Part I—Chapter 1: Fucosylation in the Brain

Carbohydrates in cellular communication

The cell surface is a fluid, dynamic structure composed of many different biomolecules, including proteins, lipids, and carbohydrates. Most often, carbohydrates are found on the cellular membrane covalently bound to lipids and proteins, where they form glycolipids and glycoproteins, respectively. These membrane-bound carbohydrates serve as markers that distinguish individual cells and are crucial elements in cell-cell recognition and communication. Many glycoproteins are known to be involved in numerous biological processes including cell differentiation, neuronal growth, interneuronal recognition, and signal transduction.[1, 2] In the brain, about 80% of glycoproteins are found in the microsomal fraction and in synaptic membranes.[3]

The reorganization of synaptic membranes (synaptic remodeling) allows for the formation of memory traces and involves changes in the synthesis of glycoproteins as well as posttranslational modifications to existing glycoproteins.[4] This synaptic remodeling is thought to underlie information processing in the brain and leads to memory formation. One of the leading models for how memory traces are mechanistically formed in the brain is long-term potentiation (LTP), a process where high-frequency stimulation of a nerve cell causes an abrupt and sustained increase in the efficiency of synaptic transmission of the signal.[4] LTP has been found to occur in both vertebrates and invertebrates in a variety of neural systems, ranging from the mammalian peripheral nervous system to the arthropod neuromuscular junction to subcortical mammalian nuclei, such as the amygdala.[5]

Changes in neuronal morphology have been directly associated with LTP in various systems. Inducing LTP in cultured rat hippocampal slices caused new spines to appear on post-synaptic dendrites, whereas no significant spine growth was found in regions where LTP was blocked.[6] Similar results were seen in hippocampal slices from spatially trained rats, where a significant increase in dendritic spine density was observed in trained rats compared to nontrained rats.[7] Such synaptic remodeling could involve glycosylation of synaptic proteins, as supported by the presence of protein glycosylation machinery in dendrites.[8] Interestingly, the presence of glycosylation machinery in dendrites suggests that the synthesis of glycoproteins at the synapse may be dynamically regulated. Moreover, protein glycosylation has been shown to be necessary for maintaining hippocampal LTP. Treating hippocampal slices with different protein glycosylation inhibitors (tunicamycin, brefeldin A, swainsonine) during induction of LTP prevented maintenance of LTP and caused postsynaptic potentials to return to baseline levels within 100 minutes after induction of LTP.[9] However, postsynaptic potentials from control slices remained elevated for more than 200 minutes following induction of LTP.[9] These findings provide a direct link between learning, LTP, synaptic remodeling, and protein glycosylation.

Evidence for the importance of fucose α(1-2) galactose glycoproteins in the mammalian brain

The formation of synaptic connections and synaptic remodeling involves the recognition of different molecules present at the membrane, including various proteins, lipids, carbohydrates, and other small molecules. We are particularly interested in the

sugar L-fucose (Figure 1.1), one of the small molecules enriched in the synaptosomal fraction.[10]

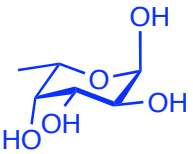

Figure 1.1. The monosaccharide L-fucose

L-Fucose (Fuc) is one of the 9 standard carbohydrate building blocks commonly found in mammals, and fucose-containing oligosaccharides play important roles in many biological processes, including blood transfusion reactions, host-microbe interactions, and cancer pathogenesis.[11] Fucose is appended to various biomolecules by fucosyltransferases, of which thirteen have been identified in the human genome.[11] All currently identified fucosyltransferases utilize guanosine diphosphatyl-fucose (GDP-fucose) as the monosaccharide donor. In mammalian cells, two pathways exist for the synthesis of GDP-fucose, the *de novo* pathway and the salvage pathway (Figure 1.2).

The *de novo* pathway converts GDP-mannose to GDP-fucose via three enzymatic reactions carried out by two enzymes. First, GDP-mannose-4,6-dehydratase (GMD) converts GDP-mannose to GDP-4-keto-6-deoxymannose through oxidation of the hydroxyl group at C-4 of the mannose ring and reduction of the hydroxyl group at C-6. Next, the dual functional epimerase-reductase enzyme known as the FX protein converts GDP-4-keto-6-deoxymannose to GDP-fucose. In the salvage pathway, GDP-fucose is synthesized from free fucose via a two-step process. In the first step, free fucose derived from extracellular or lysosomal sources is transported into the cytosol and converted to fucose-1-phosphate via fucose kinase. Next, GDP-fucose pyrophosphorylase catalyzes the reversible condensation of fucose-1-phosphate with GTP to form GDP-fucose. All

GDP-fucose, synthesized from either pathway, must then enter the Golgi apparatus to be used for fucosylation reactions by the fucosyltranferases.

