Lectin-Binding Glycoconjugates in the Subfornical Organ of the Rat

By

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Summary: In the rat subfornical organ (SFO), lectin-binding glycoconjugates were histochemically examined by 9 biotinylated lectins using a streptavidin-biotin peroxidase system. The strong or moderate bindings of *Canavalia ensiformis* (Con A), *Lens culinaris* (LCA) and *Phaseolus vulgaris* erythroagglutinin (ePHA) indicated overall distributions of high mannose, intermediate, hybrid N-linked, and non-bisected and bisected, bi/triantennate N-linked complex oligosaccharides in the rat SFO. Nonsialylated terminal N-acetylglucosamines were detected throughout this organ, as revealed by its stainabilities with *Triticum vulgaris* (WGA) and *Limax flavus* (LFA). In this organ, *Ricinus communis* (RCA-1) specifically bound to vessel-associated structures, whereas *Arachis hypogaea* (PNA) reacted with selected neurons in the central and rostral regions of this organ. *Dolichos biflorus* (DBA) and *Ulex europaeus* (UEA-1) did not stain any histologic structures in the rat SFO. The results obtained in this study provide a basis for comprehensive analyses of glycoconjugates in the rat SFO.

The subfornical organ (SFO) is known to be one of the circumventricular organs located in the third ventricle between the interventricular foramina of the lateral ventricles (Fig. 1) (Hofer, 1959). It is characterized by the absence of blood-brain barrier capable of receiving blood-borne substances, such as angiotensin II (Sirett *et al.*, 1977), atrial natriuretic factor (ANF) (Quirion *et al.*, 1984), steroid hormone (Stumpf *et al.*, 1992) and somatostatin (Krisch, 1992). In the rat, the SFO has been subdivided into three (rostral, central and caudal) regions (Fig. 2) (Dellman and Simpson, 1976). The rostral and caudal regions are dominated by nerve projections interspersed with a few neurons and glial cells, whereas the central region contains most of the neuronal perikarya, glial cells and highly developed vessel systems. This organ has been reported to participate in the control of body fluid balance and in the neuroendocrine functions (Miselis *et al.*, 1979; Summy-Long and Severs, 1979; Lepetit *et al.*, 1992), however, its exact functions still remain to be elucidated.

Lectins are proteins or glycoproteins of non-immune natures, which bind to specific sugar residues, sugar linkage, or oligomers in glycoconjugates. They have been introduced into the histochemistry of carbohydrates and have widely been used as valuable probes for

the specific detection of oligosaccharide moieties in glycoconjugates (Spicer and Schulte, 1992). In mammalian neural tissues, glycoconjugates have been postulated to play a role in a number of important physiological activities, such as cell to cell interactions, myelination, synapse formation, impulse conduction and storage and release of hormones and biogenic amines (Margolis and Margolis, 1979). Despite these, however, only few attempts have so far been made to histochemically study glycoconjugates contained in the SFO.

The aim of the present study was to demonstrate the localizations and histochemical properties of specific sugar residues of glycoconjugates in the SFO of the rat by means of techniques of light microscopic lectin histochemistry.

Materials and Methods

Lectins

The following biotinylated lectins were obtained from Honen Corporation (Tokyo, Japan); *Canavalia ensiformis* (Con A), *Phaseolus vulgaris* erythroagglutinin (ePHA), *Lens culinaris* (LCA), *Triticum vulgaris* (WGA), *Ricinus communis* (RCA-1), *Arachis*

hypogaea (PNA), *Dolichos biflorus* (DBA) and *Ulex europaeus* (UEA-1). Biotinylated *Limax flavus* (LFA) lectin was purchased from EY Laboratories (CA, USA) (Table 1).

Tissue preparations

Brains were dissected out from 6 adult male Sprague-Dawley rats weighing 250–330 g (8–10 weeks of ages) following the sacrifice of the donor animals by decapitation under Nembutal anaesthesia. The entire brain from each rat was immediately sliced on sagittal planes at a thickness of 5 mm and then slices containing the SFO were fixed in Bouin's fluid for 6 hours at room temperature. Following fixation, the fixed tissues were washed and dehydrated in 100% ethanol series, cleared in xylene and embedded in paraffin wax. Sections were cut at 5 µm in thickness and mounted on glass slides without any adhesives. Tissue sections were then dewaxed with xylene, hydrated in an ethanol series of descending concentrations and subjected to the lectin histochemical methods as described below. The stained sections were dehydrated in a graded ethanol series, cleared in xylene and mounted in Harleco synthetic resin (HSR) (Kokusai Shiyaku, Kobe, Japan).

