ELSEVIER

Hearing Research 78 (1994) 49–57

HEARING RESEARCH

The distribution of *N*-acetylgalactosamine in the cochlear nucleus of the gerbil revealed by lectin binding with soybean agglutinin ☆

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Received 26 April 1993; revision received 5 December 1993; accepted 31 March 1994

Abstract

A horseradish peroxidase conjugated lectin from *Glycine max* (soy bean agglutinin; SBA) was used to characterise the distribution of *N*-acetylgalactosamine in the cochlear nucleus of the mongolian gerbil. SBA bound differentially to a variety of structures within the cochlear nucleus. Specific SBA labelling was associated with large non-granule neurones of variable size and shape throughout the cochlear nucleus. Compared to adjacent Nissl-stained sections 80% of the non-granule cells in the dorsal cochlear nucleus (DCN) and more than 90% of the non-granule cells in the ventral cochlear nucleus (VCN) bound SBA. The variation in location, size and shape as well as the high percentage of the labelled neurones suggest that cells of several, if not all, non-granule cell types, which have been described for the cochlear nucleus according to the usual Nissl schemes, are SBA positive. Granule cells did not bind SBA because all SBA-labelled cells had diameters above 10 μm. Diffuse labelling, not systematically associated with cells or fibres, was high in the molecular and fusiform cell layers of the DCN and that part of the granule cell area located close to the surface of the VCN. Darkly labelled granules (up to 2 μm diameter) were prominent in the area of the VIIIth nerve root. After long SBA incubations, they were also present in VCN and to a lesser degree in DCN. The results are discussed with respect to findings in other brain areas and the possible co-localisation of gamma aminobutyric acid (GABA), parvalbumin and *N*-acetylgalactosamine.

Key words: Gerbil; Brain stem; Cochlear nucleus; Lectin; *N*-acetylgalactosamine; Glycoconjugate

1. Introduction

Lectin histochemistry has been used as a tool to characterise the distribution of specific glycoconjugates (Alroy et al., 1988). The differential distribution of glycoconjugates in tissues and cell types as well as during differentiation and development (Damjanov, 1987; Hofmann and Meyer, 1991; Holthöfer and Virtanen, 1987; Pfenninger and Maylie-Pfenninger, 1981) indicates that glycoconjugates are of some functional importance. Glycoconjugates are probably involved in cell-cell interactions. In the case of tumour pathology this was demonstrated by the presence of specific sugar compositions in the affected tissue which correlated with tumour characteristics (Gabius and Bardosi, 1991). However, the detailed mechanisms of glycoconjugate functions(s) remain to be resolved. Despite the growing interest in the field, the characterisation of the distribution of specific lectin binding sites in the brain is not very detailed at present.

It has been shown that characteristic distributions of glycoconjugates in the mammalian brain can be demonstrated by lectin cytochemistry (Nakagawa et al., 1986). Nakagawa et al. (1986) described in detail the binding patterns of lectins, which are specific for *N*-acetylgalactosamine, in the cortex of rat, mouse, dog and man. Extracortically, they found weak to strong binding in large cells at different brain locations. In the cerebral cortex of the rat, it was shown that lectins that are specific for *N*-acetylgalactosamine labelled a parvalbumin-immunoreactive sub population of gamma aminobutyric acid (GABA) containing neurones (Kosaka and Heizmann, 1989).

☆ Presented in part at the 16th Midwinter Meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, FL, USA, February, 1993.

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SSDI 0378-5955(94)00070-7

In recent years a growing number of studies used the mongolian gerbil as a model to investigate a variety of aspects related to their hearing. These studies include aspects of development and ageing of cochlear function (Arjimand et al., 1988; Harris et al., 1990; Norton et al., 1991; Schmiedt et al., 1990; Tarnovski et al., 1991; Woolf and Ryan, 1984) and the occurrence of pathological changes in the cochlear nucleus (Czibulka and Schwartz, 1991; Czibulka and Schwartz, 1993; Faddis and McGinn, 1993; Ostapoff and Morest, 1989). The cochlear nucleus with its complex structural organisation in mammals is the first relay station of the ascending auditory pathway where the afferent fibres from the cochlea terminate. Several subdivisions and a variety of cell types have been described with different methods (e.g. Cant, 1992; Fleckeisen et al., 1991; Moore, 1986; Osen, 1969; Webster and Trune, 1982). The aim of the present study was to test if the distribution *N*-acetylgalactosamine can be used as a marker for specific cochlear nucleus structures in the mongolian gerbil.

