Acta Medica Okayama

Volume 50, *Issue* 6 1996 *Article* 5

DECEMBER 1996

Perineuronal Sulfated Proteoglycans, Cell Surface Glycoproteins and Dark Neurons in the Cingulate Cortex of Newborn and Adult Rats

Mari Tsubouchi[*] Yutaka Tsubochi[†] Sayoko Hitomi[‡]

Aiji Ohatsuka[**] Takuro Murakami[††]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama Univeristy,
[**]Okayama University,
[††]Okayama University,

Perineuronal Sulfated Proteoglycans, Cell Surface Glycoproteins and Dark Neurons in the Cingulate Cortex of Newborn and Adult Rats*

Mari Tsubouchi, Yutaka Tsubochi, Sayoko Hitomi, Aiji Ohatsuka, and Takuro Murakami

Abstract

Many neurons in the adult rat cingulate cortex possess perineuronal sulfated proteoglycans detectable with cationic iron colloid and aldehyde fuchsin, or cell surface glycoproteins reactive to lectin Vicia villosa or soybean agglutinin. The perineuronal sulfated proteoglycans develop three to four weeks after birth. The cell surface glycoproteins develop at earlier stage or two to three weeks after birth. Dark or active neurons begin to appear three to four weeks after birth. These findings indicate that the brain matures after birth or during weaning period.

KEYWORDS: rat brain, perineuronal sulfated proteoglycans, cell surface glycoproteins, dark neurons

---

*PMID: 8985468 [PubMed - indexed for MEDLINE]

Acta Med Okayama 1996; 50(6): 313-317

Perineuronal Sulfated Proteoglycans, Cell Surface Glycoproteins and Dark Neurons in the Cingulate Cortex of Newborn and Adult Rats

Mari Tsubouchi*, Yutaka Tsubouchi, Sayoko Hitomi, Aiji Ohtsuka and Takuro Murakami

Department of Anatomy, Okayama University Medical School, Okayama 700, Japan

Many neurons in the adult rat cingulate cortex possess perineuronal sulfated proteoglycans detectable with cationic iron colloid and aldehyde fuchsin, or cell surface glycoproteins reactive to lectin *Vicia villosa* or soybean agglutinin. The perineuronal sulfated proteoglycans develop three to four weeks after birth. The cell surface glycoproteins develop at earlier stage or two to three weeks after birth. Dark or active neurons begin to appear three to four weeks after birth. These findings indicate that the brain matures after birth or during weaning period.

Key words: rat brain, perineuronal sulfated proteoglycans, cell surface glycoproteins, dark neurons

Our previous light microscopic studies of the rat brain sections stained with cationic iron colloid revealed the existence of many neurons with an intensely negative-charged surface coat (1-3). Similar neurons have been observed in the human brain (4) and in the brains of cows, cats, rats, mice and other animals, including some lower vertebrates such as bullfrogs and fish (5, 6).

Our recent histochemical and electron microscopic studies of the human, rat and mouse brains showed that the surface coat consists of sulfated proteoglycans distributed as extracellular matrix in the perineuronal tissue spaces (7-12). These studies also showed that the neurons were frequently coated with cell surface glycoproteins reactive to lectin *Vicia villosa* or soybean agglutinin (7-12).

The present study reinvestigates the rat brain (12), and demonstrates that even in this animal, the sulfated proteoglycans and cell surface glycoproteins develop after birth. It also demonstrates that the dark or active neurons with shrunken cell bodies begin to appear after birth. The cingulate cortex was the object of our study as it contains many neurons with perineuronal sulfated proteoglycans or cell surface glycoproteins (12).

Materials and Methods

Newborn (0-5 weeks after birth) and adult (16 weeks after birth) Wistar rats were killed by ether anesthesia between 9 and 10 o'clock a.m. while the animals were sleeping. From these animals, 1-2 mm thick blocks traversing the cingulate cortex were removed. These blocks were fixed with 4.0 % paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2-7.4) for 6 h or longer, embedded in paraffin and cut into sections. These sections were deparaffinized with xylene.