Lectin histochemistry

Dewaxed and hydrated tissue sections were immersed in 0.3% H₂O₂ in methanol for 30 min to suppress the activity of endogenous peroxidase, rinsed in phosphate

buffer saline (0.01 M, pH 7.4) (PBS), treated with 0.5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo, USA)-PBS for 15 min and reacted with a biotinylated lectin (Table 1) solution diluted 1:40 with BSA-PBS at room temperature for 1 h. The slides were then rinsed in PBS, incubated with streptavidin-horse-radish peroxidase (Seikagaku Corp., Tokyo, Japan) at room temperature for 10 min, rinsed in PBS and incubated in diaminobenzidine (DAB) (Katayama Chemical Co., Osaka, Japan)-H₂O₂ medium (DAB 30 mg, 30% H₂O₂ 26 µl, 0.05 M Tris HCl buffer 150 ml) (pH 7.6) at room temperature for 15 min. The specificities of lectin binding reactions were substantiated by incubation of control tissue sections in lectin solutions containing respective inhibitory sugars (Table 1).

Results

In the present study, the histochemical reactions obtained were evaluated in terms of the three regions of the SFO (Fig. 2), as described in the "Introduction".

Con A

In the SFO, the lectin Con A (Fig. 3) uniformly bound to all the histologic structures in the three regions, including neuronal perikarya, their dendrites and axons, afferent and efferent nerve projections, glial cells, ependyma, vascular walls, and perivascular spaces. The

Table 1. Biotinylated lectins used in the present study

origins	abbreviations	specificities	inhibitory sugars
<i>Canavalia ensiformis</i>	Con A	α-D-glucosyl and α-D-mannosyl residues in high mannose, intermediate and small complex N-linked sequences	0.2M α-methyl-D-mannoside
<i>Phaseolus vulgaris</i> erythroagglutinin	ePHA	Bisected bi- and triantennate complex N-linked sequences	None
<i>Lens culinaris</i>	LCA	α-D-mannose in non-bisected bi- and triantennate, complex N-linked sequences	0.2 M α-methyl-D-mannoside
<i>Triticum vulgaris</i>	WGA	Di-N-acetyl chitobiosyl, N-acetylglucosaminyl and some sialyl residues	0.1 M di-N-acetyl chitobiose
<i>Ricinus communis</i>	RCA-1	Galactosyl β1,4 N-acetylglucosaminyl sequences > β-galactosyl terminals > α-galactosyl terminals	0.1 M D-galactose
<i>Arachis hypogaea</i>	PNA	Galactosyl β1,3 N-acetylgalactosaminyl > N-acetyl-lactosaminyl sequences	0.1 M D-galactose
<i>Dolichos biflorus</i>	DBA	α-N-acetylgalactosaminyl terminals	0.1 M N-acetyl-galactosamine
<i>Ulex europaeus</i>	UEA-1	α-L-fucosyl terminals	0.1 M α-L-fucose
<i>Limax flavus</i>	LFA	Certain sialyl terminals	None

neuronal perikarya and ependyma were stained more vividly, as compared with the rest of Con A reactive histologic structures.

ePHA

ePHA (Fig. 4) bound in various intensities to the same histologic structures as those exhibiting positive Con A reactions. In the neuronal perikarya, however, this lectin did not react so markedly as Con A.

LCA

LCA (Fig. 5) bound to neuronal and glial networks in all the regions of the SFO. The neuronal perikarya and ependymal cells were stained more strongly. The blood vessels contained in all the regions did not exhibit any strong reactions with this lectin (Fig. 5).

WGA

WGA (Fig. 6) moderately stained all the histologic structures in the SFO, however, some neuronal and glial cells and ependymal cells exhibited strong positive reactions.

RCA-1

Characteristically, RCA-1 (Figs. 7, 8) stained vividly vessel-associated structures, certain neuronal and glial cells and ependymal cells throughout this organ. In particular, such positive reactions were widely distributed in the central and caudal regions. This lectin did not bind to other histologic structures than those mentioned at all.

PNA

The lectin PNA (Figs. 9, 10) strongly bound to selective neuronal perikarya, their dendrites and axons, nerve projections in both the central and rostral regions of the SFO.

LFA

The lectin LFA (Fig. 11) exhibited doubtful reactions throughout the organ, whereas the epithelial cells of the choroid plexus were stained moderately by this lectin.

DBA, UEA-1

Both the lectins DBA (Fig. 12) and UEA-1 (Fig. 13) did not stain any histologic structures in this organ, whereas ventral hippocampal commissure in contact with the SFO exhibited moderate positive reactions.

Controls

If each lectin was pre-absorbed with the appropriate inhibitory sugars, any positive reactions was not obtained throughout all the histologic structures composing the SFO (Fig. 14).

Discussion

In light microscopic histochemistry of glycoconjugates using paraffin sections, lectins are generally known to visualize oligosaccharides of glycoproteins, but not glycosaminoglycans with a possible exception of keratan sulfate (Spicer and Schulte, 1992).

