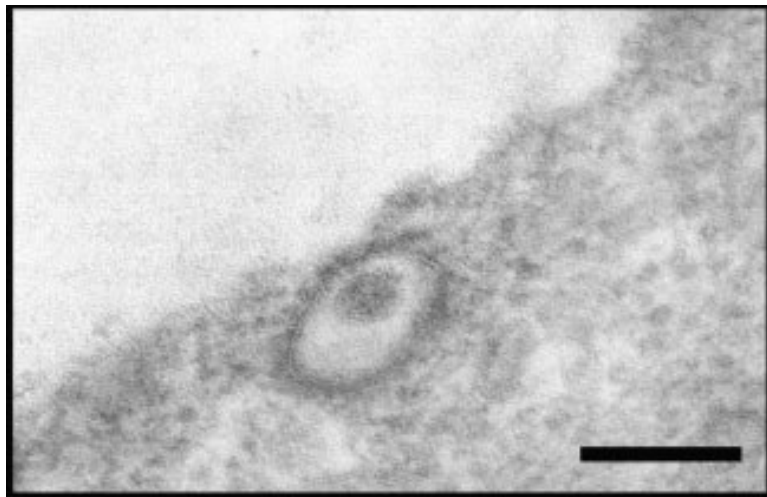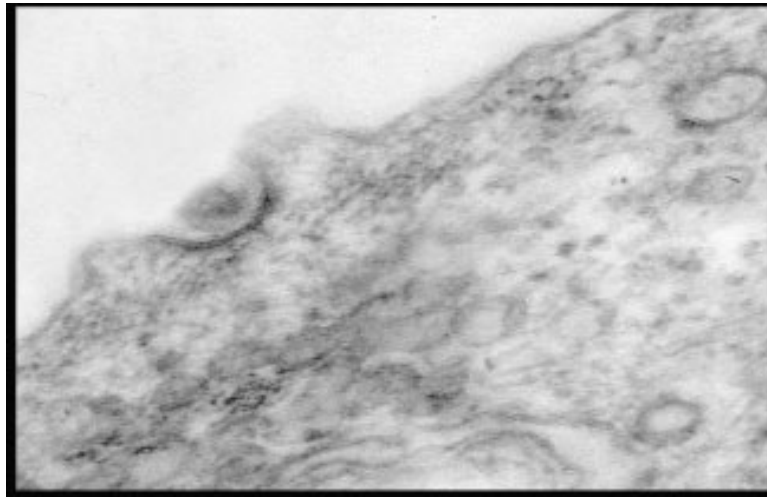# Sialic acid as a receptor for viruses and bacterial toxins

M.M. van Buuren, 13th of December 2009

About the cover

The cover shows two microscopic pictures of the internalization of an Arena virus. The first is the location of the virus in a clathrin coated pit, the second is the location of the virus in a clathrin coated vesicle.

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Biomedical Sciences – Immunity & Infection

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Index

Abstract

In order to develop strategies to stop infections, detailed information is needed about the attachment and internalization of viruses. This review discusses viruses; SV40, Rota virus, Arenavirus and Influenza A virus, and the bacterial toxin; Cholera Toxin, which all are, or thought to be, dependent on sialic acids for infection of target cells. First general information on sialic acids and sialic acid containing structures as glycosphingolipids and glycoproteins is discussed. Subsequently, several endocytic routes are reviewed that can be employed by the virus to be internalized. Finally the different viruses and toxins are discussed with regard to their sialic acid dependency, attachment-, entry receptors and endocytic pathways. In the discussion more questions are raised that need to be answered to fully understand the matter of sialic acid dependent internalization of viruses.

1. Introduction

The life cycle of a virus is dependent on its ability to infect one living cell after another. The virus exploits the cell for replication and it can actually be said that the host assists the virus in the processes of attachment, penetration, uncoating, entry, replication and release. The result is the multiplication of the initial virus particle into several progeny virus particles(Flint et al. 2004).

Attachment of the virus to the target cell is accomplished through the binding of cell specific receptors. The interaction between the attachment receptors and the virus is highly specific. Therefore, infection of certain cell types and species is determined by the characteristics of this interaction(Marsh, Helenius 2006).

