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Abstract

Neurons of cerebellar nuclei in the rat brain had a marked surface coat which was stained with cationic iron colloid or aldehyde fuchsin. Neurons with a similar surface coat were also noted in the retrosplenial cortex. The surface coat was stained doubly with cationic iron colloid and aldehyde fuchsin. Digestion with hyaluronidase eliminated the stainability of the surface coat to both agents. Combined digestion with chondroitinase ABC, heparitinase and keratanase eliminated the cationic iron colloid staining but did not interfere with the aldehyde fuchsin staining. Electron microscopy of ultrathin sections revealed that the iron particles were deposited in the perineuronal tissue spaces. These findings indicate that the surface coat consists of sulfated proteoglycans which occupy, as the extracellular matrix, the perineuronal tissue spaces. Many neurons in the retrosplenial cortex were labeled with lectin Vicia villosa agglutinin. Double staining revealed that these lectin-labeled neurons are usually reactive to cationic iron colloid. Few neurons in the cerebellar nuclei were labeled with lectin V. villosa agglutinin.

KEYWORDS: perineuronal sulfated proteoglycans, cationic iron colloid staining, aldehyde fuchsin staining, lectin VVA labeling, rat brain

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Neurons of cerebellar nuclei in the rat brain had a marked surface coat which was stained with cationic iron colloid or aldehyde fuchsin. Neurons with a similar surface coat were also noted in the retrosplenial cortex. The surface coat was stained doubly with cationic iron colloid and aldehyde fuchsin. Digestion with hyaluronidase eliminated the stainability of the surface coat to both agents. Combined digestion with chondroitinase ABC, heparitinase and keratanase eliminated the cationic iron colloid staining, but did not interfere with the aldehyde fuchsin staining. Electron microscopy of ultrathin sections revealed that the iron particles were deposited in the perineuronal tissue spaces. These findings indicate that the surface coat consists of sulfated proteoglycans which occupy, as the extracellular matrix, the perineuronal tissue spaces. Many neurons in the retrosplenial cortex were labeled with lectin *Vicia villosa* agglutinin. Double staining revealed that these lectin-labeled neurons are usually reactive to cationic iron colloid. Few neurons in the cerebellar nuclei were labeled with lectin *V. villosa* agglutinin.

Key words: perineuronal sulfated proteoglycans, cationic iron colloid staining, aldehyde fuchsin staining, lectin VVA labeling, rat brain

Our previous light microscopic studies of the rat brain and spinal cord sections stained with cationic iron colloid revealed the occurrence of numerous neurons with an intensely negatively charged surface coat (1-3). Similar neurons have been recognized also in the human brain (4) and in the brains of the cow, cat, rat, mouse and other animals, including some lower vertebrates such as the bullfrog and fish (5, 6).

Our recent histochemical and electron microscopic studies of the human visual cortex and the mouse brain showed that the surface coat consisted of sulfated proteoglycans which were digested with hyaluronidase and chondroitinase ABC/heparitinase/keratanase, and distributed in the perineuronal tissue spaces (7-9). These studies also showed that the neurons were frequently coated with cell surface glycoproteins reactive to lectin *Vicia villosa* or soybean agglutinin (7-9).

The present study reinvestigates the rat brain, and confirms that the surface coat even in this animal is composed of perineuronal sulfated proteoglycans.

Materials and Methods

Adult male Wistar rats were sacrificed under ether anesthesia. From these animals, 2- to 3-mm-thick blocks traversing the retrosplenial cortex or cerebellar nuclei were removed. The blocks were fixed in 4 % paraformaldehyde or a mixture of 4 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 6 h or longer.

The paraformaldehyde-fixed blocks were embedded in paraffin, and cut into 10- to 15-μm-thick sections. The paraformaldehyde/glutaraldehyde-fixed blocks were embedded in LR White resin, and cut into ultrathin sections.

Light microscopic and histochemical studies. The sections from the paraffin-embedded specimens were deparaffinized with xylene.

The deparaffinized sections were stained with cationic iron colloid (10), aldehyde fuchsin (11) or lectin *V. villosa* agglutinin (VVA) (12). Some sections were stained doubly with cationic iron colloid and aldehyde fuchsin or with lectin and cationic iron colloid (8). Some sections were

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methylated (13) and saponified (14) prior to the cationic iron colloid or aldehyde fuchsin staining.