Con A, LCA and ePHA have been used to identify various classes of N-linked oligosaccharides (Table 1) (McMahon *et al.*, 1989). Thus, the strong or moderate positive reactions of Con A, LCA and ePHA throughout the rat SFO suggest that high mannose, intermediate and hybrid N-linked, and non-bisected and bisected, bi/triantennate complex N-linked oligosaccharides are widely distributed in the rat SFO. WGA specifically reacts with N-acetylglucosamine and certain sialic acid residues (Debray *et al.*, 1981) and incidentally binds to keratan sulfates (Toda *et al.*, 1981). In contrast, LFA exhibits a specificity exclusively towards sialic acid residues (Miller *et al.*, 1982). Therefore, the strong or moderate stainings with WGA and doubtful reaction with LFA are taken to suggest the presence of glycoconjugates with N-acetylglucosamine but without sialic acids in this organ. RCA-1 binds to galactose (β 1,4) N-acetylglucosamine and terminal galactose (Debray *et al.*, 1981). PNA reacts not only with terminal galactose (β 1,3) N-acetylgalactosamine but with galactose (β 1,4) N-acetylglucosamine (Lotan and Sharon, 1978). In the rat SFO, therefore, oligosaccharides with galactose (β 1,4) N-acetylglucosamine and/or terminal galactose are localized in the vessel-associated structures, certain neuronal and glial cells and ependymal cells, whereas those with terminal galactose (β 1,3) N-acetylgalactosamine are contained in the selected neurons of the central and rostral regions of this organ. DBA exhibits a specificity towards terminal N-acetylgalactosamine of O-glycosidically linked oligosaccharides (Baker *et al.*, 1983). UEA-1 specifically reacts with α -Fucose at a branch or terminal position of oligosaccharides (Alroy *et al.*, 1988). In the SFO, oligosaccharides with such carbohydrate moieties were devoid of any histologic structures involved.

In mammalian nervous system, certain lectins have been shown to interact with both neuronal and glial cells. For instance, Con A and RCA were reported to bind to post-synaptic densities of isolated cortical synaptic membrane (Gurd, 1977). Further, PNA stained most small neurons in sensory ganglia and myelinated fibers in the white matter of the spinal cord (Streit *et al.*, 1985), whereas *Vicia villosa* lectin with a specificity for terminal N-acetylgalactosamine reacted with most GABAergic neurons in the thalamic reticular nucleus of rodent brains (Nakagawa *et al.*, 1986). In neuroglia, RCA-1 and *Griffonia simplicifolia* lectin have been presumed as a microglial cell marker (Mannoji *et al.*, 1986; Boya *et al.*, 1991). In addition, receptors with

different functions have been reported to show affinity towards various lectins in mammalian nervous system. Nicotinic acetylcholine receptors from rat brain exhibited affinities towards Con A, WGA and RCA (Salvatera *et al.*, 1977). Glutamate binding proteins containing synaptic membrane (Michaelis, 1975) and opiate receptors (Gioannini *et al.*, 1982) were visualized by Con A and WGA respectively. Since the SFO has been recorded to consist of neurons and glia with a variety of receptors for neurotransmitters and circulating substances (Sirett *et al.*, 1977; Quirion *et al.*, 1984; Palkovits *et al.*, 1992; Weindl *et al.*, 1992), the present lectin staining reactions could perhaps be correlated with the structures and functions of these receptors. In mammalian SFO, the rostral region has been reported to consist of functionally important fibers containing output pathways concerned with the maintenance of body fluid and cardiovascular homeostasis (Sposito and Gross, 1987; Johnson *et al.*, 1992). In the rat SFO, therefore, the PNA-reactive glycoconjugates localized in this region could possibly be correlated with the transmission of informations received.

The results obtained in the present study are believed to be useful informations for the elucidation of the true physiological functions of the rat SFO. However, further critical studies with electron microscopic lectin histochemistry may be needed for a thorough understanding of the true physiological activities of the mammalian SFO.

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Explanation of Figures

Plate I

Fig. 1. A diagrammatic illustration of a sagittal section through the region of the subfornical organ (SFO) in the rat brain.
SFO: subfornical organ

Fig. 2. A mid-sagittal section indicating the three regions in the rat subfornical organ (SFO). 3cp, choroid plexus of the third ventricle; vhc, ventral hippocampal commissure. UEA-1 stained. (×90)

Plate II

Figs. 3–14. Light microscopic localization of lectin binding sites in the rat SFO, Sagittally sectioned.

Fig. 3 Con A (×200)	Fig. 4 ePHA (×200)
Fig. 5 LCA (×200)	Fig. 6 WGA (×200)
Fig. 7 RCA-1 (×200)	Fig. 8 RCA-1 (×400)
Fig. 9 PNA (×200)	Fig. 10 PNA (×400)
Fig. 11 LFA (×200)	Fig. 12 DBA (×200)
Fig. 13 UEA-1 (×200)	
Fig. 14 PNA in the presence of 0.1 M D-galactose (Control) (×200)	