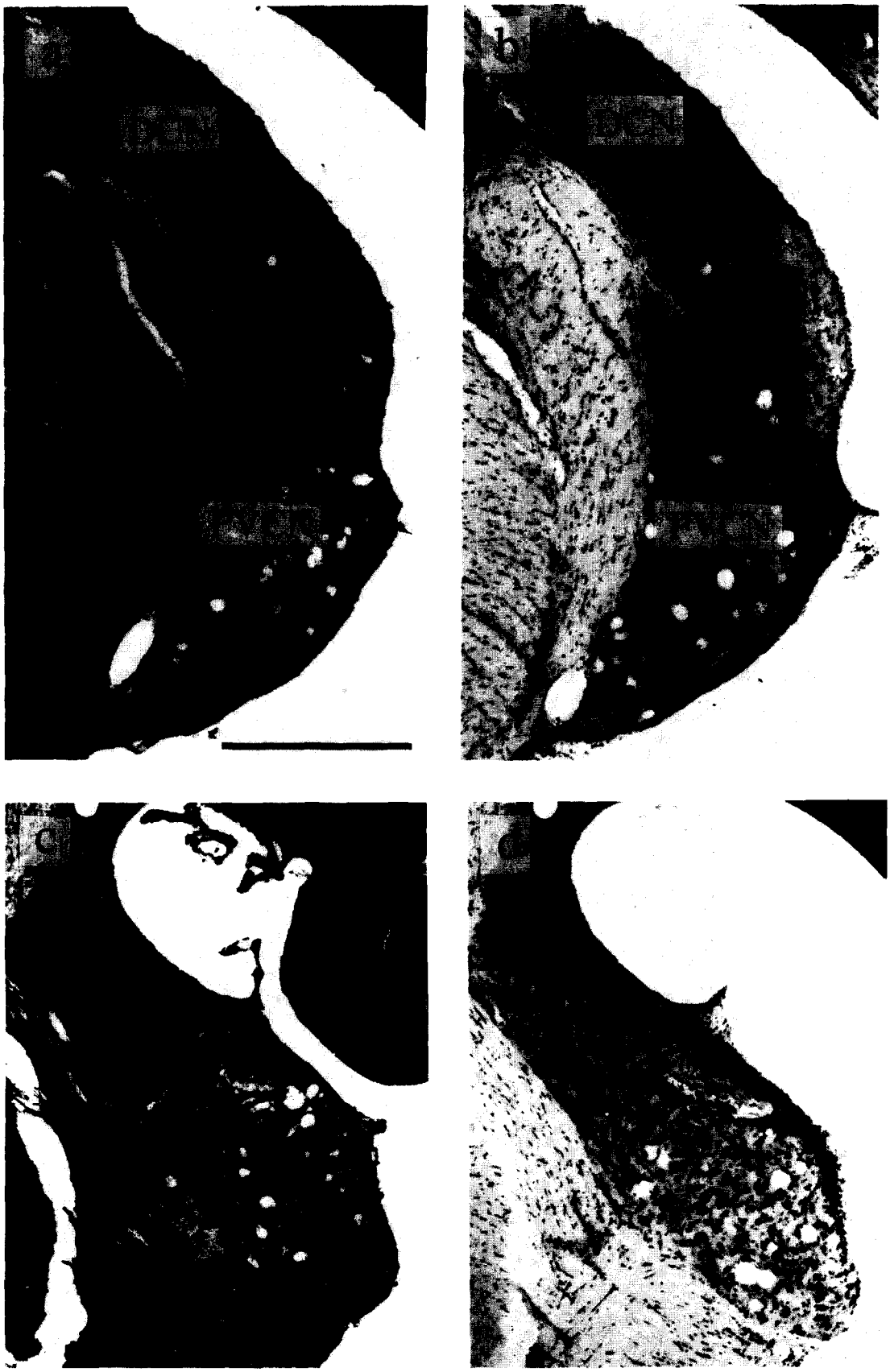
2. Materials and methods

Fourteen adult gerbils (4 male and 10 females) aged 3, 5 or 9 months were anaesthetised by intraperitoneal injection of a lethal dose of Nembutal (0.2 ml; 60 mg/ml). When deeply anaesthetised, they were transcardially perfused with approximately 50 ml Tyrodes solution (154.5 mM NaCl; 2.7 mM KCl; 1.1 mM $MgCl_2$; 3.5 mM Na_2HPO_4; 8.3 mM NaH_2PO_4; 5.5 mM Dextrose), followed by approximately 500 ml of 4% paraformaldehyde in 0.1 M, pH 7.4 sodium phosphate buffer over 30 min. The brains were dissected from the skull and infiltrated by agitation in 30% sucrose solution in 0.1 M, pH 7.4 sodium phosphate buffer at 4°C for 16–24 h. Serial coronal sections at 25 μm were cut through the brainstem on a cryostat. Selected sets of sections were used for lectin histochemistry, Nissl staining or as controls.

