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GLYCOPINION

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It is now recognized that a variety of cell surface molecular interactions are involved in the development and maintenance of the complex structures and networks of the central nervous system. A growing number of cell surface glycoproteins are believed to be important in cell–cell and cell–matrix interactions [1]. The cellular and organizational complexity of the brain indicates that, if protein glycosylation is functionally important, it will hold many clues for our understanding of the role of complex carbohydrates in recognition processes.

Much of the evidence for the functional importance of glycoproteins is based on structural and functional similarities and differences of molecules believed to mediate the interactions. One clue came from the raising of a monoclonal antibody, L2, which was shown to recognize a carbohydrate epitope common to a number of neural adhesion molecules including L1 (NILE, Ng-CAM), *N*-CAM (neural cell adhesion molecule), MAG (myelin associated glycoprotein) and the J1 family [1, 2], and which was also present on sub-populations of lymphoid cells, including natural killer cells (HNK-1 epitope [3]).

Sulphate-3-GlcUA β1 → 3 Gal β1 → 4 GlcNAc β1 → 3 Gal β1 →

Figure 1. Structure of the 'L2 tetrasaccharide' isolated from parent 'L2-glycolipid'; GlcUA = glucuronic acid.

The importance of this L2/HNK-1 epitope in cellular interactions was initially suggested by studies in which the L2 or HNK-1 antibodies inhibited neural cell adhesion or neurite outgrowth in assays *in vitro* [4, 5]. More specifically and directly, L2 tetrasaccharide (Fig. 1) isolated from peripheral nerve glycolipid recognized by the L2 antibody [as in 6] was shown to inhibit not only cell–cell but also cell–substrate interactions [7], suggesting that the L2/HNK-1 carbohydrate epitope was involved as the functional ligand. However, not all glycoforms of neural cell adhesion molecules that are potentially able to carry the L2/HNK-1 epitope are in fact L2-positive; indeed other carbohydrate epitopes (e.g. L3), also believed to be functionally significant, have been identified on some molecules [8], allowing the possibility that differential glycosylation is a means of diversifying the function of a protein.

Such evidence suggests that a wider knowledge of the glycosylation of neural proteins would lead to a better understanding of their role within the brain, and of the fundamental significance of glycoforms for the function of a glycoprotein.

Some of the questions raised by this article written by Dr David Wing include:

- Certain carbohydrate structures, such as the L2/HNK-1 epitope and the polysialic acid chains, are developmentally regulated. To what extent does the glycosylation of other functionally important glycoproteins change during development?
- Does the conserved glycosylation seen in brain *N*-CAMs across species (murine versus bovine) suggest an association with the maintenance of important functions in neural cells/tissue? Further support for this idea comes from the conservaton of glycosylation in Thy-1 from rat and murine brain, despite some 20 changes in amino acid residues. In contrast, a different spectrum of glycoforms is associated with rat Thy-1 when the same protein backbone is expressed in thymocytes.
- A number of neural glycoproteins contain repeat immunoglobulin-like domains which are differently glycosylated in different molecules (Fig. 2). What influences do variations in primary or tertiary structure have on the array of glycoforms in such glycoproteins?
- The sLe x and Le x structures are important ligands for the selectins in the haematolymphoid system (Fig. 3). Is the Le x structure an important ligand in the CNS? What candidate receptors are there? Could any functions of the Le x carbohydrate motif be influenced by the way it is presented both by the protein and within the oligosaccharide chain?
- What is the significance of the anionic (particularly sulfated) species of glycans in neural tissue? Do they impart anti-adhesive properties to the cells in some circumstances, actively discouraging inappropriate cell–cell recognition and adhesion?
- As the functions of sugars on neural glycoproteins are elucidated it will become clear which glycan structures are essential for the integrity of essential cellular interactions in both the central and peripheral nervous systems. How important, for example, is the correct glycosylation of P0 for the maintenance of compact myelin in peripheral nerves? Which therapeutic routes would be most effective in maintaining essential glycan structures during disease?

Neural glycoproteins: do they provide specific clues for our understanding of glycan functions?

