Intensely Positively Charged Perineuronal Nets in the Adult Rat Brain as Detected by Staining with Anionic Iron Colloid

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Summary. Ferric chloride, when boiled with ammonium thiocyanate, ammonia and cacodylic acid, is converted into a fine anionic iron colloid which consists of 1.0–1.5 nm electron dense granules and gives a distinct Prussian blue reaction (OHTSUKA and MURAKAMI, 1986). Light microscopy of tissue sections stained with this fine anionic iron colloid at pH values of 6.0, 7.0 and 8.0 showed that the healthy adult rat brain contains a considerable number of neurons which possess an intensely positively charged perineuronal net. This net was most clearly demonstrable by staining with the anionic iron colloid at a pH value of 8.0, at which ionizations of almost all cationic sites of the tissue elements were obliterated. Transmission electron microscopy of ultrathin sections stained at a pH value of 8.0 showed that the anionic iron colloid was preferentially deposited in the perineuronal tissue spaces. These findings indicate that the intensely positively charged perineuronal net contains some strongly basic substances such as guanidino compounds, and occupies the perineuronal (perisynaptic) tissue space.

Our studies in humans, rats, mice and certain other animals (MURAKAMI et al., 1993, 1994, 1996, 1997, 1999a,b; TAKAHASHI-IWANAGA et al., 1998; OHTSUKA et al., 2000) have shown that many neurons in the mature central nervous system possess an intensely negatively charged perineuronal net which is clearly visualized by staining with our fine cationic iron colloid (MURAKAMI et al., 1986). We recently found that some other neurons in the mature brain possessed an intensely positively charged perineuronal net which was strongly reactive to our fine anionic iron colloid (OHTSUKA and MURAKAMI, 1986). The present paper introduces some light and electron microscopic findings of this perineuronal net in the adult rat brain.

MATERIALS AND METHODS

Light microscopy

Paraffin-embedded brain blocks containing the retrosplenial cortex were prepared from healthy adult male Wistar rats which were vascularly perfused with 4% paraformaldehyde (MURAKAMI et al., 1993). The blocks were cut into 10–15 μ m thick sections and deparaffinized with xylene.

The deparaffinized sections were immersed in our fine anionic iron colloid (a diluted mixture of boiled ammonium thiocyanate–ferric chloride–ammonia–cacodylic acid) at pH values of 6.0, 7.0 or 8.0 (OHTSUKA and MURAKAMI, 1986), and treated for Prussian blue reaction. After this treatment, the sections were counter stained with nuclear fast red (OHTSUKA and MURAKAMI, 1986) and observed with a light microscope (Olympus, BX50).

Electron microscopy

Small blocks were prepared from LR White resin-embedded specimens containing the retrosplenial cortex from other healthy adult male Wistar rats which were vascularly perfused with a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and then immersion-fixed for 6 h (TSUBOUCHI et al., 1996).

The blocks were cut into ultrathin sections and incubated in our fine anionic iron colloid at pH values of 6.0, 7.0 or 8.0 for 6 h at room temperature (OHTSUKA and MURAKAMI, 1986). They were then stained with osmic acid (OHTSUKA and MURAKAMI, 1986) and observed with a transmission electron microscope (Hitachi, H-7100).

Fig. 1. A section of the retrosplenial cortex from healthy adult rat brain, stained with our fine anionic iron colloid at a pH value of 6.0. Note that some neurons show a stronger Prussian blue reaction on their surface (*large arrowheads*). $\times 200$

Fig. 2. A closer view of a part of Figure 1. Intense perineuronal Prussian blue reaction is clearly seen (*large arrowheads*). $\times 700$

RESULTS

Light microscopy

The sections treated with our fine anionic iron colloid at a pH value of 6.0 showed a homologous and strong Prussian blue reaction (Fig. 1). However, some neurons (their cell bodies and main processes) in the retrosplenial cortex possessed a marked perineuronal net which showed a stronger Prussian blue reaction than the surrounding tissues (Figs. 1, 2).

The sections stained at a pH value of 7.0 gave a reduced Prussian blue reaction (Fig. 3). However, those perineuronal nets showing a stronger Prussian blue reaction at a pH value of 6.0 maintained this reaction even at a pH value of 7.0 (Figs. 3, 4).

At the pH value of 8.0, the Prussian blue reaction of the tissue elements almost disappeared (Fig. 5). However, those perineuronal nets showing a stronger Prussian blue reaction at pH values of 6.0 and 7.0 maintained their intense Prussian blue reaction (Figs. 5, 6). The neuropil regions also exhibited a strong Prussian blue reaction at this pH value of 8.0 (Fig. 6).

Electron microscopy

Our anionic iron colloid consisted of 1.0–1.5 nm granules and yielded distinct dark images under the transmission electron microscope (Fig. 7).