The histological processing for the demonstration of *N*-acetylgalactosamine was performed on floating sections following a modified protocol of Hofman and Meyer (1991). The lectin from *Glycine max* (soybean agglutinin, SBA) was selected because it has been shown to selectively bind to *N*-acetylgalactosamine in other preparations of neuronal tissue (Nakagawa et al., 1986; Hofman and Meyer, 1991; Key and Giorgi, 1986). Endogenous peroxidase was inactivated by treatment in 0.5% H_2O_2 in 0.1 M, pH 7.4 sodium phosphate buffer for 30 min. After 2 washes in 0.1 M, pH 7.4 sodium phosphate buffer, sections were incubated in 1% bovine serum albumin (Sigma: A7638) in 0.1 M, pH 7.4 sodium phosphate buffer for 1 hour to block non-specific binding sites. After a wash in 0.1 M, pH 7.4 sodium phosphate buffer, sections were transferred into a solution of the horseradish peroxidase conjugated SBA (Sigma: L9508 and L1270) in 0.1 M, pH 7.4 sodium phosphate buffer. The lectin concentration was 10 or 20 μg/ml. Incubation was either 4 h at room temperature or overnight at 4°C. In one series of controls, incubation was performed in 0.1 M, pH 7.4 sodium phosphate buffer without any lectin addition. In another series of controls *N*-acetylgalactosamine (Sigma A2795) was added to the lectin solution at a concentration of 0.1 M and incubated for 30 min before it was applied to the sections. After 3 washes in 0.1 M, pH 7.4 sodium phosphate buffer, sections were subjected to a nickel-intensified diamino benzidine reaction. Sections were incubated for 15 min in a solu-

Fig. 1. Sections through the cortex of the gerbil. A control section (a) treated with preincubated lectin (preincubation of 20 μg/ml SBA with 0.1 M *N*-acetylgalactosamine for 30 min and incubation of the section for 4 h), but otherwise unstained shows no specific labelling (compare to b). Structures are only recognisable due to the very high contrast print. In comparison to the control the untreated SBA (20 μg/ml for 4 h) selectively labelled a number of non-pyramidal neurones (b), resembling results obtained in other species by Nakagawa et al. (1986). Surface of the cortex is to the top. Scale Bar is 200 μm.

Fig. 2. Overview showing the cochlear nucleus at low magnification. The SBA binding pattern (10 μg/ml for 4 h; a, c) and the Nissl staining pattern (b, d) are shown for adjacent sections from the DCN/PVCN area (a, b) and the AVCN (c, d). The molecular and fusiform cell layer of DCN and the granule cell area on the ventrolateral surface of PVCN show heavy SBA background labelling. Many cells show distinct SBA labelling in PVCN and DCN (a) as well as in AVCN (c). Background labelling is also increased near the surface of AVCN (c). Scale Bar is 500 μm. Dorsal is to the top and lateral to the right of the figure.


a
b
c
c

tion containing 0.05% diamino benzidine, 0.02% nickel ammonium sulphate and 0.001% H_2O_2 in 0.1 M, pH 7.4 sodium phosphate buffer. After 4 washes in 0.1 M, pH 7.4 sodium phosphate buffer, sections were mounted on gelatine-coated slides, air dried, washed in xylene and coverslipped using Entellan (Merck, No. 7961). For comparison, Nissl staining was performed on some selected sections. These sections were defatted at least over night in xylene and hydrated through a graded series of ethanol (100, 80, 70, 50%, distilled water). They were stained for 10 min in a 1% aqueous solution of cresyl fast violet (Fluka No. 61120). Sections were then rinsed in distilled water and 70% ethanol. They were differentiated in 96% ethanol containing 1% acetic acid, shortly rinsed in 3 changes of 96% ethanol, 2 changes of 100% ethanol and 2 changes of xylene. From the xylene sections were coverslipped using Entellan.

For a quantitative analysis adjacent SBA-labelled and Nissl-stained sections through the dorsal cochlear nucleus (DCN) -postero-ventral cochlear nucleus (PVCN) complex and the antero-ventral cochlear nucleus (AVCN; e.g. Fig. 2) were selected in 5 animals. Photographic reconstructions of the sections at a final magnification of 200x were used to determine the numbers of non-granule cells. Cells were counted separately for the following regions of the cochlear nucleus: (1) the molecular layer of DCN; (2) the fusiform layer of DCN; (3) the central region of DCN; (4) the PVCN; and (5) the AVCN.

3. Results

In the controls that were incubated without the addition of horseradish peroxidase-conjugated SBA, no staining was detectable. Due to the destruction of endogenous peroxidase, even erythrocytes, which usually show heavy labelling after diamino benzidine reactions, were unstained. Thus, all the staining found in the SBA sections was due to the horseradish peroxidase bound to the sections via the conjugated lectin. In the second series of controls where the SBA had been preincubated with 0.1 M *N*-acetylgalactosamine specific labelling was also absent (Fig. 1a). Fig. 1b shows a typical section through the cerebral cortex of the gerbil after SBA staining to demonstrate the similarity of the present results with those of previous studies in other mammalian species (Nakagawa et al., 1986).