The initiation of endocytic routes can be induced by the interaction of the attachment receptors and the virus, however, it often occurs that other receptors are responsible for viral entry, the so called entry receptors. Entry of the virus can be accomplished by different endocytic pathways, which are normally employed to take up essential nutrients, macro-molecules or have a function in membrane recycling and signalling. The different pathways are Clathrin Mediated Endocytosis (CME), caveolar dependent endocytosis, clathrin and caveolae independent endocytosis and finally the dependence on dynamin can play a major role in the endocytic pathway. All of the above pathways will be discussed in detail, but are here introduced shortly. From the various routes, the CME has been studied thoroughly. In CME, a broad variety of transmembrane receptors and cargo is packed into Clathrin Coated Vesicles (CCVs) and then transported deeper into the cell's interior. The caveolar/raft pathway is a clathrin independent endocytic pathway and is cholesterol sensitive. Both the CME and the caveolar dependent pathway are dynamin dependent. Dynamin is a fission protein and causes the endocytic vesicles to bud from the plasma membrane. Finally it is also possible to enter the cell through clathrin and caveolae independent endocytosis, which are not necessarily dynamin dependent. The arf6- and GEEC pathways are both examples of clathrin and caveolae independent endocytosis(Doherty, McMahon 2009).

Several viruses, including SV40, Rota virus and Influenza A virus use sialic acid moieties as attachment and entry receptors. It is thought that also Arenaviruses use sialic acids for infection of their target cells, but there is no scientific proof for that yet. Additionally, Cholera Toxin enters its target cell after binding to these sugar residues. Structures like glycolipids and glycoproteins are often sialidated and can thus serve as cellular receptors for the above mentioned viruses and toxin. Therefore, glycolipids and glycoproteins will be discussed. To understand the mechanism of entry via the sialic acid residues, literature on the specific viruses and toxin is reviewed, with a particular emphasis on Influenza A. The impact of Inluenza A on the world population in the last 100 years and the impact it still has results in the fact that it is necessary to learn about Influenza A.

2. Sialic acid containing structures

The first event of viral infection is binding to the cellular receptors. This matter determines host range, cellular tropism and also disease potential of the virus. It is known that the Influenza A virus, among other viruses, binds to structures on the plasma membrane that contain sialic acids. Sialic acids are terminating sugar residues characteristically found on glycosphingolipids, N-glycans and O-glycans(Varki et al. 2009). This chapter will discuss the structure of these sialic acid containing structures as well as their physiological function.

2.1. Sialic acid
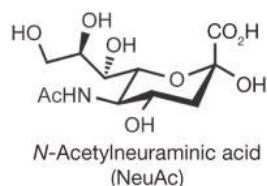
Figure 1 | Structural basis of sialic acid (NeuAc)(Varki et al. 2009)

The monosaccharide sialic acid is metabolically derived from two 'primary' sialic acids; N-acetylneuraminic acid and 2-keto-3-deoxynomonic acid. *N*-Acetylneuraminic acid (NeuAc) is the exclusive sialic acid in humans, its molecular structure can be found in figure 1. In other mammals *N*-glycolylneuraminic acid (NeuGc) is also abundant. Hydroxylation of NeuAc results into NeuGc. The enzyme that mediates the hydroxylation reaction contains a specific mutation in humans, which results in the lack of NeuGc.

The variety in recognition of the sialic acids residues by viruses mainly results from the different α linkage that can possibly be formed between the C-2 of sialic acid and the underlying sugar moiety. The common linkages are attachment of sialic acid to the galactose residues on the C-3 (as shown in figure 2) or C-6 position or to the N-acetylgalactosamine residues on the C-6 position. Mediation of attachment of one sialic acid residue to another is often via the C-8 position.
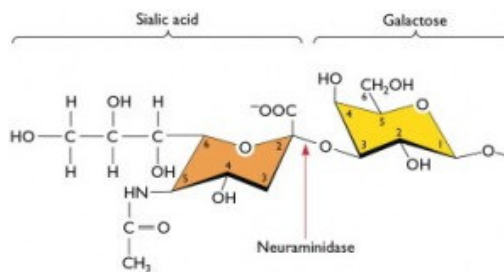
Figure 2 | An α2-3 linkage of sialic acid to galactose(Anonymous)

Additionally, a variety of modifications can cause diversity of sialic acids. Various positions of the core structure can carry one or more substitutions at the hydroxyl groups, namely: acetyl-, methyl-, sulphate-, lactyl- or phosphate-groups(Varki et al. 2009). In the scope of this thesis it is merely important to know that sialic acid can be present at the terminal position of glycolipids and glycoproteins.

