's or Kopsch's silver nitrate and is identical to Golgi's reticular coating (18, 19). Our previous studies of human, rat and mouse brain samples showed furthermore that the silver staining of the surface coat is erased by hyaluronidase digestion and not by chondroitinase ABC/keratanase/heparitinase digestions, indicating that Golgi's or Kopsch's silver nitrate stains the core proteins of proteoglycans (8, 9, 17).

The present study together with our previous studies of the mouse brain (20, 21) showed that the perineuronal proteoglycan is stained with Gömöri's ammoniacal silver and that it can be stained with this ammoniacal silver and cationic iron colloid. The present study also showed that Gömöri's ammoniacal silver staining is erased by collagenase digestion. These findings indicate that the proteoglycan surface coat contains some additional collagenous elements (reactive to Gömöri's ammoniacal

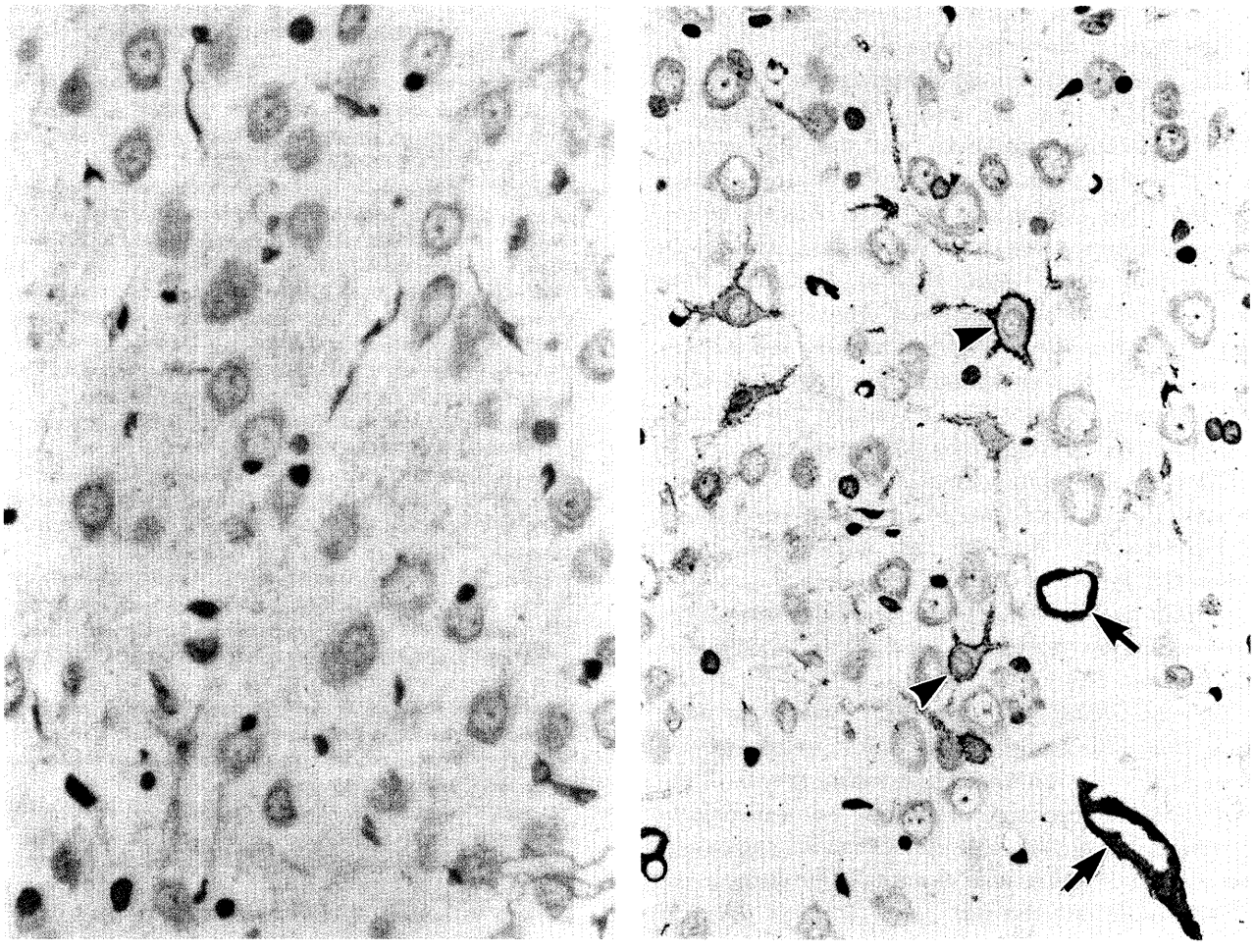

Fig. 5 A retrosplenial section incubated with collagenase and stained with Gömöri's ammoniacal silver. **B** shows a control section stained with silver without preliminary collagenase incubation. In **A**, no neurons react to Gömöri's ammoniacal silver. In contrast, **B** shows that many neurons react to the ammoniacal silver (single arrowhead). The single arrow indicates the reticular network surrounding the vascular walls. **A** and **B**, × 400.

silver) linked to the cell surfaces.

The present study showed further that the perineuronal proteoglycan surface coat is stained supravitally with Ehrlich's methylene blue and that it is stained doubly with this methylene blue and aldehyde fuchsin. These findings indicate that the perineuronal proteoglycan surface coat is identical to Cajal's superficial reticulum or red superficial (44), or réseasaux péricellulaires (45). Golgi's reticular coating and Cajal's pericellular reticulum are defined as perineuronal meshwork structures which are demonstrated by means of Golgi's or Kopsch's silver nitrate or Ehrlich's methylene blue (8, 19, 44, 45). Many researchers have stained adult rat or mouse brain sections with lectin *Vicia villosa*, soybean or *Wisteria floribunda* agglutinin and have discovered another perineuronal meshwork structure reactive to these lectins which detected the cell surface glycoproteins (26, 46–59). Distribution of the neurons with such a lectin-labeled meshwork in the rat brain and spinal cord has been compared with that of the neurons with surface coats reactive to Mowry's colloidal iron hydroxide (50, 51, 60). Brückner *et al.* (50, 51) and Blümcke *et al.* (54) showed by means of electron

microscopes that the lectin-binding sites were scattered throughout the perineuronal tissue spaces.

The present study showed that the neurons reactive to methylene blue were not always labeled with lectin *Wisterial floribunda* agglutinin and that the neurons reactive to this lectin were not always stained with methylene blue. Similar findings were obtained in our previous double staining of human, rat and mouse brain samples with lectin (lectin *Wisteria floribunda*, *Vicia villosa* or soybean agglutinin) and cationic iron colloid (5-9, 12, 14-16). We believe that the nerve cell surfaces are stained only with methylene blue or iron colloid when the cell surface glycoproteins are occupied thoroughly by the perineuronal proteoglycans; that the cell surfaces are stained solely with lectin agglutinin when the cell surface glycoproteins are thoroughly free from the perineuronal proteoglycans; and that the cell surface are doubly stained with methylene blue and lectin agglutinin or with iron colloid and lectin agglutinin when the cell surface glycoproteins are reacting in part to the perineuronal proteoglycans (20).

Recently, Müller (61) supravitally stained an adult mouse brain with methylene blue and reported that this dye is attracted to anionic sites or sulfate groups of the perineuronal proteoglycans. However, our experiments indicated that methylene blue and Gömöri's ammoniacal silver react to the collagenous elements of the perineuronal proteoglycan surface coat (20, 21).

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