SBA gave a distinct staining pattern in the cochlear nucleus (Fig. 2). The best concentration for a differential staining was 20 μg/ml for the peroxidase-conjugated SBA, but similar results were also obtained with 10 μg/ml. With the short (4 h) incubation in the lectin solution, staining was generally less intense than with the long (14 h) incubation. The pattern of labelling was, however, similar and showed only small variations across animals. Quantitative cell counts from adjacent Nissl- and SBA-stained sections revealed that the incubation parameters (lectin concentration and incubation time) had no systematic effect on the percentage of cells labelled by SBA (Table 1).

There were three different types of SBA labelling in the gerbil cochlear nucleus. The first type was a diffuse background staining that varied systematically within the cochlear nucleus. On the lightmicroscopical level, the background was not systematically associated with recognizeable structures like individual cells or fibres. The molecular and fusiform cell layers of the DCN showed a distinct, heavy background labelling that decreased from the fusiform cell layer towards the central region of the dorsal cochlear nucleus (DCN; Fig. 2a,

Table 1
The numbers of non-granule cells (> 10 μm) that were counted in adjacent Nissl- and SBA-stained sections

	G1		G2		G3		G4		G5		Total	
	Nissl	SBA	Nissl	SBA	Nissl	SBA	Nissl	SBA	Nissl	SBA	Nissl	SBA
Concentration	–	10 μg/ml	–	10 μg/ml	–	20 μg/ml	–	20 μg/ml	–	20 μg/ml	–	–
Incubation time	–	4 h	–	4 h	–	14 h	–	4 h	–	4 h	–	–
DCN	319	230	379	293	334	268	247	187	248	238	1527	1216
Molecular	17	10	36	12	38	13	36	10	35	16	162	61
Fusiform	121	103	152	111	130	104	101	89	93	90	597	497
Deep	181	117	191	170	166	151	110	88	120	132	768	658
PVCN	136	112	166	156	116	117	100	82	143	142	661	609
AVCN	372	366	445	436	401	347	373	370	441	405	2032	1924
Total	827	708	990	885	851	732	720	639	832	785	4220	3749

Sections through the DCN/PVCN complex and through the AVCN were selected from 5 animals (G1–G5; indicated in the top row of the table) for the analysis. The second row indicates Nissl- or SBA-stained material. The lectin concentration and the incubation times for the different specimens are given in row 3 and 4 of the table. The following rows show the cell counts obtained in different areas of the cochlear nucleus in individual specimens. The bottom row and the two right columns give the total numbers obtained from the 5 specimens.