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Glycosylation profiles in neural adhesion molecules have been studied in a comparative way as an initial step to widening our knowledge of possible structural–functional relationships. Studies of the neutral and de-sialylated glycan classes of the neural adhesion molecules L1, *N*-CAM, MAG and J1 used Bio-Gel P4 gel permeation chromatographic profiles to pattern the molar proportions of structures having different hydrodynamic volumes [9]. For *N*-CAM, a comparison was made between bovine and murine sources of the molecule, and a remarkable conservation of *N*-linked glycosylation was seen across the species. This conservation of glycosylation dominated any influence on glycan processing of non-identical amino acid sequences in the different *N*-CAM molecules. A comparison of these glycosylation patterns with the other adhesion molecules, L1, MAG and J1 from murine brain, still revealed the same spectrum of chromatographic fractions, although with the pattern varying in a quantitative way ([9] and Table 1).

These results illustrated that neural tissue possessed a significant conservation of its *N*-linked glycosylation processing, and that this was only partially modified at the level of the individual protein type. In addition to this degree of conservation, neural glycosylation has also been shown to be tissue specific from studies of Thy-1 molecules in the rat. Despite identical amino acid sequences in Thy-1 from both the brain and the thymocyte, distinctive *N*-glycosylation patterns (even at individual glycosylation sites) were observed between the tissue sources ([10] and Table 1). In general, Thy-1 molecules did not conform to the earlier statistical analysis of 50 glycoproteins by Pollack and Atkinson [11], showing that more highly processed oligosaccharides routinely occurred at glycosylation sites near the NH_2 terminus, while oligomannose structures were found nearer the carboxyl terminus.

Table 1. Comparison of *N*-linked neutral/desialylated glycan profiles in neural molecules: molecular, species and tissue specificity. The table is based on references [9] and [10] (and unpublished observations by the same authors). Very similar = profiles (from Bio-Gel P4 chromatograms) that can be superimposed (i.e. qualitative and quantitative identity). Different = profiles that cannot be superimposed.

Molecule	*Species*	*Tissue*	N-*linked neutral/ desialo-glycan profile*
N-CAM	mouse/bovine	brain	very similar
N-CAM/MAG/ J1/L1	mouse	brain	different
THY-1	rat	brain/thymocyte	different
THY-1	mouse/rat	brain	very similar

In the neural molecules studied above, oligosaccharides eluting at a hydrodynamic volume of 12.8 glucose units (g.u.) were prominent on the Bio-Gel P4 chromatographic system [9]. Parallel studies on whole brain tissue *N*-linked glycans allowed the preparation of greater quantities of material. Definitive structural analysis of this 12.8 g.u. fraction from the whole tissue preparation showed that, although heterogeneous, a major component carried the Le x determinant on a biantennary oligosaccharide, truncated in one arm, and possessing a bisecting GlcNAc and a core fucose [12].

N-CAM, L1 and MAG, like Thy-1, are all surface glycoproteins belonging to the immunoglobulin superfamily [13–15]. Molecular modelling of Thy-1 has shown that the *N*-linked oligosaccharides are well exposed in relation to the protein [16]. This indicates that the glycans and their epitopes may well be displayed to advantage as potential ligands.

The most notable feature of the glycosylation pattern of J1 (160/180 kDa enriched), in contrast to the neural adhesion molecules *N*-CAM, L1 and MAG, was its array of smaller glycans [9]. Within this class of glycans, assignments were made based on analysis of sequential exoglycosidase digestions, indicating the presence, for example, of sialyl lactosamine linked to a reducing terminal hexose, with mannose dominant [9]. The J1 family of glycoproteins (including tenascin) possesses many repeat fibronectin typeIII domains [17]. The display of potential carbohydrate ligands on J1 may therefore show differences from the domains of the neural adhesion molecules

belonging to the immunoglobulin superfamily. The possibility of a variety of potential carbohydrate epitopes in '*O*'-linkage and in relatively high local concentrations must be considered for J1 in view of this preliminary structural analysis of the glycans, known amino acid sequence [17] and the existence of '*O*'-glycosidic mannose-linked glycans carried elsewhere in the brain on the glycoprotein of chondroitin sulfate proteoglycans [18].