2.2. Glycolipids

A major group of glycolipids are glycosphingolipids, which are merely found in the vertebrate brain, where they also were discovered. Later on it became clear that glycosphingolipids reside in all cell types of the body. They are part of the plasma membrane, where they exclusively reside in the noncytosolic monolayer. Glycosphingolipids are not evenly distributed, but assembly into micro domains, also called 'lipid rafts'(Varki et al. 2009, Alberts et al. 2002b). These lipid rafts have a diameter of 10-200nm and are heterogeneous membrane domains enriched in glycosphingolipids, cholesterol, selected proteins, like GPI-anchored proteins and transmembrane signalling proteins(Lajoie, Nabi 2007). It has been suggested that the formation of lipid rafts results in the clustering of signalling molecules and therefore it is thought that this clustering can initiate signalling(Varki et al. 2009).

The base of glycosphingolipids is the lipid moiety ceramide, which consists of a long-chain amino alcohol (sphingosine) in amide linkage to a fatty acid. The sugar chain is attached to ceramide. Although ceramide can vary in hydroxylation, length and saturation the differentiation between the various glycosphingolipids results from differences in the glycan chain. GT1b is an example of a glycosphingolipid and depicted in figure 3.
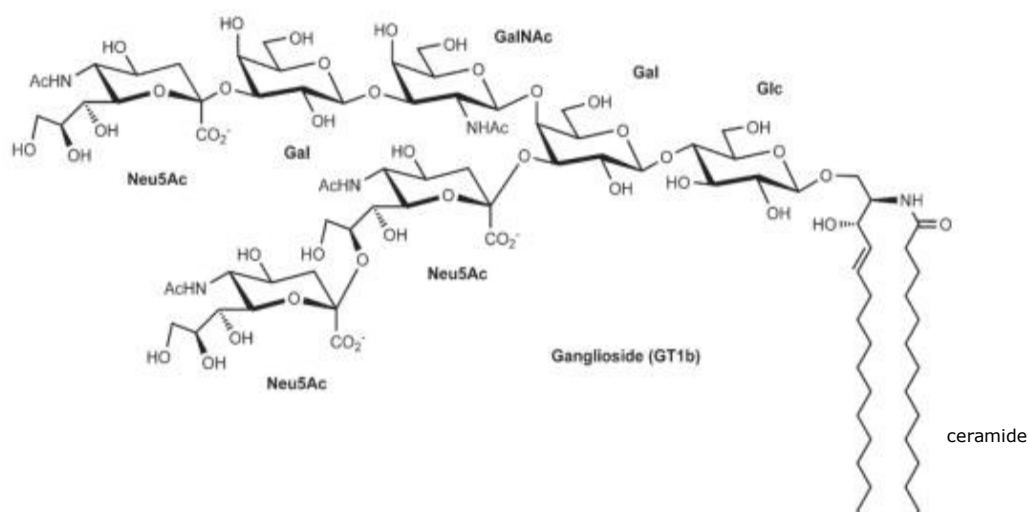
Figure 3 | A glycosphingolipid is built on a ceramide lipid moiety connected to a sugar chain, GT1b is depicted(Varki et al. 2009).

The family of glycosphingolipids can be divided into five subfamilies, based on their neutral core structures; lacto-, neolacto-, ganglio-, globo-, and iso-globo series. The diverse subfamilies are differentially expressed in specific tissues, the ganglio series for example, are mainly found in the brain. All sialylated glycosphingolipids are traditionally called Gangliosides, like GT1b, independently of the core structure. The nomenclature of ganglioside series is as follows: "G refers to gangliosides series, the second letter refers to the number of sialic acid residues (mono, di, tri, etc.), and the number refers to the order of migration of the gangliosides on thin-layer chromatography."(Varki et al. 2009).

