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A sugar rush for developmental biology

Catherine L. R. Merry[1,*] and Christopher M. Ward[2]

The EMBO Workshop on Glycoscience and Development, organised by Philippe Delannoy, Yann Guérardel, Tony Merry and Jean-Claude Michalski, was held in the picturesque, contemplative environment of Les Minimes, a converted seventeenth century Flemish convent in Lille, France, in December 2007. A cross-section of researchers, both confirmed 'glycomaniacs' and those newer to the field, discussed and debated recent advances in the field of glycobiology. Presentations ranged from the clinical applications of glycobiology to novel approaches for unravelling carbohydrate biosynthesis in developmental settings and models, such as the fruit fly, nematode and zebrafish.

Introduction

Glycoscience is the study of complex sugars, and of the molecules that display them and the proteins to which they bind. These post-translational modifications can be small and simple, such as the addition of a single sugar, e.g. *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), or they can be very large and complex, as with modifications involving glycosaminoglycans, which can be more than 100 units in length and highly heterogeneous. Many complex sugars are attached to either proteins or lipids, which often tether them to cell surfaces, where they play key roles in cell recognition events, such as in inflammation, cancer and tissue patterning. The biosynthesis of glycoconjugates (the sugar and its protein/lipid moiety) requires the coordinated activity of a cohort of enzymes (including glycosyltransferases, epimerases, etc.), which work without a genetic template, and can create the myriad of structures that fulfil a variety of crucial functions, such as the biosynthesis of blood group antigens and the regulation of Notch signalling. Historically the preserve of a dedicated few, glycoscience is now a 'hot' topic, both academically and commercially, and technological advances such as the generation of conditional knock-out animals and higher resolution structural studies have been key to this advance.

Throughout the meeting, a range of topics was covered, from which three themes emerged: technological advances, evolutionary biology and development. Technological advances are particularly welcomed, as, frequently, for a field in which there is no method for template-driven amplification or for rapid global sequencing, these lead to exciting and novel findings (Merry and Merry, 2005). As discussed in more detail below, Ajit Varki (University of California, San Diego, USA) provided an introduction to the second theme of evolutionary biology, explaining how, 'Nothing in Glycobiology makes sense, except in the light of evolution' (Varki, 2006), which was followed by a wealth of supporting evidence. The discussions of the role of glycoscience in development ranged from fertilisation and implantation to the specifics of organ formation. Here, we have predominantly focused on those presentations and themes that particularly concern the many ways in which glycoscience affects development.

Technological advances

As mentioned above, technological advances in glycoscience have lead to many exciting discoveries. Complex sugars are difficult to study using common structural analytical tools, such as mass spectroscopy, as they are highly heterogeneous and labile under ionising conditions. Jerry Hart (Johns Hopkins Medical School, Baltimore, USA) explained how modified mass spectrometric analyses, combined with enzymatic characterisation, have been of significant benefit. This approach has enabled the discovery that *O*-GlcNAcylation is much more widespread and dynamically regulated than was previously thought (Hart et al., 2007), re-enforcing the view that this modification is as important as phosphorylation for the sensing of nutrient levels and stress within cells. Similar problems with detection have previously restricted the analysis of heparan sulphate (HS). However, as highlighted by Claire Johnson (University of Manchester, Manchester, UK), a panel of phage-display-derived antibodies, able to detect subtle differences in HS patterning, can be combined with flow cytometry to characterise cell-surface HS in a rapid and non-destructive manner (Johnson et al., 2007). Another method introduced was that of high-resolution magic angle spinning (HRMAS) NMR for the analysis of polysaccharides in an impure state. Described enthusiastically by Guy Lippens (University of Lille, Lille, France) as a 'lousy' method (as it requires relatively large amounts of material), it is, however, suited for complex biological samples, as it can generate data from material without prolonged and wasteful purification steps. This allows ingenious experiments to be undertaken, such as the analysis of live bacteria, using pulse-chase with ^{13}C to follow the biosynthesis of glycans as the bacteria multiply (Hanoulle et al., 2006). HRMAS NMR has also been used to study the prodrug ethionamide, used for the treatment of multidrug-resistant tuberculosis, helping to uncover its activation mechanism and to detect intermediates that are unstable when removed from the cellular environment (Hanoulle et al., 2006).