The deparaffinized sections were stained with cationic iron colloid at a pH value of 1.0-1.5 (13), aldehyde fuchsin (14), lectin *Vicia villosa* agglutinin (VVA) or soybean agglutinin (SBA) (7). Some sections were stained doubly with cationic iron colloid and aldehyde fuchsin, or with lectin and cationic iron colloid (pH 1.0-1.5) (8). In the lectin staining (labeling), peroxidase activity was demonstrated with diaminobenzidine (DAB) (15) or amino-ethylcarbazol (AEC) (16). Controls for lectin labeled sections consisted of adjacent sections treated with phosphate buffer containing no agglutinin (7).

Results

Adult rats. The cingulate cortex of adult rats contained many neurons which were coated with perineuronal sulfated proteoglycans reactive to cationic iron colloid and aldehyde fuchsin (Fig. 1). In the sleeping animals, the neurons were usually light though some neurons were dark (Fig. 1). In the light neurons, nuclear fast red preferentially stained the nucleolus and nucleolus

* To whom correspondence should be addressed.

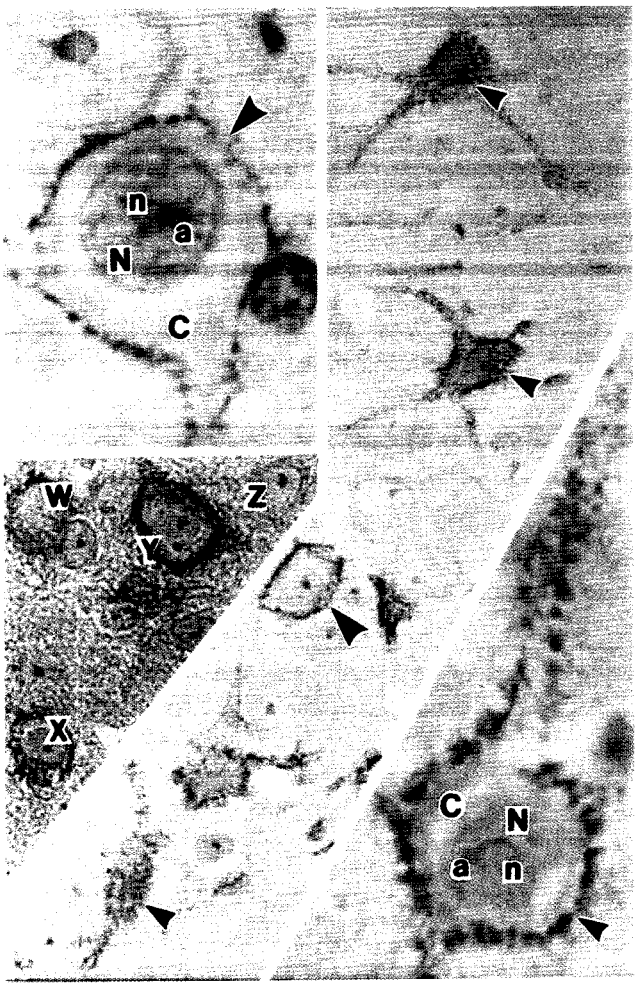

Fig. 1

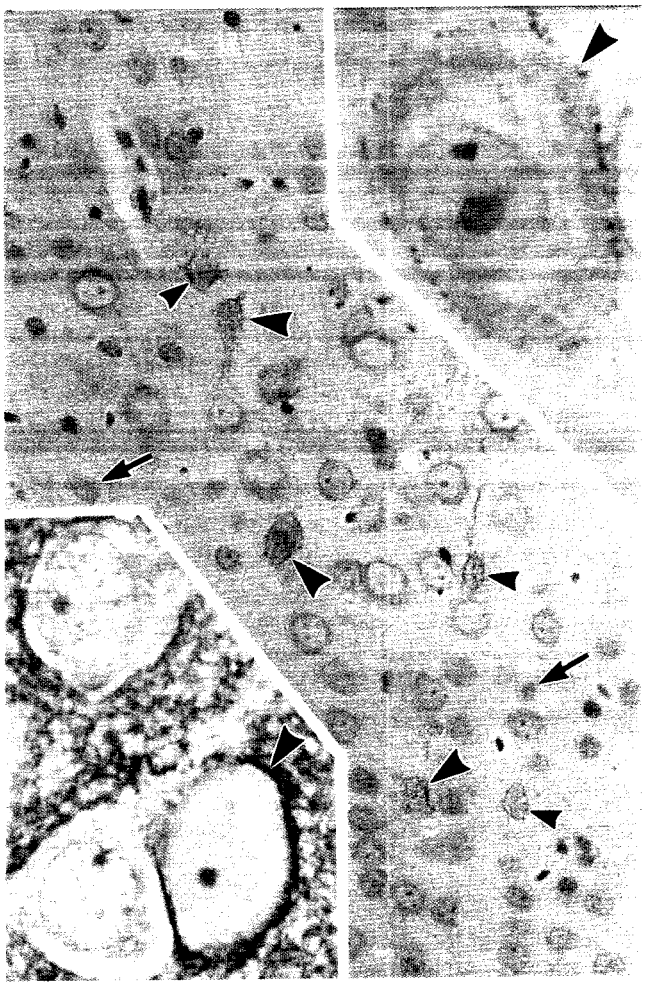

Fig. 2

Fig. 1. A light micrograph survey of the adult rat cingulate cortex, stained with cationic iron colloid and nuclear fast red. Many neurons are coated with perineuronal sulfated proteoglycans reactive to cationic iron colloid (large and small arrowheads). Some neurons are darkened, and stained with nuclear fast red (small arrowheads). Upper-left inset shows a closer view of a light neuron with the perineuronal proteoglycans (large arrowhead). In the light neuron, nucleolus (**n**) and nucleolus associated chromatin (**a**) are reactive to nuclear fast red; nucleus (**N**) and cytoplasm are not stained with this dye. Right inset shows