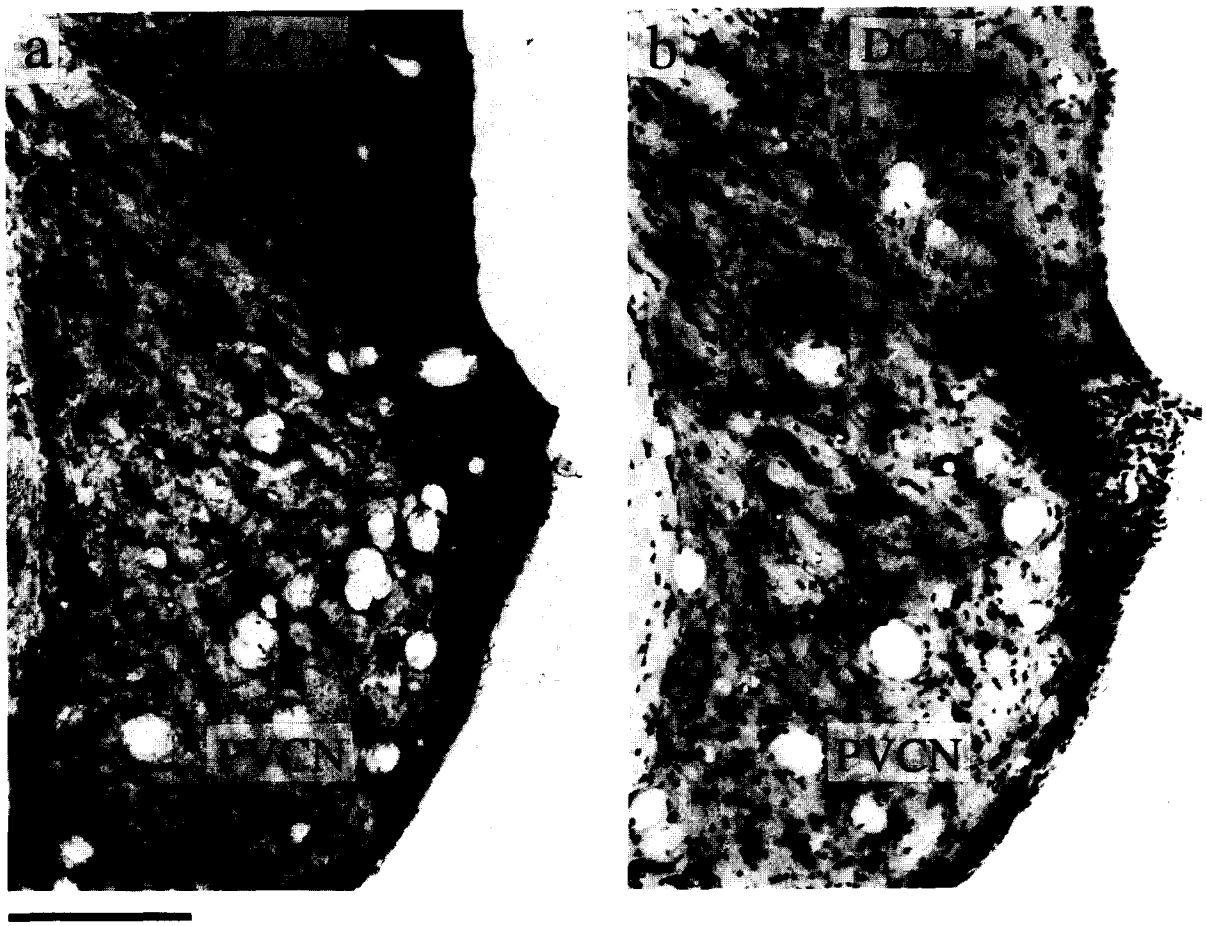


Fig. 3. Comparison of a SBA- (10 μg/ml for 4 h; a) and Nissl-stained (b) detail from DCN and PVCN. The diffuse background labelling in the molecular layer of DCN and the granule cell area on the ventrolateral surface of PVCN is obvious. However, the background is absent in the granule cell layer separating DCN and PVCN (indicated by the arrows). Despite the presence of many granule cells in the Nissl stained section (b) there are none such cells labelled by SBA (a). However, many other, non-granule cells are distinctly labelled by SBA. The extremely dark appearance of the edge of the SBA-stained section (a) is an artefact due to curling up of tissue during the drying process. The orientation of the sections is as in Fig. 2. Scale bar is 200 μm.

3a, 5a). The highest level of diffuse background staining in the ventral cochlear nucleus (VCN), comparable to that found in the molecular layer of DCN, was present close to the surface of the cochlear nucleus (Fig. 2a, c). The granule cell layer separating DCN and the posterior ventral cochlear nucleus (PVCN) did not show high background labelling (Fig. 2a, 3a).

The second type of labelling consisted of small (< 2 μm), very heavily labelled granular structures that were scattered within the auditory fibres of the nerve root in all animals (Fig. 4). In animals with long lectin incubation times, these intensely labelled granules were also present in the VCN (Fig. 5d) and to a lesser degree in the DCN. After short SBA incubation, there were less of these granules labelled across the cochlear nucleus, or they were completely missing (e.g. Fig. 5c) except for the nerve root.

The third type of staining was associated with individual neurones (Fig. 5). Staining intensity was variable, but many non-granule cells in DCN and VCN displayed intense SBA binding. Labelling was restricted to the soma and only a short segment of the proximal dendrites, giving many cells a rather irregular outline (Fig. 5b, 6). At high magnifications the surface

Fig. 4. The area of the nerve root in a SBA stained (20 μg/ml for 4 h) section at high magnification. Dark, heavily-labelled granules and intensely-labelled interstitial cell are scattered among the nerve fibres of the VIIIth nerve. Scale bar is 100 μm.