Glycoscience and evolution

Ajit Varki began his talk by explaining that humans can be considered as a 'knock-out model' for the function of a specific sugar modification, that of *N*-glycolylneuraminic acid (Neu5Gc). This form of sialic acid is widely expressed in mammals, including in great apes, but is not found in humans because of an inactivating mutation in the gene (CMP-*N*-acetylneuraminic acid hydroxylase) that is required to form Neu5Gc from the related sialic acid Neu5Ac, a mutation that occurred in our ancestors ~3 million years ago (Varki, 2007). These residues are often displayed at the termini of sugar chains that act as receptors for a wide variety of binding proteins, such as haemagglutinins, selectins and siglecs. Varki explained how pathogens to which humans are selectively sensitive tend to preferentially bind the excess of Neu5Ac (e.g. *Plasmodium falciparum*), whereas those that tend to infect animals (e.g. *E. coli* K99), even when humans are in close proximity, preferentially bind Neu5Gc. However, Neu5Gc can be found in human cells and tissues, and in carcinomas, following its uptake from an extrinsic source. Circulating antibodies against Neu5Gc

[1]Materials Science Centre, The University of Manchester, Grosvenor Street, Manchester M1 7HS, UK. [2]Centre for Molecular Medicine, Lab. 3.722 Stopford Building, Faculty of Medical and Human Sciences, The University of Manchester, Manchester M13 9PT, UK.

*Author for correspondence (e-mail: catherine.merry@manchester.ac.uk)

are found in all normal adults, which are very specific for these Neu5Gc-containing epitopes. This antigen-antibody reaction is hypothesized to cause chronic inflammation and to increase the risk of cancer and other diseases. This is of particular significance because the levels of Neu5Gc to which we are exposed are already high, and are likely to increase in the near future. For example, major contributors are diet, with red meat and dairy products being particularly high in Neu5Gc, and biotechnology products that have used animal cells and sera as a source of Neu5Gc for metabolic uptake by cultured cells. This discussion came as a timely reminder of the benefits of a vegan diet prior to the serving of veal and crème caramel at lunch!

Continuing with the theme of glycoscience and evolution, and choosing the provocative title of 'How is a Worm more complex than a Fly', Iain Wilson (Universität für Bodenkultur, Vienna, Austria) argued that major advances in recent years have led to the generation of comprehensive genomic information for a variety of species. However, our knowledge of the glycomic repertoire of most organisms is far from complete. By comparing and contrasting two familiar species, the fruit fly (*Drosophila melanogaster*) and the nematode worm (*Caenorhabditis elegans*), Wilson discussed the occurrence of both specific structures and, more generally, the total number of discernable *N*-glycans in the two species. Asparagine (*N*-)linked-glycans are structurally diverse, ranging from the relatively simple high-mannose type to hybrid and complex *N*-glycans. Synthesised within the endoplasmic reticulum and Golgi, they participate in protein folding and, once displayed at their final destination, they play essential roles, such as defining the circulatory lifetime of hormones, tissue patterning during embryogenesis, immune function and inflammation. Within the fly, a total of 42 discrete *N*-glycans have been identified, with high mannose and truncated *N*-glycan structures being the major contributors to the profile. Conversely, wild-type worms have possibly 65 different structures, some of which remain uncertain (Paschinger et al., 2008). Therefore is the worm more complex, at least in terms of glycans, than the fly? Wilson argues that this is evidence against the Victorian concept of the 'Great chain of being', leading from simplistic organisms to the more complex, and that, in fact, diversity exists all the way along the chain.

This diversity can cause problems when investigating glycoconjugates, particularly as genetically simple developmental model systems are often sought-after, bypassing the problems of redundancy associated with complex systems. Vlad Panin (Texas A&M University, Texas, USA) introduced us to the benefits of *Drosophila* for the study of sialyltransferase activity, as it encodes a single enzyme [DSiaT (ST6Gal – FlyBase)] (in place of the twenty found in humans) that shares greatest homology with the ST6Gal family of mammalian sialyltransferases. DSiaT is highly conserved among distant species within the *Drosophila* genus, suggesting significant evolutionary conservation of DSiaT-mediated functions. This is evident from the study of DSiaT mutant flies, which reveal it to be required for the development and function of the *Drosophila* central nervous system (CNS); however, the relatively low level of sialylated glycans within the fly CNS (Koles et al., 2007) indicates that they might be serving highly specialised functions. In particular, a possible link to memory was highlighted, with DSiaT expression found in projection neurons, as well as in a subset of other types of neurons. Panin also discussed the locomotor abnormalities, neuromuscular junction morphology and other neurological phenotypes of DSiaT mutants, which further indicate that DSiaT is indispensable for the development and function of the *Drosophila* CNS.