a closer view of a dark neuron with the perineuronal proteoglycans (small arrowhead). In the dark neuron, the nucleus (**N**) and cytoplasm (**C**) as well as nucleolus and nucleolus associated chromatin are stained with nuclear fast red. Lower-left inset shows the adult rat cingulate cortex as doubly stained with lectin VVA (DAB) and cationic iron colloid. The neuron **W** is stained with iron colloid; the neuron **X** is stained with lectin; the neuron **Y** is stained doubly with lectin and iron colloid; the neuron **Z** is neither labeled with lectin nor stained with iron colloid. × 400. Upper-left inset: × 1,200; lower-left inset: × 500; right inset: × 1,200.

Fig. 2. Newborn rat cingulate cortex (4 weeks after birth), stained with cationic iron colloid and nuclear fast red. Many neurons are coated with perineuronal sulfated proteoglycans reactive to cationic iron colloid (large and small arrowheads). Some of these neurons are darkened and stained with nuclear fast red (small arrowheads). Some neurons without perineuronal surface coats are also darkened (small arrows). Upper inset shows a neuron with perineuronal sulfated proteoglycans (large arrowhead) as observed in the newborn rat cingulate cortex (4 weeks after birth). Lower inset shows the developing perineuronal sulfated proteoglycans as stained with aldehyde fuchsin (large arrowhead) (newborn cingulate cortex, 4 weeks after birth). × 350. Upper inset: × 1,500; lower inset: × 1,300.

associated chromatin (Fig. 1 upper-left inset). In the dark neurons, nuclear fast red stained the cytoplasm and nucleus as well as the nucleolus and nucleolus associated chromatin (Fig. 1 right inset).

The cell surfaces of many neurons, including the dark neurons, were labeled with lectin VVA or SBA. Double