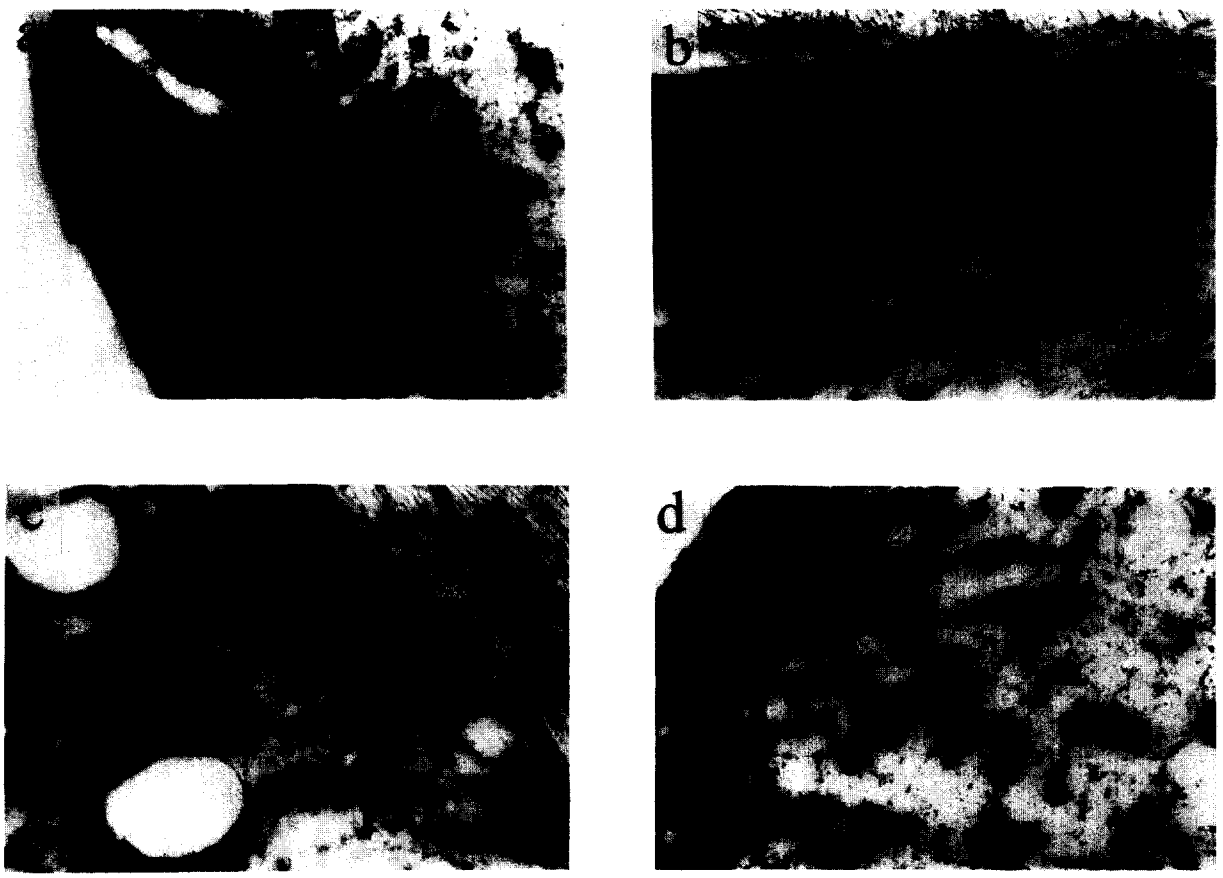


Fig. 5. High-power pictures of SBA labelled cells from different regions of the cochlear nucleus. In the DCN (20 μg/ml for 14 h; a) a cell is shown that is labelled above the rather high background in the molecular layer (arrow). The extremely dark appearance of the edge of the section is an artefact due to curling up of tissue during the drying process. Many other cells are labelled in the fusiform and central layers of DCN. In the dorsal PVCN (20 μg/ml for 4 h; b) large neurones, including parts of their proximal dendrites, are labelled by SBA. In the ventral PVCN (20 μg/ml for 4 h; c) many cells of differing size are SBA positive. In AVCN (20 μg/ml for 14 h; d) many cells show intense SBA labelling. Scale bar is 50 μm.

of labelled cells appeared granular and not very smooth (e.g. Fig. 5b, 6b). In the 25 μm sections, where the somata of many cells were completely contained within the section, it was not easy to determine which cellular structures had accumulated the label. However, some cells were only partially contained in individual sections. Focusing on the cut surface of these cells made it possible in some cases to determine which cellular structures had actually been stained. The best views were obtained from some large cells that had been intensely labelled in the dorsal part of PVCN and where diffuse background staining was very low (Fig. 6). The labelling appeared restricted to the surface of the neurones and neither their cytoplasm nor the nucleus had accumulated obvious label; no stained structures were discernible in the inside of the transsected cells (Fig. 6a). The cell surface appeared as a mesh of irregularly-stained granules, due to the fact that the density of labelling on the cell surface had a quite irregular distribution (Fig. 6b).

The discrimination of the cell types as described for the cochlear nucleus on the basis of Nissl-stained material (Fleckeisen et al., 1991; Osen, 1969, Cant, 1992; Morest et al., 1990) was not unequivocally possible in the SBA labelled sections. However, all SBA positive cells were rather large (> 10 μm), indicating that granule cells that had diameters of 5–8 μm (as determined from the Nissl stained sections) did not exhibit significant SBA binding. Close inspection of the fusiform cell area of DCN and the granule cell area of VCN confirmed this impression (Fig. 3a, 5a). Small, intensely SBA-positive cells could not be identified in these areas despite the presence of many granule cells in corresponding areas of adjacent Nissl-stained sections (Fig. 3b). In these areas, diffuse background was rather high and might have obscured small labelled cells. However, in other areas of VCN with very low background, no small SBA-positive cells were detectable, although they were frequently visible in Nissl stained material (eg. arrow in Fig. 3). Furthermore, in the