Introducing us to his work with fantastic views of Chesapeake Bay, Gerardo Vasta (University of Maryland Biotechnology Institute, Baltimore, USA) shared his passion for both the basic functional aspects of galectins (a family of β-galactoside-binding proteins) and environmental protection, targeted to the restoration of oyster beds. Once again turning to model systems, Gerardo initially explained how galectins in zebrafish (*Danio rerio*) have a high homology with human galectins, in both their structure and their carbohydrate-binding specificity. Using morpholino technology to isolate the function of specific family members, he reported that the knock down of Drgal-L2 (Lgals1l2 – Zebrafish Information Network) resulted in defects in muscle fiber organization and tail morphology, supporting its potential roles in directing cell-laminin interactions, and in the ability of the notochord (where it is highly expressed) and its derived structures to respond to sonic hedgehog (Shh) and Wnt. A second family member, Drgal-L4, with a similar expression pattern, when knocked down together with Drgal-L2, produces a more severe phenotype, with additional defects in heart and blood cell development. It was therefore suggested that galectins within the developing notochord, by binding to endogenous glycans, can regulate the correct patterning response of cells within the adjacent tissues of the neural tube and somites, particularly those requiring bone morphogenetic protein (BMP) and Wnt signalling, such as heart and blood. Moving to an invertebrate model system, the eastern oyster, Gerardo also discussed the functional diversity of galectins, which in the oyster also bind exogenous ligands, such as glycans on the microalgae they feed upon, internalized by filtering litres of water per hour, which they process by intrahaemocytic digestion. This activity, however, makes them particularly susceptible to microbial pathogens and parasitic infections. One parasite in particular, *Perkinsus marinus*, may have evolved its surface carbohydrates to be strongly recognized by the oyster galectins (Tasumi and Vasta, 2007). By exploiting the role of galectin in recognising 'non-self', it therefore gains a selective advantage over the phytoplankton for infecting its oyster host.

Glycoscience and early development

Introducing early mammalian development and the events surrounding fertilisation, Pamela Stanley (Albert Einstein College of Medicine, New York, USA) described how the large hydrodynamic volume occupied by membrane-tethered glycans on glycoproteins and glycolipids makes them 'The Molecular Frontier' of the cell. One of the most critical frontiers encountered in development is that of the zona pellucida (ZP), the complex extracellular matrix that envelops mouse oocytes and ovulated eggs. In trying to dissect the role of complex and hybrid *N*-glycans, core 1-derived *O*-glycans or *O*-fucose glycans (see Fig. 1) in the function of the ZP, Stanley was faced with the problem of tackling the compensatory effects of maternal transcripts. To overcome this, maternal and zygotic mouse mutants were generated, by crossing floxed alleles of *Mgat1* (encoding *N*-acetylglucosaminyltransferase I, essential for the hybrid and complex branching of *N*-glycans), *C1galt1* (encoding T-synthase, which transfers Gal to *O*-GalNac to generate core 1 and 2 *O*-glycans) and *Pofut1* (encoding protein *O*-fucosyltransferase 1, which transfers fucose to epidermal growth factor-like repeats) with ZP3-Cre mice. Using this approach, $Mgat1^{-/-}$ eggs, decorated with *N*-glycans that lack terminal Gal and GlcNAc, had previously been generated and found to be developmentally compromised, but were readily fertilised (Shi et al., 2004). So to investigate the potential role of an alternative source of terminal Gal and GlcNAc residues, the Stanley group generated $C1galt1^{-/-}$ eggs, lacking core 1-derived *O*-glycans (Williams et al.,