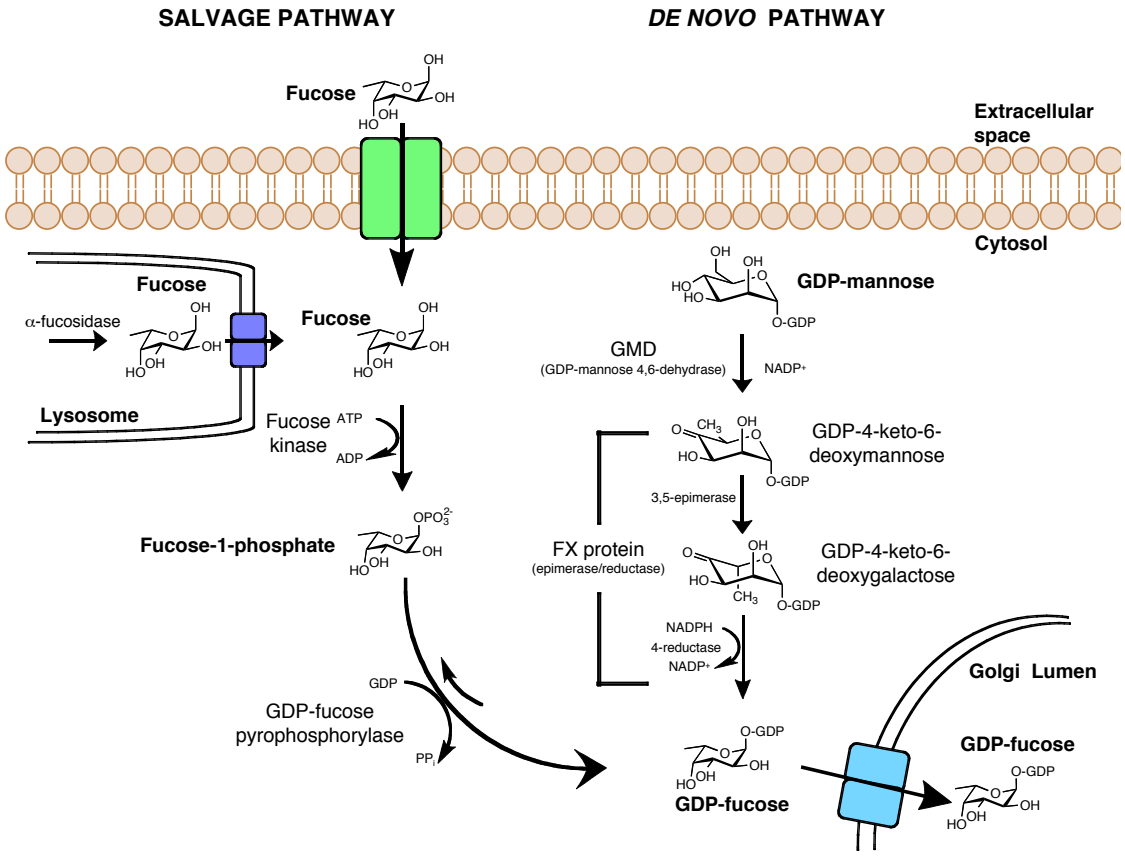

Figure 1.2. In mammalian cells, the biosynthesis of GDP-fucose occurs through two distinct pathways. The *de novo* pathway converts GDP-mannose to GDP-fucose via three enzymatic reactions carried out by two proteins, GMD and the FX protein. The salvage pathway utilizes free fucose found in the cytosol to synthesize GDP-fucose using the enzymes fucose kinase and GDP-fucose pyrophosphorylase. GDP-fucose generated by either pathway is transported into the Golgi lumen to become available to the fucosyltransferases.