Furthermore, some sections were digested with hyaluronidase or with chondroitinase ABC, heparitinase and keratanase (8). These enzyme-digested sections were stained with the cationic iron colloid, aldehyde fuchsin or lectin VVA (8). In each experiment, a control section was prepared and incubated in solution containing no enzyme (8).

The sections treated above were observed with a light microscope.

Electron microscopy. The ultrathin sections from the LR White resin-embedded specimens were incubated in cationic iron colloid with pH values of 1.0-1.5, exposed to osmium vapor and observed with a transmission electron microscope (15).

Results

Light microscopy of the sections stained with cationic iron colloid revealed that the retrosplenial cortex and cerebellar nuclei of rat brain contained many neurons with an intensely negatively charged surface coat which was reactive to cationic iron colloid with pH values of 1.0-1.5 (Fig. 1). The dark neurons characterized by their shrunken cell body are occasionally coated with a similar surface coat (Fig. 1). This surface coat showed fine meshwork structures (Fig. 1 Inset).

The surface coats in the retrosplenial cortex and cerebellar nuclei were stained with aldehyde fuchsin (Fig. 2), and stained doubly with cationic iron colloid and aldehyde fuchsin (Fig. 2 Upper inset). The reaction of the surface coats to cationic iron colloid was abolished by

Fig. 1

Fig. 2

methylation and was not reversed by saponification (Fig. 3, 3 Inset). The reaction of the surface coats to aldehyde fuchsin was neither abolished by methylation nor interrupted by saponification.

In the retrosplenial cortex, the cell surfaces of many neurons were labeled with lectin VVA. In this cortex, thus, the neurons labeled with lectin VVA were usually stained with cationic iron colloid though they sometimes showed no reaction to cationic iron colloid (Fig. 2 Lower inset). In the cerebellar nuclei, however, no neurons were labeled with lectin VVA.

Preliminary hyaluronidase digestion obliterated the cationic iron colloid staining of the surface coats (Fig. 4, 4 Inset). This hyaluronidase treatment also abolished the aldehyde fuchsin staining of the surface coats. No obliteration in these stainings was noted in the control or non-hyaluronidase-treated sections (Fig. 4 Inset). Pretreatment with hyaluronidase did not obliterate lectin VVA labeling. Even in the control sections, lectin VVA labeling was not abolished.

Successive digestions with chondroitinase ABC, heparitinase and keratanase almost completely eliminated the

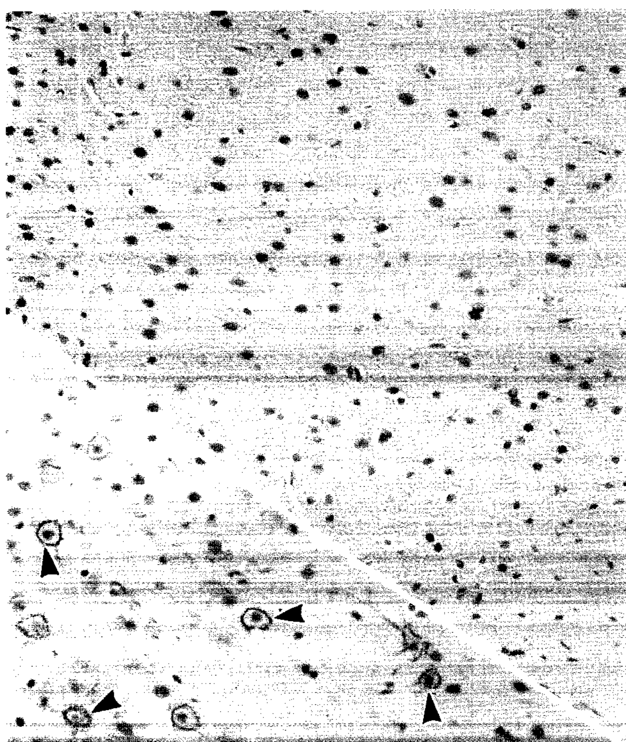

Fig. 3 Retrosplenial section, stained with cationic iron colloid after methylation and saponification. Note that no cell is reactive to cationic iron colloid. Inset shows a control section, in which many neurons are reactive to cationic iron colloid (arrowheads). × 100, Inset: × 100.