2.2.1. Synthesis of glycosphingolipids

The synthesis of glycosphingolipids is a stepwise process; the first sugar is added to ceramide. Subsequently sugars are transferred from their nucleotide sugar donor to the growing sugar chain. Typically β-linked galactose or glucose is the first sugar of the growing chain and therefore closest to ceramide, this results in a galactolipid (GalCer) or a glucolipid (GlcCer). Sialylated galactolipids are seldom extended with additional sugar residues and are mostly sulphated. Most of the subfamilies of glycosphingolipids are built on the glucolipids. An illustration of the biosynthesis of the ganglio-series can be found in figure 4(Varki et al. 2009).

Figure 4 | The biosynthesis of the glycosphingolipid ganglio-series(Varki et al. 2009).
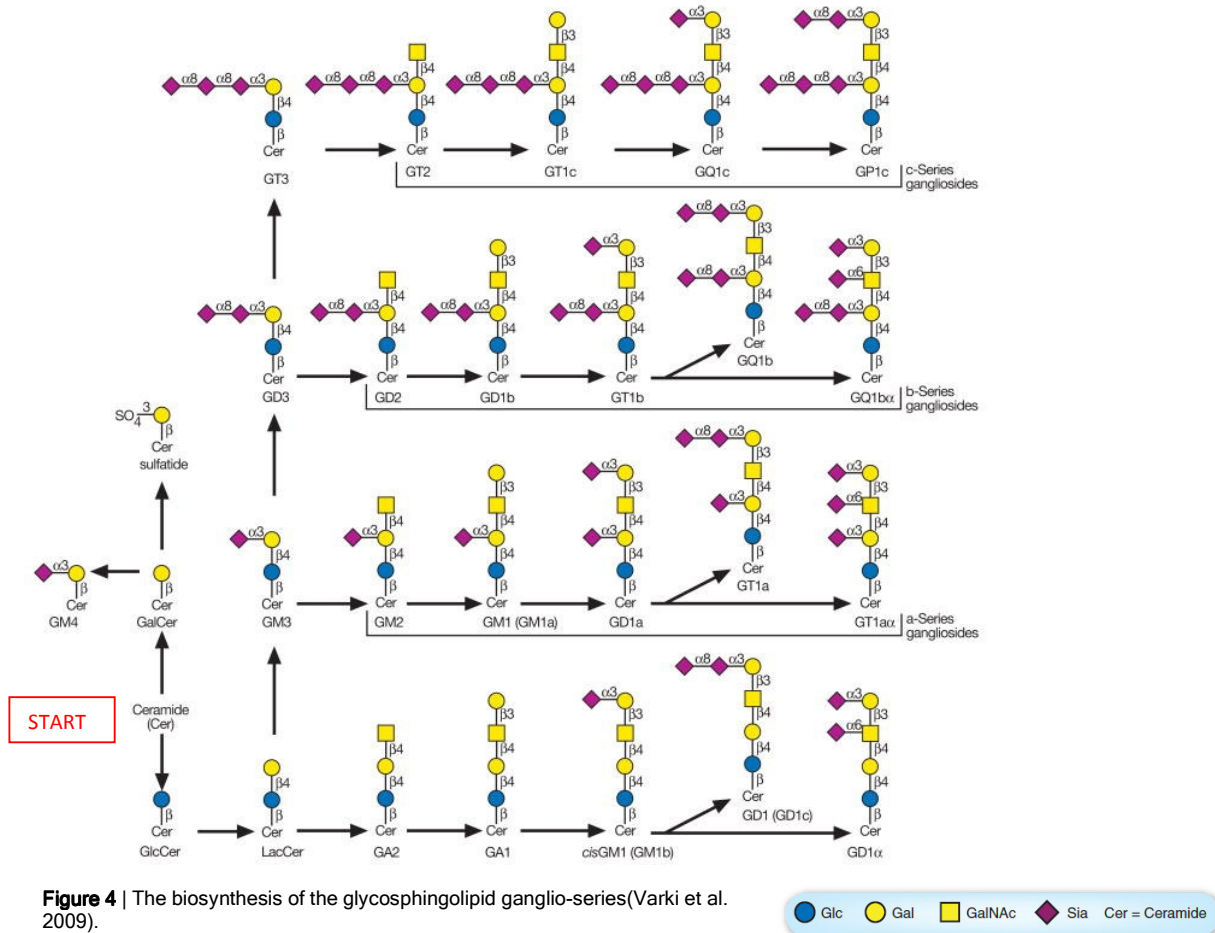
The synthesis of glycosphingolipids is mediated by various glycosyltransferases. Synthesis of GlcCer occurs on the cytoplasmic face of the Endoplasmatic Reticulum (ER) and then flips into the Golgi lumen where the glycan chain is elongated. On the contrary GalCer synthesis occurs on the luminal face of the ER, traffics through the Golgi, and there it may be sulphated or sialylated. In both cases the orientation on the plasma membrane is in accordance with the final orientation during synthesis. They will reside in the non-cytosolic layer and therefore face the extracellular milieu(Varki et al. 2009).