In the brain, L-Fuc is usually found as the terminal residue on carbohydrate chains attached to proteins via *N*- or *O*-linkages to asparagine or serine/threonine residues, respectively.[12, 13] Frequently, L-Fuc is attached to the C-3 and C-6 positions of *N*-acetylglucosamine (GlcNAc) or to the C-2 position of galactose (Gal) (Figure 1.3).[12] Several studies support the idea that oligosaccharides containing terminal fucose α(1-2)

galactose (Fucα(1-2)Gal) moieties contribute to information storage and processing in the brain.

The importance of Fucα(1-2)Gal is supported by behavioral and electrophysiological studies using the unnatural sugar analog 2-deoxy-D-galactose (2-dGal). In these studies, the sugar analog 2-dGal competes with Gal for incorporation into carbohydrate chains.[14] Once inserted, 2-dGal inhibits the formation of a specific 1-2 glycosidic linkage with fucose because it lacks the hydroxyl group at the C-2 position (Figure 1.4).

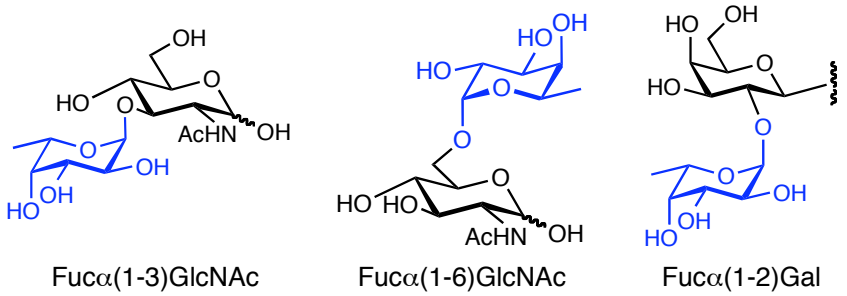

Figure 1.3. Common fucose-galactose linkages found on the terminal ends of carbohydrate chains

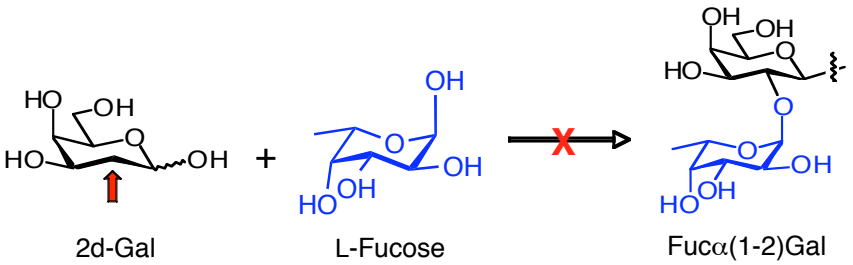

Figure 1.4. Incorporation of 2-deoxy-D-Galactose (2-dGal) inhibits formation of Fucα(1-2)Gal linkages. 2-dGal is missing the hydroxyl group at the C-2 position (red arrow) and thus prevents the incorporation of fucose into carbohydrate chains.

Fucose incorporation into hippocampal glycoproteins was found to be significantly reduced in trained rats injected with 2-dGal compared to saline-treated rats.[15, 16] Furthermore, treating rats with 2-dGal either 30 minutes before or 15 minutes

after retrieval testing caused amnesia in a passive avoidance response.[17] Similar experiments performed in trained chicks also resulted in decreased fucose incorporation and amnesic effects in animals treated with 2-dGal.[18, 19] In electrophysiological studies, hippocampal LTP maintenance was also suppressed upon treatment with 2-dGal.[20] These studies underscore the importance of Fucα(1-2)Gal glycoproteins in long-term memory formation.

Although many studies have looked at the amnesic effects of 2-dGal in the brain, studies have yet to examine the impact of 2-dGal on synaptic remodeling or neuronal structure. However, related small molecules have been shown to elicit varying effects in cell culture. Incorporation of the sugar analog 2-deoxy-2-fluoro-D-galactose in cultured rat hepatocytes inhibited *N*-glycosylation of membrane proteins.[21, 22] However, addition of the analog 2-deoxy-2-fluoro-D-glucose only partially inhibited *N*-glycosylation.[22] Furthermore, neither analog affected *O*-glycosylation.

Additional support for the importance of fucose-expressing glycoproteins in learning and memory can be found in studies examining the levels of fucose incorporation following learning tasks. When incorporation of [^{14}C]-labeled fucose was monitored in trained chicks, a linear increase of fucose could be seen in forebrain slices for up to 3 hours after training.[3] Furthermore, a 26% increase in tritium-labeled fucose 24 hours following learning in trained chicks, as compared to nontrained chicks, was observed in a separate study.[23] Collectively, these findings support the view that glycoproteins expressing the Fucα(1-2)Gal epitope play a critical role in neuronal communication.