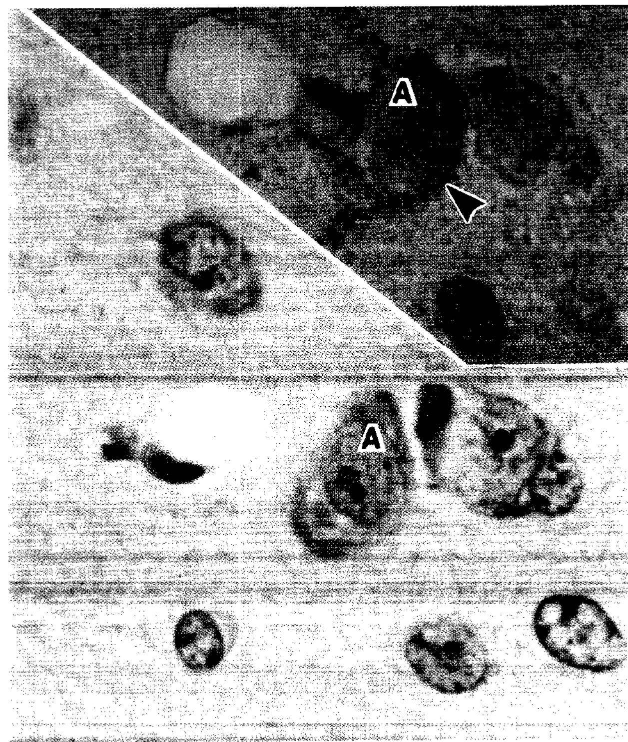

Fig. 4 Retrosplenial cortex, stained with cationic iron colloid after hyaluronidase digestion. No cell is reactive to the colloid. Inset shows a control section (an adjacent section of main figure). The cell labeled **A** is reactive to the colloid (arrowhead). This neuron is identical with neuron **A** in the main figure. × 500, Inset: × 500.

Fig. 1 Survey light micrograph of a adult rat brain section traversing the retrosplenial cortex. Many neurons are covered by an intensely negatively charged surface coat which shows a strong Prussian blue reaction (thick arrowheads). Thin arrowheads indicate dark neurons also revealing their surface coats. Inset shows highly magnified neurons with intensely negatively charged surface coats. The surface coats are formed by meshworks (thick arrowheads). × 350, Inset: × 1,000.

Fig. 2 The surface coats as stained with aldehyde fuchsin (arrowheads) (main or middle figure). Upper inset shows the surface coats as doubly stained with aldehyde fuchsin and cationic iron colloid (arrowheads). Lower inset shows a section of the retrosplenial cortex as doubly treated with lectin VVA and cationic iron colloid. Note in the lower inset that neuron **A** is doubly stained with lectin and iron colloid (arrow), whereas neuron **B** is solely stained with the colloid (arrowhead). × 1,000, Upper Inset: 1,000, Lower Inset: × 900.