2.2.2. Physiological functions of glycosphingolipids

As discussed earlier, glycosphingolipids are found in the plasma membrane. The two main functions of glycosphingolipids are the mediation of cell-cell interaction (*trans* regulation) and the modulation of activities of proteins in the same plasma membrane (*cis* regulation) (Varki et al. 2009).

Cell-cell interaction can be accomplished by the binding of complementary molecules. This interaction is mediated through the recognition of the glycan chain by Glycan-Binding-Proteins (GBP). GBP's can be subdivided into two major groups; glycosaminoglycan-binding proteins and lectins. The glycosaminoglycan-binding proteins recognize sulphated glycosaminoglycans. Lectins usually recognize the terminal parts of the glycan chains via Carbohydrate-Recognition Domains (CRDs). Most lectins contain two or more CRDs. It is necessary that multiple CRDs are involved in the interaction between a lectin and a glycan, in order to achieve a high affinity bonding (Varki et al. 2009).

The function of the glycosphingolipids involved in cell-cell interaction is illustrated in the research of Sun *et al.* in which the GM2/GD2 synthase was knocked out in mice. In these mice an axon degeneration and demyelination was observed. Myelin-Associated Glycoprotein (MAG) is an important glycoprotein in the myelin sheet of neurons and complementary to gangliosides GD1a and GT1b. It is a member of the Siglec family of sialic-acid-dependent carbohydrate-binding proteins. A decrease in the protein expression of MAG was observed in the GM2/GD2 synthase knock out mice, whereas the mRNA levels were comparable to the wild type controls. This was ascribed to a decreased stabilization of the protein because its ligands GD1a and GT1b could not be expressed and it is thought that the interaction between these two is necessary to retrieve stabile MAG protein. The decreased stability of MAG causes the demyelination of the axons(Sun et al. 2004).

The *cis* regulation of glycosphingolipids is illustrated by analysis of the role of GM3 in insulin resistance. A decreased ability of cells to respond to physiological levels of insulin is defined as insulin resistance. This is characteristic for type II diabetes. The addition of exogenous GM3 to adipocytes resulted in a decreased phosphorylated state of the insulin receptor. The addition of GM3 to adipocytes eventually resulted in a decreased insulin sensitive uptake of glucose. However, the depletion of GM3 resulted in a hyper-phosyphorylated state of the insulin receptor. Previous studies reviewed in this paper showed that GM3 inhibits auto-phosphorylation of the Epidermal Growth Factor (EGF) and insulin receptor. The process of auto-phosphorylation occurs after receptor dimerization, so it is thought that GM3 interferes with the insulin receptor by inhibiting the clustering of the receptors, which is necessary for downstream signalling. But scientific evidence is necessary to confirm this hypothesis(Tagami et al. 2002).

2.3. Glycoproteins

Glycoproteins are a type of glycoconjugates in which one or more glycans are covalently attached to a polypeptide chain, in general via an *N*- or *O*- linkage(Varki et al. 2009). Many glycoproteins are part of the plasma membrane where they are involved in cell adhesion. However, glycoproteins can also be soluble and are then secreted from cells(Berg, Tymozcko & Streyer).