It is interesting to note that glycoproteins present at synapses have been previously implicated in cellular recognition and adhesion steps during synaptic development. A well-studied example is the neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily that is glycosylated with polysialic acid (PSA) residues. NCAM is crucial in the development and regeneration of the nervous system and is also involved in synaptogenesis.[24, 25] Interestingly, the PSA residues present on NCAM greatly affect these functions. Enzymatic removal of PSA with endoneuraminidase (endo N) inhibited LTP in the hippocampus and disrupted neuron migration and axon outgrowth.[26 -- 29] Polysialylated NCAM also helps maintain membrane fluidity and neural plasticity by increasing intermembrane repulsion and inhibiting cellular adhesion.[30]

Evidence for the existence of Fucα(1-2)Gal lectins in the mammalian brain

In addition to the role of fucose-containing glycoproteins, there is evidence that proteins which specifically recognize fucose may also be involved in regulating neuronal communication. The importance of lectins (proteins that recognize carbohydrates) that specifically recognize the Fucα(1-2)Gal moiety in memory formation has been demonstrated in several behavioral studies using an antibody specific to the Fucα(1-2)Gal epitope.

The monoclonal antibody A46-B/B10, which recognizes the trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc[31] (Figure 1.5), was injected intrahippocampally to rats trained on a brightness discrimination task. Administration of the antibody both before and after training did not interfere with learning but drastically reduced the retention of

the task in relearning sessions, whereas an antibody selective for other trisaccharides had no amnesic effect.[32] Although the potential molecular mechanism(s) by which the antibody causes amnesia has not been elucidated, one potential explanation is that the antibody prevents the interaction between Fucα(1-2)Gal-containing glycoproteins and lectins. These studies support the notion that Fucα(1-2)Gal lectins may be involved in modulating neuronal communication.

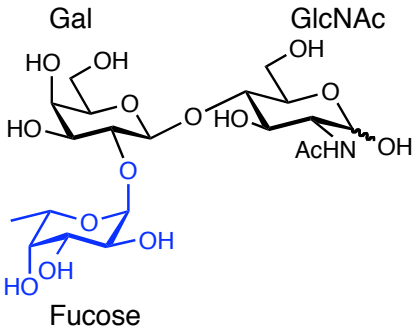

Figure 1.5. The trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc recognized by antibody A46-B/B10

Studies using free fucosyl saccharides provide further evidence for the importance of Fucα(1-2)Gal lectins in the brain. Several reports have shown that free fucosyl saccharides enhance memory retention and LTP. For instance, rats injected with L-Fuc or the trisaccahride L-Fucα(1-2)Galβ(1-4)Glucose (2'-Fucosyllactose) exhibited prolonged, enhanced potentiation following induction of LTP compared to control animals injected with saline or lactose.[33] Higher potentiation following LTP induction was also observed in rat hippocampal slices incubated with L-Fuc and 2'-Fucosyllactose.[34] Importantly, the observed effects were stereo- and regiospecific as neither D-fucose or 3'-Fucosyllactose showed any effect on LTP or memory consolidation.[34] While these effects may also be due in part to fucosylation of

glycoproteins, another potential explanation is that these fucosyl saccharides could be interacting with specific Fucα(1-2)Gal lectins on the cell surface to promote LTP.

The binding of lectins to specific carbohydrate motifs is well precedented in mediating cellular recognition events, including leukocyte adhesion, microbial phagocytosis, and neuronal outgrowth.[35 -- 37] One well-studied example is the galectin family of lectins, which specifically recognize galactose-containing oligosaccharides. Galectins are found on both the cell surface and the extracellular matrix, as well as in the cytoplasm and nucleus, and are involved in cell adhesion, cell growth, and apoptosis.[38] Notably, galectin-3 mediates cell adhesion in a carbohydrate-dependent manner, as treatment of cells with either endo-β-galactosidase (a protein that cleaves the galactose moieties from the protein) or a polyvalent carrier of terminal β-galactosides inhibited adhesion.[39]