These results reflect earlier studies (see [19] for references) in which general structural features of protein-bound glycans in nervous tissue were studied. Apart from the novel feature of the *O*-glycosidic mannose-linked units, other distinctive features that could be found in molecules involved in cell adhesion and could also be highly enriched in nervous tissue include the presence of outer-arm fucose, polylactosamine and polysialic acid chains, and an '*O*'-linked disaccharide containing galactose in a-linkage. Polylactosamine chains, for example, were carried on glycans of the core protein of the brain chondroitin sulfate proteoglycan and were shown to bear the HNK-1 epitope [20].

In another study, glycans were isolated from a preparation of whole brain tissue (murine) by increasing the scale of the procedure for oligosaccharide release from glycoproteins through anhydrous hydrazinolysis [21]. A general feature of this brain glycan 'library' was that 80% of the oligosaccharides were anionic with all common acidic

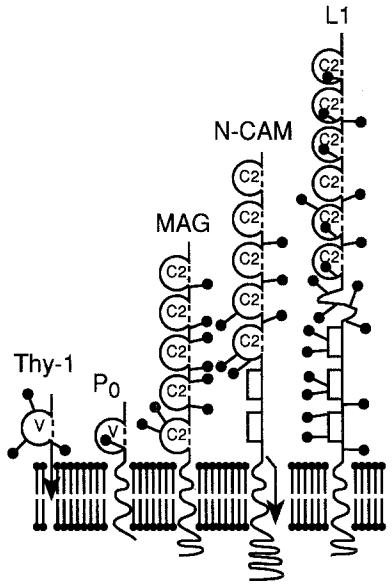

Figure 2. Representative neural molecules belonging to the immunoglobulin superfamily. Immunoglobulin-like domains are shown as part circles, fibronectin-like type III domains by rectangles (three to five being indicated in the case of L1), *N*-linked oligosaccharides by ♆. All are depicted attached to a membrane bilayer either by a glycosylphosphatidyl-inositol lipid anchor, indicated by an arrow, or by a transmembrane hydrophobic domain.

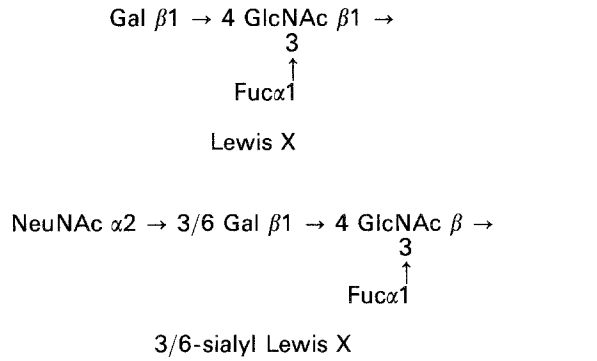

Figure 3. Structures of the Lewis x (Le x) and 3/6 Sialyl Lewis x (sLe x) determinants; NeuNAc = *N*-acetylneuraminic acid.

groups represented. Although not all of these glycans would have been exposed on the cell membrane, the importance of protein glycoconjugates in recognition processes in nervous tissue will depend on the further structural analysis of the array of acidic glycans present in brain.

Functional aspects

The importance of complex carbohydrates in neural glycoproteins may be indicated not only by the nature of the glycosylation itself, as described above, but also by functional considerations. The following topics have been selected, based on current interests in these glycoproteins. While it is not intended to be a comprehensive list, attempts are made to integrate a number of aspects of glycosylation with function.

P0

P0 is one of the smallest members of the immunoglobulin superfamily [14], is an adhesion molecule that can be recognized by the L2 monoclonal antibody, and is believed to be involved in peripheral nerve myelin compaction [22] and possesses one *N*-linked glycosylation site [23, 24]. It is known that the L2/HNK-1 epitope, as identified on glycolipids from peripheral nerves, possesses a glucuronic acid terminal, sulfated in the 3-position [6]. It is of interest, therefore, that 'L2-positive' P0, isolated by immunoaffinity chromatography, was shown to possess several sulfated structures, varying in their charge-carrying capacity, amongst its glycoforms [25]. Sulfate incorporation into the glycans of P0 has been associated with the onset of myelination, as shown by Poduslo [26]. It would be of interest to know if the degree of sulfation is critical in this process.