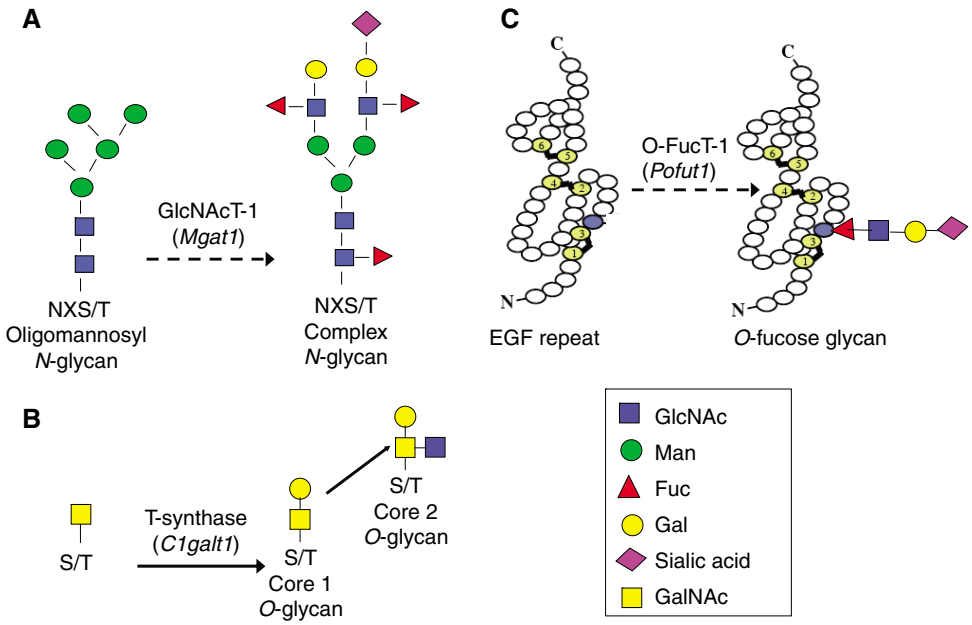

Fig. 1. Mammalian glycan structures. Mammalian glycans discussed in the text are depicted using sugar symbols. (**A**) *N*-glycans; (**B**) mucin *O*-glycans; and (**C**) *O*-fucose glycans. The glycosyltransferase that initiates the transition shown is represented by its biochemical abbreviation and by its gene name (in parentheses). *N*-glycans (A) occur at NXS/T (where X is any amino acid) sequences, and *O*-glycans (B) at Ser or Thr (S/T). (C) *O*-FucT-1 transfers only to EGF (epidermal growth factor) repeats with a particular consensus sequence (Cys-Cys bonds, 1-6, are highlighted in yellow). A dashed line signifies that several reactions must occur before obtaining the product shown. A solid line designates a single reaction. Maternal and zygotic mutants of each glycosysyltransferase are discussed in the text. Fuc, fucose; Gal, galactose; Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine. Image courtesy of Pamela Stanley.

2007). These eggs were again fertilised, with embryos surviving to ~E13.5. In a conclusive experiment, eggs lacking complex and hybrid *N*-glycans, as well as core-1-derived *O*-glycans (*C1galt1*$^{-/-}$/*Mgat1*$^{-/-}$) were generated and again found to be fertile, thereby proving that terminal Gal or GlcNAc residues on *N*- or *O*-glycans displayed by the zona pellucida protein ZP3 are not essential for fertilisation. The experimental system also allowed Stanley to demonstrate that, surprisingly, maternal and zygotic *Pofut1* mutant blastocysts develop normally, indicating that canonical Notch signaling is not required for preimplantation development.

The establishment of a stable maternal-fetal interface is critical for pregnancy success and hence, by inference, speciation. John Aplin (University of Manchester, Manchester, UK) introduced us to the three types of interface that occur in eutherian mammals: haemochorial (human, rat and mouse), endotheliochorial (carnivores) and epitheliochorial (pigs and sheep, and evolutionarily the most recent). In epitheliochorial species, a prominent glycocalyx is present at the long-lived adhesive interface between trophoblast and uterine epithelium. This led to the question of whether fetomaternal compatibility could be specified by a type of glyco-code. Aplin described how the glycan composition of the glycocalyx in epitheliochorial species might be conserved over long evolutionary periods; furthermore, in species exhibiting haemochorial placentation, there is evidence for the convergent evolution of the glycome (the entire cell complement of sugars) at the placental surface. In humans, there is evidence that the epithelium acts as a barrier to prevent karyotypically abnormal embryos from implanting. Many human embryos (~40%) are karyotypically abnormal. Aplin described how, although these are observed prior to implantation, the proportion found post-implantation is much lower, suggesting that the process of implantation might itself select against karyotypically abnormal embryos. A possible mechanism involves MUC1, a key glycan-bearing component of the interface, which is present on the maternal epithelium prior to implantation and is cleared from the site directly under the embryo in humans. Aplin stressed how fertilisation and implantation involve tightly orchestrated carbohydrate-mediated, long-lived and short-lived cellular interactions (Aplin, 2006). For obvious reasons, these are particularly difficult to study in humans, and Aplin described how in vitro model systems could be used to provide valuable insights into these mechanisms.