In the sections treated with the anionic iron colloid at pH value of 6.0, the colloidal iron granules were deposited most densely in the perineuronal tissue

Fig. 3. A section of the healthy adult rat retrosplenial cortex, stained with our fine anionic iron colloid at a pH value of 7.0. *Large arrowheads* indicate the intensely positively charged perineuronal surface nets which react with our colloid and show an intense Prussian blue reaction. $\times 450$

Fig. 4. A closer view of the lower right area in Figure 3. The intense perineuronal Prussian blue reaction is clearly observed (*large arrowheads*). $\times 1,300$

spaces, while they were diffusely deposited in other tissue elements (data, not shown).

In the sections treated with the anionic iron colloid at pH value of 7.0, our colloidal iron granules were again distributed most densely in the perineuronal tissue spaces. Depositions of the iron granules in other tissue elements were markedly less in amount than in the specimens treated at pH value of 6.0.

In the sections treated with our anionic iron colloid at pH value of 8.0, the colloidal iron granules were distributed preferentially in the perineuronal tissue spaces (Fig. 7). Such preferential depositions of the iron granules were also observed in the neuropil tissue spaces (Fig. 7). Other areas showed few depositions of colloidal iron granules.

DISCUSSION

The present study shows that some neurons in the mature rat brain possess an intensely positively charged perineuronal net which was strongly reactive to our fine anionic iron colloid (OHTSUKA and MURAKAMI, 1986). Preferential depositions of our fine anionic iron colloid in the perineuronal and neuropil tissue spaces at a pH value of 8.0 indicate that the intensely positively charged perineuronal net occupies these tissue spaces and embeds the synapses. As far as we know, no previous authors have reported such intensely positively charged perineuronal nets (CELIO et al., 1998; YAMAGUCHI, 2000).

Ferric oxide-potassium ferrocyanide (GASIC et al.,

Fig. 5. A retrosplenial section, stained with our fine anionic iron colloid at pH value of 8.0. The intense perineuronal Prussian blue reaction is maintained (*large arrowheads*). $\times 500$

Fig. 6. A closer view of the retrosplenial section stained with our anionic iron colloid at pH value of 8.0. In addition to the intense perineuronal Prussian blue reaction (*large arrowheads*), some intense Prussian blue reaction (*small arrowheads*) is seen in the neuropil regions. $\times 1,500$

1968) and chondroitin sulfate (SENO et al., 1983) colloids have been used for histochemical or cytochemical detection of cationic sites in tissues. In a concomitant experiment in this study, however, it was difficult to clearly demonstrate the intensely positively charged perineuronal nets with those previously used reagents consisting of coarse or large granules (5–20 nm).

In another concomitant experiment of ours, we stained some serial sections from the retrosplenial cortex of an adult rat brain with our fine anionic iron colloid (OHTSUKA and MURAKAMI, 1986), our fine cationic iron colloid (MURAKAMI et al., 1986), FUJITA's highly concentrated aldehyde fuchsin (FUJITA, 1957), or lectin agglutinin (*Vicia villosa* or *Wisteria floribunda* agglutinin) (NAKAGAWA et al., 1992; HÄRTIG et al.,

1992). The results of these experiments have shown that the neurons stained with our fine anionic iron colloid are not identical to the neurons stained with our fine cationic iron colloid and aldehyde fuchsin nor to the neurons labeled with lectin *Vicia villosa* or *Wisteria floribunda* agglutinin. These findings indicate that the intensely positively charged perineuronal nets are independent from the intensely negatively charged perineuronal nets (i. e., perineuronal nets of proteoglycans) and also from the nerve cell surface glycoproteins (MURAKAMI et al., 1994, 1996, 1997, 1999b).

A noteworthy finding is that our intensely positively charged perineuronal (or perisynaptic) nets can be stained even at such a high pH value of 8.0 in which ionization of almost all cationic sites of the tissue

Fig. 7. A transmission electron micrograph of an ultrathin section of the retrosplenial cortex from a healthy adult rat, stained with our fine anionic iron colloid at a pH value of 8.0. Colloidal iron granules are preferentially deposited in the perineuronal tissue spaces (*large arrowheads*). Colloidal iron granules are also preferentially deposited in the neuropil tissue spaces (*small arrowheads*). *N* nerve cell, *P* neuropil. $\times 25,000$

elements are apparently obliterated. This fact indicates that these nets contain some strongly basic substances such as guanidino compounds. Chromatographically, these compounds with the guanidinium group can be stained by treatment with a mixture of NaOH, urea, and alpha-naphthol alcohol and then with hydrobromide (SAKAGUCHI, 1924). We are now trying to improve this method for staining tissue sections or for confirming our idea that the intensely positively charged perineuronal nets contain the guanidino compounds.

It has been generally believed that the guanidino compounds are neurotoxic and cause some such diseases as epilepsy (MORI, 1987, 1996; MORI et al., 1996). The present study, as demonstrated in Figures 1-7, strongly suggests that the guanidino compounds contribute to the formation of the extracellular matrix or perineuronal (perisynaptic) nets of some

neurons in the mature brain of healthy animals. The detailed distribution of these intensely positively charged perineuronal nets will be reported elsewhere.

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