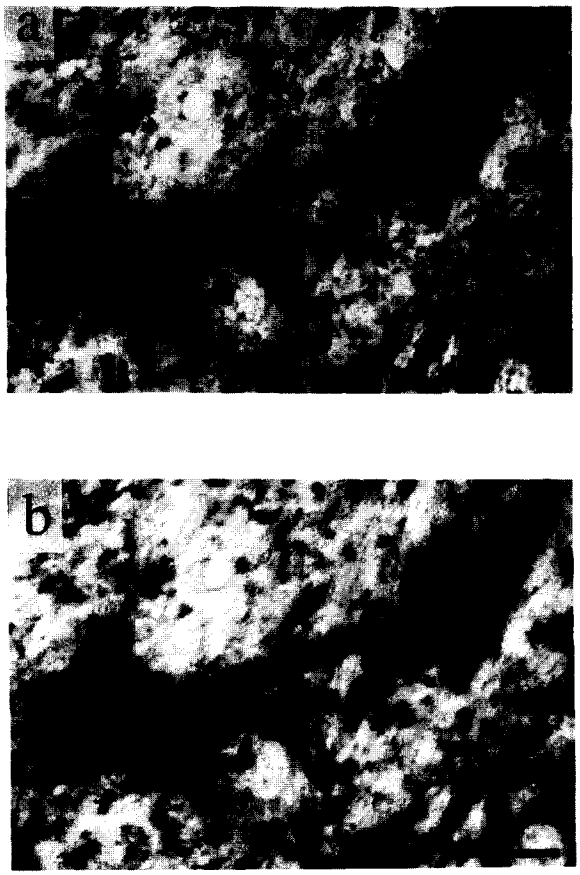


Fig. 6. High-power view of a presumed octopus cell in the dorsal PVCN. The labelling (20 μg/ml for 14 h) appears restricted to the surface of the cell. Focusing on the surface of the section (a) reveals an unstructured cytoplasm and intense labelling of the cell surface. Focusing in the same cell on the cell surface within the section (b) reveals a mesh of variable labelling density that causes a very inhomogenous appearance of the cell surface. Scale bar is 20 μm.

fusiform cell layer of DCN there were, despite the high background, other larger cells clearly SBA positive above the background (arrow in Fig. 5a). These findings demonstrate that granule cells did not bind SBA.

In the SBA-stained material it was very difficult to identify different cell types because the nucleus and cytoplasm were unstained. Thus, only octopus cells and spherical cells could be clearly identified in the SBA-labelled sections of the VCN due to their location, size and shape. The most conspicuous cells in the SBA stained sections were octopus cells in the dorsal and central portions of PVCN, which appeared quite irregular due to the label on proximal dendrites, (Fig. 3a, 5b, 6). At the most rostral tip of the anterior ventral cochlear nucleus (AVCN) there were many SBA positive spherical cells (Fig. 5d).

The quantitative comparison of cell numbers in adjacent SBA- and Nissl-stained sections (Table 1) revealed that most of the cells in the gerbil cochlear nucleus bind SBA. In AVCN and PVCN the numbers of SBA stained cells were on average 96% and 92% of the number of non-granule cells in adjacent Nissl-stained sections. Based on the high proportion of cells labelled by SBA it can be concluded that also the other cell types described for the ventral cochlear nucleus (e.g. multipolar and globular) bind SBA.

In the DCN a comparison of non-granule cell numbers in corresponding areas of adjacent SBA- and Nissl-stained sections revealed that on average only 80% of the cells bound SBA (Table 1). However, the difference was most pronounced for the molecular layer, where the number of cells found in SBA-stained sections was on average only 38% compared to that of adjacent Nissl stained sections. The relatively small round cells in the molecular layer that were labelled by SBA (arrow in Fig. 5a) resemble cartwheel or stellate cells. Obviously SBA labelled only a subpopulation of these cells in the molecular layer. In the fusiform cell layer and in the deep region of the DCN the majority of cells (83% and 86% respectively) appeared labelled by SBA as compared to Nissl-stained sections, indicating that probably all cell types in this area bound SBA. Due to their size and location fusiform cells and giant cells could be identified in SBA-stained sections of the DCN.

