The Intensely Positively Charged Perineuronal Net in the Adult Rat Brain, with Special Reference to Its Reactions to Oxine, Chondroitinase ABC, Hyaluronidase and Collagenase

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Summary. Light microscopic observations of healthy adult rat brain sections stained with anionic iron colloid indicated that 5–10% of neurons in the hippocampal subiculum and all neurons in the medial cerebellar nucleus possessed an intensely positively charged perineuronal net. This net was demonstrated to react to oxine, and therefore suggested to consist of guanidino compounds. It was further shown that the intensely positively charged perineuronal net, in accordance with the intensely negatively charged perineuronal net of proteoglycans, was digested by chondroitinase ABC, hyaluronidase, and collagenase, but not by endo- α -N-acetylgalactosaminidase. This finding suggested that the former positively charged net might be linked to the latter negatively charged one.

Our previous studies in humans, rats, mice and 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 brain and spinal cord possess an intensely negatively charged perineuronal net of sulfated proteoglycans which is clearly visible by staining with our fine cationic iron colloid (MURAKAMI et al., 1986) and is usually expressed together with the nerve cell surface glycoproteins labeled with lectin *Vicia villosa* or *Wisteria floribunda* agglutinin (HÄRTIG et al., 1992; SEEGER et al., 1994). In addition to that negatively charged one, we recently found another perineuronal net (MURAKAMI et al., 2001a) which is intensely positively charged and is detectable by staining with our fine anionic iron colloid (OHTSUKA and MURAKAMI, 1986).

The present study reinvestigates the rat brain, and indicates that the latter positively charged perineuronal net is reactive to oxine with an affinity for guanidino compounds. It also demonstrates that this net is digested by chondroitinase ABC, hyaluronidase and collagenase, but not by endo- α -N-acetylgalactosaminidase.

The hippocampal subiculum and medial cerebellar nucleus of adult rats comprise the materials of this study, as our preliminary study indicated that these brain portions contain many neurons reactive to oxine and anionic iron colloid.

MATERIALS AND METHODS

Paraffin-embedded brain blocks containing the hippocampal subiculum or medial cerebellar nucleus 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.

Histochemical stainings

The deparaffinized sections were immersed in a fine anionic iron colloid solution (a diluted mixture of boiled ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid) at pH values of 5.0–7.5 (OHTSUKA and MURAKAMI, 1986), and treated for Prussian blue reaction. After this treatment, some of the sections were counterstained with nuclear fast red (OHTSUKA and MURAKAMI, 1986) and observed with a light microscope (Olympus, BX50).

Some deparaffinized sections were treated with an oxine/brome method (MORI, 1980) modified after SAKAGUCHI (1925) for biochemical detection of guanidino compounds. Briefly, the sections were washed in distilled water, and sprayed with 10% NaOH solution for 0.5–1.0 sec and with 0.1% oxine (8-hydroxyquinoline) in acetone for 0.5–1.0 sec. The sections were then dried in air at room temperature. The dried sections were sprayed with 0.3% brome in 5N NaOH solution for color reaction by oxine. The sections thus treated were immersed in ethanol, embedded in balsam, and observed with the light microscope.

Enzyme digestions

Some deparaffinized sections were incubated with 5 U/ml chondroitinase ABC (*Proteus vulgaris*) (Seikagaku Kogyo, Tokyo) in 0.04 M Tris-HCl buffer (pH 8.0) containing 0.05 M sodium acetate and 0.02% bovine serum albumin for 5 min at 37 °C (MURAKAMI et al., 1994), with 100 TRU/ml hyaluronidase (*Streptomyces hyalurolyticus*) (Seikagaku Kogyo, Tokyo) in a 0.02 M sodium acetate-acetic acid buffer (pH 6.0) containing 0.21% NaCl for 3 h at 60 °C (MURAKAMI et al., 1994), with 0.005% collagenase (*Cl. histolyticum*) (Elastin Products Co., Missouri) in distilled water for 60 min at 37 °C (MURAKAMI et al., 1999a), or with 0.35 U/ml endo-alpha-N-acetylgalactosaminidase (*Alcaligenes sp.*) (Seikagaku Kogyo, Tokyo) in 0.1 M citrate buffer (pH 4.5) for 6 h at room temperature (MURAKAMI et al., 1999b).

The sections thus incubated in enzymes were stained with our fine anionic iron colloid or with MORI's oxine (see above), and observed with the light microscope.

Control sections for the enzyme digestions were rinsed with respective buffer or distilled water containing no enzymes.

RESULTS

Histochemical stainings

Five–10% of neurons in the hippocampal subiculum and all neurons in the medial cerebellar nucleus possessed an intensively positively charged perineuronal net on their cell bodies and main processes which was reactive to anionic iron colloid at pH values of 5.0–7.5 and showed a Prussian blue reaction stronger than the surrounding tissues (Fig. 1, Inset).

In the oxine staining, 5–10% of neurons in the hippocampal subiculum and all neurons in the medial cerebellar nucleus were reactive to oxine, and showed a perineuronal color (brome) reaction on their cell bodies and main processes (Fig. 2, Inset).

Enzyme digestions

Results of the enzyme digestion assays were similar in the hippocampal subiculum and the medial cerebellar nucleus. They are summarized in Table 1.

Sections incubated with chondroitinase ABC, hyaluronidase or collagenase showed that the anionic iron colloid staining and the oxine staining were erased (Figs. 3, Inset, 4, Inset). In contrast, sections incubated with endo-alpha-N-acetylgalactosaminidase revealed that the anionic iron colloid staining and the oxine staining were not erased.

The control sections among all enzyme digestion assays showed similar staining patterns: the anionic staining and the oxine staining were similarly not erased.