Although membrane microdomains (or lipid rafts) and their role in cell-cell interactions are relatively well investigated in development, the inclusion of large numbers of glycolipids within these regions is frequently overlooked. Ken Kitajima (Nagoya University, Nagoya, Japan) explained how a combination of sugar- (specifically Lewis X glycans) and protein (cadherin)-mediated interactions is required for blastodermal cell adhesion in medaka. Lipid rafts are typically 50-200 nm in diameter and are a recognised 'hot spot' for signal transduction. When isolated from medaka embryos, these regions are enriched for cholesterol and sphinomyelin, as well as for Lewis X-containing glycoproteins and glycolipids. Also present are cadherin/catenin, Src and phospholipase-Cγ, in agreement with previous studies that have shown these domains to be involved in cell-cell interactions and in subsequent signalling events. Using chemical methods to disrupt the structure of the microdomains, Kitajima demonstrated that epiboly (the first cell migration process that occurs from blastula to gastrula) depends on intact microdomains, which can be reconstituted by adding cholesterol, which appears to allow the re-integration of Lewis X-containing glycoproteins into the rafts (Adachi et al., 2007). Kitajima also analysed fucosyltransferase mutant animals, to

investigate how microdomains isolated from mutant medaka embryos interacted with each other. By combining these genetic and chemical approaches, he concluded that cell-cell binding during epiboly depends on both fucosylated carbohydrate and protein components that reside within membrane microdomains. Indeed, the Le^x-glycoprotein and cadherin colocalise to the same microdomain, although there are likely to be intermediate proteins associated with them.

Glycoscience and organ formation

Once again the benefits of *Drosophila* as a model system for helping to unravel the complexities of glycan biosynthesis were highlighted, this time by Kelly Ten Hagen (NIH, Bethesda, USA), who investigated the developmental role of mucin-type protein *O*-linked glycosylation (Tian and Ten Hagen, 2007a). An evolutionarily conserved family of UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferases (ppGaNTases in mammals or PGANTs in *Drosophila*) initiate the formation of these glycans, which occur in tightly regulated patterns during organ development. In particular, high levels are observed along the apical and luminal surfaces of developing tubular organs, suggesting a possible role in tubulogenesis. Using a *Drosophila pgant35A* mutant, which has reduced levels of *O*-glycans, Ten Hagen demonstrated the importance of *O*-glycosylation for the organisation and polarisation of the cells that comprise the tracheal tubes. Along with a substantial reduction in *O*-glycans present, the *pgant35A* mutant has a severely disrupted apical surface and apicobasal polarity within the tracheal system, as well as loss of the diffusion barrier (Fig. 2). Moving from epithelial tube formation to another similarly highly evolutionarily conserved process, that of cell adhesion, Ten Hagen introduced the *pgant3* mutant, which displays a wing blister phenotype indicative of impaired cell-cell interactions within the developing wing. Although integrins are recognised as being a primary mediator of cell-matrix attachment during this process, it appears that *O*-glycans are also involved, with *pgant3* mutants phenocopying other cell adhesion mutants. Crucially, these systems provide a source of material to enable the proteins to which these essential modifications are attached to be isolated and identified, an ongoing interest of Ten Hagen's group.

The *Ext1* mutant mouse, which lacks HS, dies early in embryonic development prior to gastrulation. To study the role of HS in tissue formation, and to try and uncover how this complex sugar co-ordinates growth factor and morphogen signalling, as well as cell adhesion and migration, Yu Yamaguchi (University of San Diego, San Diego, USA) uses a loxP-modified conditional allele of *Ext1* together with tissue-specific Cre drivers. By ablating *Ext1* with Nestin-Cre, he generated a mouse mutant with multiple defects in brain patterning, including agenesis of the olfactory bulbs, severe cerebral hypoplasia and the failed separation of the midbrain and cerebellum. Mutant retinal axons are also misguided at the optic chiasm, where a genetic interaction was demonstrated between the axon guidance ligand Slit1 and Ext1, with HS being suggested to either increase the local concentration of Slit1 or act as a co-receptor. To study these interactions in more detail, the pathfinding of spinal cord commissural axons was analysed, allowing the dissection of environmental and cell-autonomous effects (Matsumoto et al., 2007). The enzymatic or chemical removal of HS blocked axon outgrowth in response to netrin 1, with further studies demonstrating that HS is additionally required at the surface of responsive cells, indicating that, in the case of netrin 1, HS is likely to acts as a co-receptor necessary for transducing netrin 1 signals. HS can therefore be an obligatory co-receptor or an essential environmental factor that