4. Discussion

The observation that SBA labels specifically the surface of somata and proximal dendrites agrees well with the findings of Nakagawa et al. (1986) and Kosaka and Heizmann (1989). They described that SBA labelling appeared as unevenly distributed periodic foci on the surface of the somata and dendritic shafts of nonpyramidal cells in the cerebral cortex of several mammalian species. Nakagawa et al. (1986) found no cytoplasmic staining with the exception of some cells that showed perinuclear labelling that was interpreted as Golgi zones. This description resembles very much the labelling characteristics found here in the gerbil cochlear nucleus (e.g. Fig. 6) as well as in the gerbil cortex (Fig. 1b). In the gerbil material presented here, it was not possible to determine unequivocally for all smaller cells and for areas of more intense background labelling whether only the surface of stained cells had accumulated the label. The 25 μm thick sections used in this study were not suitable for a more detailed investigation of the distribution of SBA binding sites within the cells.

The histological processing, especially the type of fixation and cryostat/vibratome versus paraffin sections (Alroy et al., 1988; Nakagawa et al., 1986), influences lectin binding to the tissue. However, independent of methodological differences, reasonable numbers

of SBA binding neurones have been demonstrated in the mammalian brain. In contrast to the situation in mammals, SBA labels the surface of only a (sub)population of olfactory neurones in eels (Franceschini and Ciani, 1991) and frogs (Hofmann and Meyer, 1991; Key and Giorgi, 1986), but does not label other cells in the central nervous system of these animals. In mice it has been shown that *Vicia villosa* agglutinin, which labels essentially the same cells as SBA (Kosaka and Heizmann, 1989), fails to produce the typical labelling pattern in 1 week old animals (Nakagawa et al., 1987). Adult-like labelling was observed by the age of 3 weeks. However, in studies of adult *Xenopus* frogs (Hofman and Meyer, 1991) as well as young postmetamorphic frogs (Key and Giorgi, 1986) no labelled cell bodies were found within the central nervous system. Thus age is unlikely to explain the difference in the distribution of *N*-acetylgalactosamine of mammalian and frog brains. Since the function of glycoconjugate expression is only poorly understood, a more detailed and comparative investigation among vertebrate orders might contribute to a better understanding of their function and would be interesting from an evolutionary point of view.

Combination of lectin histochemistry with immunocytochemistry has shown in the cerebral cortex of the rat that the majority of cells (85%) that bound SBA were labelled by antibodies against gamma aminobutyric acid (GABA) and parvalbumin (Kosaka and Heizmann, 1989). The distribution of GABA- or glutamic acid decarboxylase- (GAD) immunoreactivity in the cochlear nucleus has been studied in different species including the gerbil (Wenthold, 1991). GAD-positive neurones were found in the gerbil's AVCN, PVCN and DCN (Roberts and Ribak, 1987) with some indication that globular, spherical and fusiform cells show no GAD labelling. In addition granule cells appear not to be immunoreactive (Wenthold, 1991). The lack of GAD labelling in the above mentioned cochlear nucleus cell types of the gerbil have recently been confirmed for the guinea pig using an antibody against GABA (Kolston et al., 1992). Taking together the evidence from these studies, SBA binding in the cochlear nucleus may, at least for some cells, coexist with GAD or GABA immunoreactivity, however spherical (Fig. 5d) and fusiform cells that show quite prominent labelling by SBA appear to be not GABAergic (Roberts and Ribak, 1987; Kolston et al., 1992).

The distribution of diffuse SBA background labelling in the cochlear nucleus shows some parallels to the distribution of GABA and GAD. Microchemical analysis of GABA revealed that its concentration is highest in the superficial layers of the DCN (Godfrey et al., 1988). In the granule cell area of the VCN and in the superficial layers of DCN there is a very high concentration of GAD immunoreactive puncta (Moore, 1988; Roberts and Ribak, 1987; Wenthold, 1991). The diffuse SBA labelling found here is very pronounced in most of these areas.

The distribution of parvalbumin has been studied in different brain regions of several species (Braun et al., 1991; Seto-Oshima et al., 1990; Solbach and Celio, 1991). The occurrence of parvalbumin immunoreactive cells has been described for the VCN in the last embryonic day of rats (Solbach and Celio, 1991). For adult rats Aoki et al. (1990) found that about 30% of the neurones in DCN contain parvalbumin.

Because almost all non-granule cells in the gerbil cochlear nucleus are stained by SBA (Table 1) some of them are probably GABAergic and contain parvalbumin. However, in contrast to the situation in the cerebral cortex (Kosaka and Heinzmann, 1989) SBA binding is not indicative for GABA- and parvalbumin immunoreactivity in the cochlear nucleus of the gerbil.

Acknowledgements

I wish to thank Prof. J. Strutz for support and comments on the manuscript and Prof. G.A. Manley for his comments and correcting the English. Profs. Chl. Beck and W. Spatz supported the project by providing working facilities at the ENT-Department of the Albert-Ludwig Universität in Freiburg. I thank Prof. M. Vater for introducing me to the cytoarchitecture of the gerbil cochlear nucleus. K. Bielenberg and B. Heimer gave expert help with the histological processing. C. Cavanna provided the final photographic prints. Supported by the DFG (Str 275/2-1).

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