In addition, Filbin and Tennekoon [27] have shown that when P0 is expressed in Chinese hamster ovary cells there is cellular aggregation if normal (wild type) glycosylation of P0 is allowed to occur. No aggregation is seen in mutant cells (*N*-acetylglucosaminyltransferase I deficient) in which glycosylation processing of P0 is limited to the production

of the oligomannose class of structures only. This and related evidence [28] was taken to suggest that full oligosaccharide processing on P0 was necessary for adhesion, and that the adhesion was homophilic. However, it is not known whether sulfated glycans played a role in this particular interaction.

The studies of Schneider-Schaulies *et al.* [29] confirmed that P0 entered into homophilic adhesion, but also that the molecule could participate in heterophilic interactions. This was demonstrated by specific inhibition and competition experiments using recombinant DNA technology. Further, the homophilic interaction between recombinant P0 molecules could be mediated with at least one partner of the interacting molecules unglycosylated.

The possibility has been raised that cerebellar soluble lectin (present in peripheral nerve myelin, but first isolated from the cerebellum) may enter into recognition with a minority of P0 molecules on peripheral myelin through mannose specific interactions [30]. Studies of the glycans of P0 have shown, moreover, that oligomannose and/or hybrid structures are present on some myelin P0 glycoforms [31, 32]. Hybrid structures were more prominent in adult rat P0 when compared with P0 in 5 day-old animals which carried predominantly complex-type oligosaccharides [33]. This would be consistent with more actively myelinating Schwann cells in the young [31], and may be a factor in the interplay between homophilic and heterophilic interactions of P0.

L2/HNK-1 receptor

Despite the occurrence of the L2/HNK-1 carbohydrate epitope on a variety of glycoproteins in the nervous system and the homo- or heterophilic binding properties of these molecules, there is little definitive evidence of the nature of the binding site or receptor for the epitope. Recent evidence, however, indicates that the sulfoglycolipids, known to carry the L2/HNK-1 epitope, have been shown to bind to a 30 kDa protein of the cerebellum, thought to be amphoterin [34]. The possibility has been raised [34] that clustered lysine residues may be important as seen in the heparin-binding growth-associated molecule (HB-GAM) [35], which also binds specifically to the HNK-1 positive glycolipids and sulfatides but not to other anionic glycolipids [34]. HB-GAM appears to have the same sequence as pleiotrophin, a developmentally regulated heparin-binding cytokine [36] that induces mitogenic and neurite outgrowth activity from mixed cultures of embryonic rat brain cells.

From the studies involving neurite outgrowths over laminin surfaces and the binding properties of the L2/HNK-1 reactive sulfoglycolipids, it is clear that a receptor for the L2/HNK-1 epitope must be present on this extracellular matrix molecule [7]. It appeared that the heparin-binding site present in the laminin molecule could be recognized, and that the sulfate group was essential [37]. A feature of the *N*-linked glycans carrying the L2/HNK-1 epitope on P0 and MAG has been shown, by *Aleuria aurentia* lectin affinity chromatography, to contain core fucose, α1–6 linked to the reducing terminal *N*-acetyl-glucosamine [38]. It is not known whether this structural feature of the glycan on these glycoproteins has a bearing on receptor specificity.

Polysialylation of N-*CAM*

An unusual structural feature of *N*-CAM is its ability to associate covalently with polysialic acid which, on neuroblastoma cells, has been shown to have a minimum chain length of over 50 residues [39]. The sialic acids are α2–8 linked and attached as outer branches to tri-/tetraantennary *N*-linked oligosaccharides [40, 41]. The polysialylated chains on *N*-CAM, a notable feature of embryonic forms, appeared to have an excluded volume greater than that of the polypeptide itself – thus acting to prevent close membrane–membrane apposition [42, 43]. This is proving to be a useful model system for studying functional roles of these complex carbohydrates in cellular interactions and has been well reviewed elsewhere [44, 45].