Fig. 3

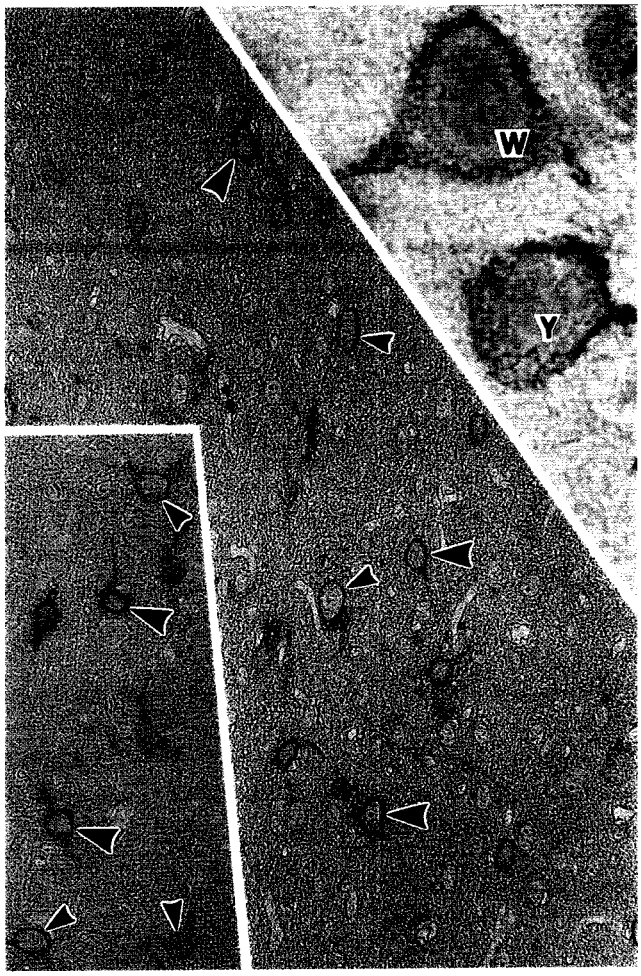

Fig. 4

Fig. 3. Newborn rat cingulate cortex (3 weeks after birth), stained with lectin VVA (DAB) and hematoxylin. Many neurons are labeled with lectin VVA (large arrowheads). Upper inset shows a closer view of a lectin VVA-labeled neuron (large arrowhead) (DAB). Lower inset shows the newborn rat cingulate cortex (3 weeks after birth), stained with lectin SBA (small arrowheads) (DAB). Some neurons are labeled with lectin SBA (small arrowheads). × 150. Upper inset: × 1,000; lower inset: × 150.

Fig. 4. Newborn rat cingulate cortex (five weeks after birth), stained doubly with lectin VVA (AEC) and cationic iron colloid. Many neurons are stained doubly with lectin VVA and iron colloid (large arrowheads). Small arrowheads indicate the neurons stained only with iron colloid. Lower inset shows the newborn rat cingulate (5 weeks after birth) as doubly stained with lectin SBA (AEC) and iron colloid. Many neurons are stained only with iron colloid (small arrowheads). Large arrowheads indicate the neurons doubly stained with lectin SBA and iron colloid. Upper inset shows a closer view of the newborn rat cingluate cortex (5 weeks after birth) as doubly stained with lectin SBA (DAB) and iron colloid. It is obvious that the neuron **W** is stained solely with iron colloid, while the neuron **Y** is stained doubly with lectin VVA and iron colloid. × 250. Upper inset: × 1,000; lower inset: × 250.

stainings indicated that the neurons labeled with lectin VVA or SBA were usually stained with cationic iron colloid though they sometimes showed no reaction to cationic iron colloid; the neurons stained with cationic iron colloid also sometimes showed no reaction to lectin VVA or SBA (Fig. 1 lower-left inset). In the control sections, no neuron was reacted to DAB or AEC.

Newborn rats. The perineuronal sulfated proteogycans began to appear at 3 to 4 weeks after birth (Fig. 2). In this stage, the proteoglycans were lightly stained with cationic iron colloid and aldehyde fuchsin (Fig. 2 upper and lower insets). The proteoglycans rapid-

ly increased their thickness. At 5 weeks after the birth, thus, the proteoglycans were clearly observable even at low magnifications (Figs. 3, 4). From 0 to 2 weeks after birth, no neuron was reactive to cationic iron colloid or aldehyde fuchsin.

The cell surface glycoproteins detectable with lectin VVA or SBA began to appear at 2 to 3 weeks after birth (Fig. 3 lower inset). These glycoproteins also developed rapidly. At 3 weeks after birth, thus, many neurons were clearly labeled with lectin VVA or SBA, or deeply stained with DAB or AEC (Fig. 3, upper inset). In the control sections, no neuron was reactive to DAB or AEC. In the stage of 0–1 week after birth, no neuron was labeled with lectin VVA or SBA.

In the doubly stained sections obtained from newborn animals 5 weeks after birth, it was confirmed that the neurons stained with cationic iron colloid were frequently labeled with lectin VVA or SBA though they occasionally showed no reaction to these lectins (Fig. 4, upper and lower insets). It was also observed in these double stainings that some neurons labeled with lectin were not stained with cationic iron colloid (data not shown).