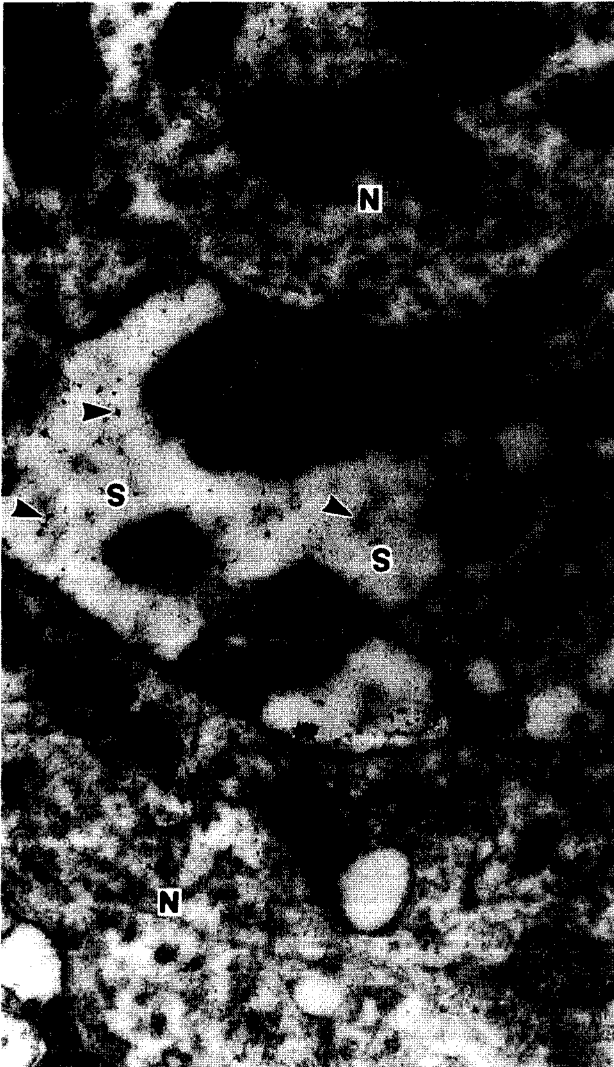

Fig. 5 Electron micrograph of a retrosplenial section, stained with cationic iron colloid and exposed to osmium vapor. The perineuronal tissue space (S) shows clustered depositions of iron particles (arrowheads). N neuronal soma. $\times$ 6,000.

cationic iron colloid staining of the surface coats. No reduction in aldehyde fuchsin staining and the lectin VVA labeling of the surface coats was noted in the sections which were preliminarily treated with chondroitinase ABC, heparitinase and keratanase. In the control sections, as well, neither the aldehyde fuchsin staining nor the lectin labeling of the surface coats were abolished.

Transmission electron microscopy of tissue sections revealed that the perineuronal tissue spaces of certain neurons showed a preferential and diffuse deposition of cationic iron colloid (Fig. 5). Such clustered iron deposits were not noted either in the neuronal somata and processes or in the neuropil regions, except for diffuse deposition of iron particles.

Discussion

The present study supplements our previous studies of the rat (1, 3), and confirms that the rat brain contains numerous neurons with intensely negatively charged surface coats which are stained with cationic iron colloid. Our recent studies of the mouse brain and spinal cord indicate that the neurons with such surface coats are widely distributed as local or relay interneurons in the central nervous system (5, 9).

The present study, together with our recent studies of the human visual cortex and mouse brain (5, 7, 8, 9), demonstrates that the surface coats are stained with cationic iron colloid and aldehyde fuchsin, and digested with hyaluronidase and chondroitinase ABC/heparitinase/keratanase. It also demonstrates that the cationic iron colloid staining of the surface coats was abolished by methylation and was not reversed by saponification. It further shows that hyaluronidase digestion eliminates both the cationic iron colloid and aldehyde fuchsin stainings of the surface coats and that the chondroitinase ABC/heparitinase/keratanase digestion does not interfere with aldehyde fuchsin staining of the surface coats although this digestion eliminated the cationic iron colloid staining of the surface coats. These findings indicate that the surface coats consist of sulfated proteoglycans and that cationic iron colloid and aldehyde fuchsin stain the sulfate groups and core proteins of proteoglycans, respectively.

Similar surface coats or meshworks were described in the rat and mouse with Golgi's silver impregnation, and as the processes of glial cells (glial nets) (16-18). Our recent double staining of human brain with Golgi's silver nitrate and our cationic iron colloid indicated that the surface coat is identical to the reticular covering of Golgi (19, 20) or glial nets of the previous authors (16-18). The details of these findings will be reported elsewhere.

The present study, together with our recent study of the mouse brain (9), demonstrates that the neurons in the cerebellar nuclei are reactive to cationic iron colloid or aldehyde fuchsin but are not labeled with lectin VVA. These findings, together with those from the human visual cortex (8, 9), indicate that cell surface glycoproteins as labeled with lectin VVA are neither structural elements

nor adhesive molecules of the sulfated proteoglycans.

Some authors have shown that the neurons labeled with lectin VVA are GABAergic interneurons (21-23). These data suggest that such neurons reactive to both cationic iron colloid and lectin VVA as observed in the retrosplenial cortex are inhibitory interneurons.

The present transmission electron microscopy of ultrathin sections revealed that the cationic iron colloid is preferentially and diffusely deposited in the perineuronal tissue spaces. Similar findings have been obtained in the human visual cortex and mouse brain (5, 7, 9). These facts indicate that the perineuronal sulfated proteoglycans surround, as the extracellular matrix, the axosomatic synapses and that their ability to function, as an insulator assures stable signal transmission at the synapses (5, 7-9).

The present study confirmed that dark neurons are common in the rat brain. Our recent study in the mouse brain suggested that dark neurons are exhausted cells which show an circadian rhythm in occurrence or are restored to normal or light neurons during sleep (9, 24, 25).

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