Carbohydrate-dependent co-operativity between adhesion molecules

A feature of the wide distribution of *N*-CAM in nervous tissue is that it is expressed on all L1-expressing cells. The possibility that *N*-CAM and L1 may form a close functional association was suggested by Kadmon *et al.* [46]. The association was shown to enhance the interaction of L1 on that cell with L1 on a neighbouring cell. It was believed that the original '*cis*' interaction between *N*-CAM and L1 was carbohydrate-dependent, based on the use of inhibitors of oligosaccharide processing in neuroblastoma cells [46].

Thy-^{1}

Although the site specificity of *N*-glycosylation of brain Thy-1 in certain species is known [10], little is understood of the function of Thy-1 despite it being a major cell-surface glycoprotein in brain and the smallest known member of the immunoglobulin superfamily [14]. It is expressed, in particular, on neurons after axonal growth has ceased [47]. Recent evidence indicated an inhibition by Thy-1 of neurite outgrowth on mature astrocytes [48], indicating the potential importance of Thy-1 and an astrocytic ligand in neuron–astrocyte interactions, possibly to suppress axonal regrowth in the CNS after injury in astrocytic-rich areas. An unusual feature of glycosylation in brain Thy-1 compared with the composition of whole brain *N*-linked oligosaccharides [21] is that the glycoprotein carries very few acidic glycans and these are restricted to the sialylated class [10]. A possible role of the glycan moieties in functional interactions has not yet been studied in detail.

J1/tenascin

Neuron–astrocyte interactions are also under the influence of other molecules of recognition, including the J1/tenascin glycoproteins [49, 50]. These glycoproteins are secreted by neuroglial cells and constitute part of the extracellular matrix in the nervous system. The possibility of a variety of glycans, in '*O*'-linkage (as described above) being carried on these molecules could enable clustering of carbohydrate ligands in ways that may be critical for certain types of interaction, particularly where dynamic processes are involved [51]. It is of interest that chondroitin sulfate proteoglycan, with a core protein bearing HNK-1 antigenic determinants, was found to be a ligand for the cytotactin molecule, a structurally distinct but related member of the extracellular matrix [52]. It seems that the function of extracellular matrix components may be modulated not only by competition for shared receptors but also by a network of molecular interactions among the components themselves. This was seen with J1/tenascin, upon which a neurite outgrowth promoting domain was distinguishable from a cell-binding site that inhibited neurite outgrowth or cell-spreading [53]. The remarkable heterogeneity of the nerve growth cone glycoproteins [54] adds further complexity to the possible array of interactions occurring during neurite outgrowth.

Conclusion

GM-1 ganglioside has been shown to be effective in the treatment of acute spinal cord injury [55] and also to ameliorate effects of experimental Parkinsonism in primates [56]. Thus, the seeds for the potential therapeutic use of glycoconjugates in nervous tissue have been sown.

Some of the described subtleties of protein glycosylation in this tissue suggest that control of activity of key glycosyltransferase/glycosidase enzymes will be important. On the synthetic processing pathways these include the recently discovered glucuronyltransferases [57, 58] for functional epitopes recognized by the L2/HNK-1 monoclonal antibodies, α1–3 fucosyltransferase for determinants of the Le x type [59], poly-α-2–8 sialosyl sialyltransferase for the synthesis of polysialylated chains [60], as well as sulfotransferases in general.

The conservation of glycosylation seen at both tissue and molecular level indicates the specificity of processing for both cell type and for particular protein conformation. The display and nature of the glycans, as illustrated for the Le x determinant, will determine the potential function of any acting carbohydrate ligand. Thus, a transient recognition is needed during processes of neural migration, but a stronger bond ultimately for adhesion. The attractive or repulsive nature of the interaction will give guidance cues. The timing of expression of ligands and/or receptors will give fine tuning to the spatial dimensions of the interactions.

It follows that a more complete understanding of the role of glycans conjugated to proteins in nervous tissue will be dependent on further structural analyses, particularly of acidic constituents. This information will be critical in influencing the directions of functional studies, as has been shown in recent years in the haematolymphoid system with the identification of the carbohydrate ligands Le x and sialyl Le x and their receptors [61].

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Letters or comments relating to this article would be received with interest by Pauline Rudd, Assistant to the Special Advisory Editor, R. A. Dwek.