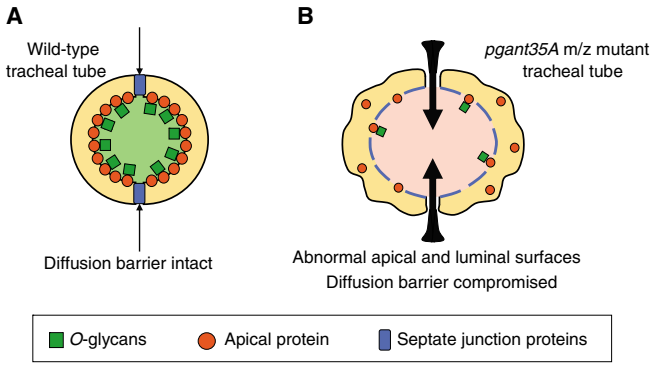

Fig. 2. The role of *pgant35A* during *Drosophila* tracheal development. (**A**) Schematic of a wild-type tracheal tube in cross section. (**B**) Cross section of a *pgant35A* maternal/zygotic (m/z) mutant tracheal tube, showing loss of apicobasal polarity and of the diffusion barrier (large arrow). Reproduced, with permission, from Tian and Ten Hagen (Tian and Ten Hagen, 2007b).

controls the distribution and degradation of ligands, depending on developmental context. Moving from the brain to bone, Yamaguchi also discussed recent findings from limb bud-targeted *Ext1* knock-out mice. Loss of HS here causes severe limb bud hypoplasia with multiple skeletal defects. The underlying cause of these defects appears to be due to the aberrant differentiation and patterning of mesenchymal condensations, which act as templates for cartilage. These data from *Ext1* conditional knock-out mice demonstrate that the role of HS in regulating the function of diffusible factors during critical developmental processes, so elegantly detailed in flies, is clearly also true in mammals.

A frequent puzzle in glycoscience is the assignment of function to the carbohydrate and non-carbohydrate elements of a glycoconjugate. The now common use of knock-out and knock-down mutants has only confounded this issue with specific glycan functions often remaining elusive. However, the function of one glycan, polysialic acid, is now much clearer, thanks to the fascinating work of Rita Gerardy-Schahn (Hannover Medical School, Hannover, Germany). Polysialic acid (PolySia) is unusual, even amongst other carbohydrates, for its high water-binding capacity and the large hydrodynamic volume it imparts to the molecules to which it is attached. One of these in particular, neural cell adhesion molecule (NCAM), dramatically switches from an adhesive molecule to an anti-adhesive molecule following the addition of PolySia (Hildebrandt et al., 2007). During embryonic and early postnatal development of the mammalian brain, NCAM and PolySia are both present at high levels. NCAM has been implicated in many crucial developmental processes, including neuroblast migration, neurite outgrowth and fasciculation, synaptogenesis and synaptic plasticity. However, the NCAM knock-out mouse has an unexpectedly mild phenotype (Cremer et al., 1994). Conversely, mice mutant for two enzymes involved in PolySia biosynthesis, ST8SiaIV and ST8SiaII, which have been generated by Gerardy-Schahn's lab, have an unexpectedly severe (and lethal) phenotype, with retarded postnatal growth and loss of the anterior commissure in the brain (despite the single mutants having a mild phenotype). This, Gerardy-Schahn explains, is because the loss of both ST8SiaV and ST8SiaII effectively causes a gain of PolySia-free NCAM function. To test this, a triple knock-out mutant was generated by these researchers that lacks both PolySia and NCAM. In these mice, anterior commissure formation is

normal. To further investigate this phenomenon, they then performed experiments to vary the level of PolySia attached to NCAM. These studies provided conclusive proof that the phenotype depends on the amount of PolySia-free NCAM that is available and suggests that the system generates NCAM only to then make it invisible by the addition of PolySia. It was suggested that this process is essential for the plasticity that is required during the complex process of building the vertebrate brain and highlights the complex relationship between glycans and their 'support act' – the proteins and lipids to which they are attached.

Conclusion

This meeting was unique in both the subject area and the diverse interests of the participants. It therefore provided an ideal opportunity for newcomers to glycobiology and development to engage in discussion with others who had many years of experience in their respective fields. The engaging posters, particularly those presented by the younger scientists, provided a focus for these discussions and clearly demonstrated the diverse range of research interests. We left Les Minimes with our heads full of new ideas and opportunities for collaboration. Glycoscience may have previously been the domain of specialists but, certainly within developmental biology, this is unlikely to be the case in the future as the functions of these essential mediators of numerous and diverse cellular interactions – from signaling to adhesion – become clear.

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