Proposed mechanisms of Fucα(1-2)Gal action

As detailed in the above studies, L-Fuc and, in particular, the Fucα(1-2)Gal saccharide appear to be critical components for long-term information storage. Despite the overwhelming evidence implicating the Fucα(1-2)Gal epitope in memory formation, relatively little is known about the proteins that display (glycoproteins) or bind (lectins) this epitope. One research group has reported the identification of several proteins to be Fucα(1-2)Gal glycoproteins.[40] However, the existence of a Fucα(1-2)Gal epitope on these proteins was not rigorously proven and was only shown through antibody binding, which can be somewhat promiscuous. Additionally, another group has reported the fucose-mediated binding of the proteoglycan versican to the adhesion protein tenascin-R.

Importantly, only the C-type lectin domain of versican, expressed as a recombinant protein, was shown to bind to insolubilized fucose.[41] Aside from these two reports, no other Fucα(1-2)Gal glycoproteins or lectins have been identified. Moreover, the functional significance of the Fucα(1-2)Gal epitope has not yet been investigated.

Based on literature findings and our own results, we have developed three working models to explain the role of fucosyl saccharides in modulating neuronal communication. In the first model, we propose that the Fucα(1-2)Gal epitope functions as a "recognition element" to mediate protein-protein interactions at the cell surface (Mechanism A, Figure 1.6). In this model, specific molecular recognition between Fucα(1-2)Gal glycoproteins and lectins would be expected to trigger intracellular signaling cascades that modulate neuronal communication. In the second model, we propose that the Fucα(1-2)Gal epitope may function as a "targeting element" to regulate the folding, function and/or trafficking of Fucα(1-2)Gal glycoproteins to the synapse (Mechanism B, Figure 1.6). By directing glycoproteins to the synapse, the Fucα(1-2)Gal epitope may position the glycoproteins to perform essential functions, as well as help recruit proteins to the synapse during cell communication events. Alternatively, it is possible that fucosyl glycoproteins may act intracellularly to modulate proteins and signaling pathways involved in synaptic plasticity (Mechanism C, Figure 1.6).

Given the complexity of the brain, it is possible that these three mechanisms are operating in parallel to mediate neuronal communication. Regardless of which model is correct, it is clear that identification of Fucα(1-2)Gal lectins and glycoproteins is necessary to understand the potential role of fucosyl saccharides in the formation and strengthening of synaptic connections. Furthermore, the discovery of new lectins and

glycoproteins will enable a molecular-level understanding of the role of fucosyl saccharides on neuronal communication and provide new insights into cell communication and synaptic plasticity in the brain.

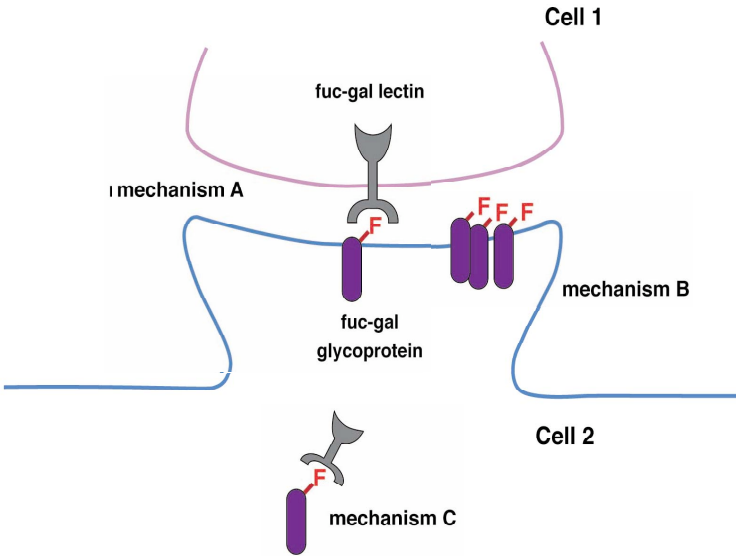

Figure 1.6. Proposed mechanisms by which the Fucα(1-2)Gal epitope acts in neurons. Fucα(1-2)Gal saccharides (**F**) may function as recognition elements to mediate protein-protein interactions between Fucα(1-2)Gal lectins and glycoproteins (mechanism A) or as targeting elements to increase the concentration of fucose at the synapse (mechanism B). Additionally, Fucα(1-2)Gal proteins may operate intracellularly to modulate proteins at the synapse.

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