DISCUSSION

The present study, together with our previous study of the adult rat retrosplenial cortex (MURAKAMI et al., 2001a), confirms the occurrence of an intensely positively charged perineuronal net in the adult rat brain, and shows that 5–10% of neurons in the hippocampal subiculum and all neurons in the medial cerebellar nucleus possess this net, which is stained with our fine anionic iron colloid (OHTSUKA and MURAKAMI, 1986). It also shows that these neurons are reactive to MORI's oxine which detects guanidino compounds (MORI, 1980; MORI et al., 1996). This finding strengthens our previous view that the guanidino compounds contribute to the formation of the intensely positively charged perineuronal net (MURAKAMI et al., 2001a). The normal occurrence of guanidino compounds in the brain has been confirmed by some biochemists (MORI, 1980; MORI et al., 1996).

The present study demonstrates that the anionic iron colloid and oxine stainings of the intensely positively charged perineuronal net of guanidino compounds is erased by chondroitinase ABC, hyaluronidase, and collagenase, but not by endo-alpha-N-acetylgalactosaminidase. A similar finding was obtained in the case of the cationic iron colloid staining of the intensely negatively charged perineuronal net of proteoglycans (MURAKAMI et al., 1994, 1996, 1999a, b). These findings suggest that the positively charged perineuronal net of guanidino compounds may be linked to the negatively charged net of proteoglycans (MURAKAMI et al., 1996, 1999b). In our previous study, we omitted enzyme-digestion experiments of the specimens, and carelessly described that the former net was independent of the latter one (MURAKAMI et al., 2001a).

Fig. 1. A section of the hippocampal subiculum from a healthy adult rat, stained with our fine anionic iron colloid at a pH value of 7.0. Note that some neurons show a strong Prussian blue reaction on their surface (*large arrowheads*). **Inset** is a section of the medial cerebellar nucleus from a healthy adult rat, stained with our fine anionic iron colloid at pH value of 7.0. Note in this **Inset** that all neurons show a stronger Prussian blue reaction on their surface (*large arrowheads*). $\times 300$, **Inset**: $\times 400$

Fig. 2. A section of the hippocampal subiculum, reacting to oxine. The reaction was demonstrated by treatment with brome-NaOH. Note that some neurons show a brome reaction on their surfaces (*large arrowheads*). **Inset** is a section of the medial cerebellar nucleus. All neurons of this nucleus show a brome reaction on their surface (*large arrowheads*). $\times 350$, **Inset**: $\times 300$

Table 1. Effects of exclusive incubation with chondroitinase ABC, hyaluronidase, collagenase and endo-alpha-N-acetylgalactosaminidase on anionic iron colloid and oxine stainings in the adult rat hippocampal subiculum and medial cerebellar nucleus.

Enzymes	ChondroitinaseABC	Hyaluronidase	Collagenase	Endo-alpha-N-acetyl-galactosaminidase
Stainings				
Anionic iron colloid staining	erased	erased	erased	not erased
Oxine staining	erased	erased	erased	not erased

Fig. 3. A section of the hippocampal subiculum, digested by hyaluronidase and stained with anionic iron colloid. **Inset** shows a section of the medial cerebellar nucleus, digested by collagenase and stained with anionic iron colloid. None of the neurons are reactive to anionic iron colloid when the specimens have been preliminarily treated with hyaluronidase (**main Figure**) or with collagenase (**Inset**). $\times 300$, **Inset** $\times 400$

Fig. 4. A section of the hippocampal subiculum, digested by chondroitinase ABC and treated with oxine. **Inset** shows a section from the medial cerebellar nucleus, digested by hyaluronidase and treated with oxine. No neurons are reactive to oxine when the specimens have been digested by chondroitinase ABC (**main Figure**) and by hyaluronidase (**Inset**). $\times 200$, **Inset** $\times 350$

In our concomitant experiments of this study, the intensely positively charged perineuronal net was doubly stained with our fine anionic iron colloid (OH-TSUKA and MURAKAMI, 1986) and FUJITA's highly concentrated aldehyde fuchsin (FUJITA, 1959) reactive to the intensely negatively charged perineuronal net-core proteins of the proteoglycans (data, not shown). These experiments or double stainings may also support our present view that the positively charged perineuronal net may be linked to the negatively charged one (see above).

The positively charged perineuronal net of

guanidino compounds as well as the negatively charged one of proteoglycans are believed to occupy the same perineuronal tissue spaces, and surround individual synapses (MURAKAMI et al., 1994, 2001a). We suggest that these intensely positively and negatively charged nets entrap interstitial tissue water, and form a gel through which perineuronal tissue fluid moves, molecule by molecule, by kinetic motion. Thus, the positively and negatively charged nets may be referred to as the "perineuronal or perisynaptic barrier" (MURAKAMI et al., 2001c).

Recent biochemical, histochemical, and immuno-

histochemical studies have shown that aggrecan, versican, phosphacan, neurocan, brevican and other agents including tenascin-R are representative elements in the construction of the perineuronal nets (RAUCH, 1997; YAMAGUCHI, 2000; BRÜCKNER et al., 2000). Our recent light microscopic studies of some sections from an adult brevican/neurocan knockout mouse have shown neither a marked reduction of the perineuronal cationic iron colloid staining of perineuronal proteoglycans nor a marked erasure of the perineuronal anionic iron colloid staining of perineuronal guanidino compounds (MURAKAMI et al., 2001b). This finding indicates that neurocan and brevican are not major perineuronal proteoglycans in the mature brain. BRÜCKNER and his associates (2000) recently studied a mutant mouse deficient in tenascin-R, and suggested that tenascin-R might exert a critical role in the maintenance of the perineuronal nets.

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