An N-glycan is a polypeptide chain which has a sugar chain covalently attached to the nitrogen atom in the side chain of an asparagine residue, in the case of an O-glycan the sugar is attached to an oxygen atom in the side chain of a serine or threonine residue. Examples of an *O*- and *N*-linked glycoprotein can be found in figure 5(Berg, Tymozcko & Streyer).
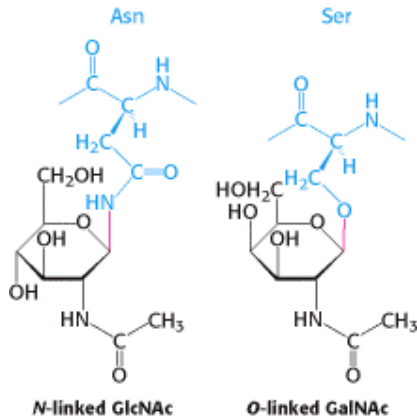
Figure 5 | *N*- and *O*- linked glycoproteins(Berg, Tymozcko & Streyer)

There is a great variety of *N*-linked glycoproteins. This variety is caused by all the sugar combinations that can be added to the common pentasaccharide core, which contains two *N*-acetylglucosamine- and three mannose residues, as can be observed in figure 6(Berg, Tymozcko Streyer).

&
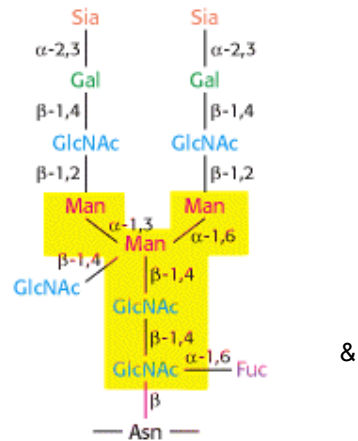
Figure 6 | *N*- linked glycoproteins consist of a pentasaccharide core(Berg, Tymozcko & Streyer).

2.3.1. Synthesis of glycoproteins

As with glycolipids, the glycosilation of glycoproteins takes place in the lumen of the ER and the Golgi. O-linked glycosilation exceptionally takes place in the Golgi, whereas the N-linked glycosilation process starts off in the ER and continues in the Golgi.

Glycosilation of the N-linked glycoproteins is a very well studied and interesting process, which is a two phase process of core glycosilation in the ER and terminal glycosilation in the Golgi. Transfer of a specific oligosaccharide is mediated via the lipid molecule dolichol phosphate, which resides in the membrane of the ER. Assembly of the N-linked glycolipid is a three step process and shown in figure 7. It starts of with the addition of two N-acetylglucosamine- and five mannose residues to dolichol phosphate. Sugar nucleotides are the donors in this matter. This monosaccharide transfer is catalyzed by various cytoplasmic enzymes. Subsequently, this combined structure of dolichol phosphate and oligosaccharide is flipped into the lumen of the ER through the ER membrane. Finally, in the lumen of the ER the sugar chain is built up to a 14-residue oligosaccharide which is then transferred to an asparagine residue of a polypeptide chain in the ER lumen. The N-linked glycoprotein is then transported to the Golgi, where the carbohydrate units are elaborated or altered. The trans-Golgi is the location where glycoproteins can be terminated with sialic acids, forming a complex glycoprotein(Berg, Tymozcko & Streyer).
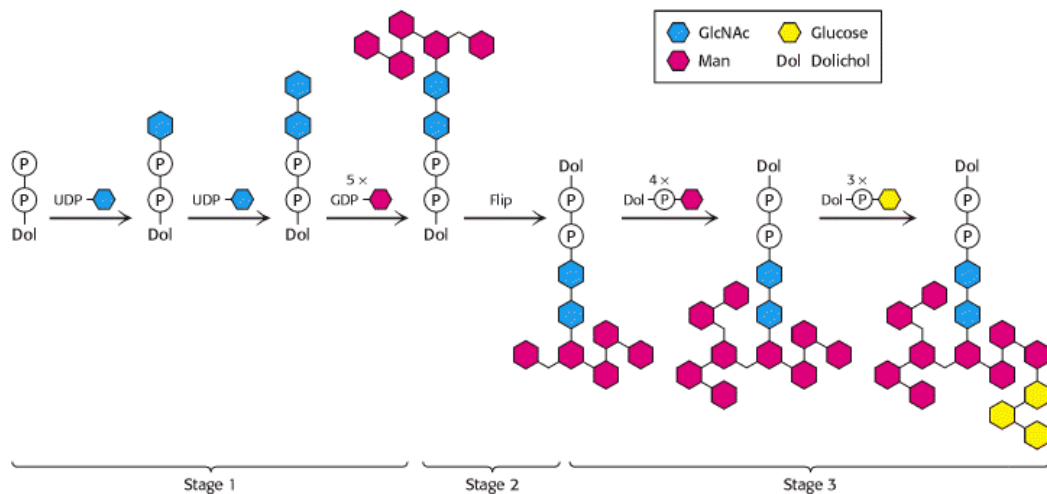
Figure 7 | Dolichol phosphate assembles the olichosaccharide chain in three steps(Berg, Tymozcko & Streyer).