The dark neurons began to appear 4 weeks after birth. No dark neurons were noted at the earlier stages or 0–3 weeks after birth.

Discussion

The present study supplements our previous studies of the rat brain (1, 3, 12), and confirms that the rat brain contains many neurons with intensely negative-charged surface coats which are stained with cationic iron colloid and aldehyde fuchsin. Our previous experiments with the human and mouse brains showed that chondroitinase ABC/heparitinase/keratanase digestions never interfere with the aldehyde fuchsin staining though they completely erase the cationic iron colloid staining (5, 7). These findings indicate that cationic iron colloid stains the sulfate groups, and aldehyde fuchsin stains the core proteins of proteoglycans (5, 7–9).

Our recent double stainings of the human and mouse brain sections show that the perineuronal sulfated proteoglycans are stained with Golgi's silver nitrate (10, 11), and confirm that they are identical with Golgi's reticular coating (17). These double stainings further indicate that Golgi's silver nitrate stains the core proteins of proteoglycans (10, 11). Previously, Golgi's reticular coating was regarded as the processes of glial cells (glial nets) (18).

Our recent studies of the mouse brain and spinal cord show that the neurons with perineuronal sulfated proteoglycans are mainly distributed as local or relay interneurons in the red nucleus, nucleus of the trapezoid body, cerebellar nuclei and certain other nuclei, including some sensory and motor nuclei such as the lateral vestibular nucleus and nucleus of the oculomotor nerve (5, 9, 12). In these studies, we have proposed that the perineuronal sulfated proteoglycans might serve as insulators in the stable transmission of the synapses or in stabilizing the neuronal circuits in the brain and spinal cord (5, 9, 12).

The present study, together with our recent study of the mouse brain (12), shows that the perineuronal sulfated proteoglycans are formed at the stages of 3 to 4 weeks after birth or during the weaning period. Also noteworthy is that the dark neurons began to appear at this stage. As discussed elsewhere, we believe that the development of proteoglycans and appearance of dark neurons indicate a certain stage of brain maturation (10).

Ramón y Cajal (1909) indicated that the dark neurons were resting neurons (19), while Hodge (1892) regarded the dark neurons as fatigued cells (cited by Ramón y Cajal, 19). Our recent observations of the mouse brain show that the dark neurons, which show a circadian rhythm in occurrence (20) and are restored to light neurons by sleep (21), are active neurons which have fully developed endoplasmic reticulum and Golgi's complexes (22). The dark neurons are not apoptotic cells since they showed no reaction to DNA nick end labeling (20).

The present study, together with our earlier study of the mouse brain and spinal cord (9), confirms that the cell surface glycoproteins labeled with lectin VVA or SBA begin to develop 2 to 3 weeks after birth or at an earlier stage than the development of proteoglycans. It also supplements our recent studies of the human and mouse brains (8, 9), and confirms that the neurons stained with cationic iron colloid are not always labeled with lectin VVA or SBA. These findings indicate that the cell surface glycoproteins are fairly independent, and that they are not adhesive molecules of the proteoglycans (8, 9). Our recent studies of the human brain further indicate that the neurons labeled with lectin VVA are not always identical to the neurons labeled with lectin SBA (8).

Acknowledgments. We are grateful to Mr. Hiromichi Kusano for his help in tissue preparation and staining. This study was supported in part by a Grant-in-Aid (08670016) from the Japanese Ministry of Education, Science,

Sports and Culture.

References

1. Murakami T, Taguchi T, Ohtsuka A and Kikuta A: Neurons with strongly negative-charged surface-coats in adult rat brain as detected by staining with cationic iron colloid. Arch Histol Cytol (1993) **56**, 13-21.
2. Murakami T, Taguchi T and Ohtsuka A: Central nervous cells with strongly negative-charged surface-coats (Japanese text with English abstract). Okayama Igakkai Zasshi (1993) **105**, 49-53.
3. Murakami T, Tsubouchi Y, Tsubouchi A, Ohtsuka A, Taguchi T and Kikuta A: The occurrence of rat spinal cord neurons with strongly negative-charged surface-coats. Arch Histol Cytol **56**, 501-504.
4. Murakami T, Taguchi T and Ohtsuka A: The occurrence in the human brain of neurons with strongly negative-charged proteoglycans. Arch Histol Cytol (1993) **56**, 23-26.
5. Murakami T: Strongly negatively charged extracellular matrix in the mouse central nervous system (Japanese text with English abstract). Okayama Igakkai Zasshi (1994) **106**, 1063-1071.
6. Murakami T, Tsubouchi M, Tsubouchi Y, Taguchi T and Ohtsuka A: The occurrence of neurons with strongly negatively charged surface coats in mammalian, avian, reptilian, amphibian and piscine brains. Acta Med Okayama (1994) **48**, 195-197.
7. Murakami T, Ohtsuka A and Taguchi T: Neurons with intensely negatively charged extracellular matrix in the human visual cortex. Arch Histol Cytol (1994) **57**, 509-522.
8. Murakami T, Hitomi S, Ohtsuka A and Taguchi T: Neurons with perineuronal sulfated proteoglycans in the human visual cortex, with special reference to their reactions to lectins. Arch Histol Cytol (1995) **58**, 357-364.
9. Murakami T, Ohtsuka A, Taguchi T and Piao DX: Perineuronal sulfated proteoglycans and dark neurons in the brain and spinal cord: A histochemical and electron microscopic study of newborn and adult mice. Arch Histol Cytol (1995) **58**, 557-565.
10. Murakami T, Ohtsuka A and Ono K: Neurons with perineuronal sulfated proteoglycans in the mouse brain and spinal cord: Their distribution and reactions to lectin *Vicia villosa* agglutinin and Golgi's silver nitrate. Arch Histol Cytol (1996) **59**, 219-231.
11. Murakami T, Ohtsuka A and Piao DX: Perineuronal sulfated proteoglycans in the human brain are identical to Golgi's reticular coating. Arch Histol Cytol (1996) **59**, 233-237.
12. Tsubouchi Y, Tsubouchi M, Hitomi S, Ohtsuka A and Murakami T: Perineuronal sulfated proteoglycans in the adult rat brain: Histochemical and electron microscopic studies. Acta Med Okayama (1996) **50**, 237-241.
13. Murakami T, Taguchi T, Ohtsuka A, Sano T, Kaneshige T, Owen RL and Jones AL: A modified method of fine-granular cationic iron colloid preparation: Its use in light and electron microscopic detection of anionic sites in the rat kidney glomerulus and certain other tissues. Arch Histol Jpn (1986) **49**, 13-23.
14. Gomori G: Aldehyde-fuchsin: A new stain for elastic tissue. Am J Clin Pathol (1950) **20**, 665-666.
15. Nakagawa T, Schulte BA and Spicer SS: Selective cytochemical demonstration of glycoconjugate-containing terminal N-acetylgalactosamine on some brain neurons. J Comp Neurol (1986) **243**, 280-290.
16. Kaplow LS: Substitute for benzidine in myeloperoxidase stains. Am J Clin Pathol (1975) 63, **451**.
17. Golgi C: Intorno alla struttura delle cellule nervose. Arch Ital Biol (1898) 30, 60-71.
18. Celio MR and Blümcke I: Perineuronal nets: A specialized form of extracellular matrix in the adult nervous system. Brain Res Rev (1994) 19, 128-145.
19. Ramón y Cajal S: Variations in the structure and shape of neurons under normal and pathological conditions, and functional implications of such variations; in Histology of the Nervous System of Man and Vertebrates, Swanson N and Swanson LW eds, Vol. I., Oxford University Press, New York and London (1995) pp 174-190.
20. Ohtsuka A and Murakami T: Dark neurons in the mouse brain: An investigation into the possible significance of their variable appearance within a day and their relation to negatively charged cell coats. Arch Histol Cytol (1996) 59, 79-85.
21. Murakami T and Ohtsuka A: Sleep restores the ratio of dark neurons to light neurons in the central nervous system (Japanese text with English abstract). Okayama Iggakai Zasshi (1995) **107**, 235-242.
22. Murakami T, Ohtsuka A and Yamana S: Dark and light neurons in the central nervous system: A light and electron microscopic study of the mouse brain (Japanese text with English Abstract). Okayama Igakkai Zasshi (1996) **108**, 303-310.

Received August 9, 1996; accepted September 18, 1996.