2.3.2. Physiological functions of glycoproteins

Glycoproteins are ubiquitously expressed and have a very broad range of functions, like involvement in cell-cell interaction and protection. Since glycoproteins, like glycosphingolipids, contain a carbohydrate moiety, binding to lectins often occurs to accomplish cell-cell interaction. And, as is the case for glycosphingolipids, multiple weak interactions are necessary to yield a binding that is strong enough(Berg, Tymozcko & Streyer). Another function of glycoproteins is protection. The group of mucins, which are highly *O*-glycosylated glycoproteins are found as trans-membrane glycoproteins and in mucous secretion. Epithelial cells in the trachea, genitourinary- and gastrointestinal tract produce gel-forming mucins that hydrate and protect the underlying epithelial cells(Varki et al. 2009).

2.4. Useful techniques

The binding to sialic acid containing structures is often a prerequisite for attachment and infection of the various viruses and bacterial toxins that will be discussed in this review. It is therefore of great importance to analyze the presence and the function of sialic acids on cellular surface molecules. Here, several techniques will be discussed that can be used to detect sialic acids or that can alter sialylation. A summary of the discussed techniques can be found in table 1.

2.4.1. Detection of sialic acids

Sialic acids are generally deteced by fluoresceinated lectins or by histochemistry. Sialic acids are specifically recognized by a lectin that is linked to a fluorochrome or biotin respectively. Fluorescent labelling of cells can be visualized using fluorescent microscopy and biotin labelling of cells can be detected using various development kits(Nicholls et al. 2007).

2.4.2. Alteration of sialylation

Some assays require the alteration the sialylation of cells. There are several methods to do this. Here, the use of various drugs, mutant cell lines and the use of neuraminidase are discussed.

2.4.2.1. Drugs

Glucosyltransferase inhibitors are regularly used drugs to influence the glycosilation or synthesis of glycoproteins or glycolipids. Glucosyltransferase inhibitors as PPMP (1-phenyl-2-hexadecanoyl-amino-3-morpholino-1-propanol)(Kovacs, Pinter & Csaba 2000), DGJ (deoxygalactonojirimycin) and DNJ (deoxynojirimycin) inhibit the glucosylation of ceramide, which is the primary building block of many glycosphingolipids, as discussed above in chapter 2.2 and can additionally be seen in figure 8.

Besides the inhibitory effect on glucosyltransferase, DNJ additionally inhibits the N-glycan processing enzymes (α-glucosidase I and II) in the ER{{228 Alonzi,D.S. 2008; }}. DGJ, for example, is a competitive inhibitor, which reduces substrate binding by glucosyltransferases. This is the enzyme that glucosylates ceramide. DGJ thereby reduces the synthesis of glycosphingolipids(Kasperzyk et al. 2004).

Figure 8 | Action of glucosyltransferase inhibitors (adapted from (Varki et al. 2009)).

2.4.2.2. Mutant cell lines

The involvement of specific sialylated structures can be analyzed with the use of various mutant cell lines. For example Lec-1, Lec-2, GM95 and C6 cell lines have been shown useful in this field of research.

The involvement of (sialylated) glycoconjugates in certain biological processes can be analyzed with the use of Chinese Hamster Ovary (CHO) cell clones Lec-1 and Lec-2. Lec-1 cells are suited to study the involvement of N-linked glycoproteins. It is a cell line deficient in GlcNAc glycosyltransferase, this causes blockage of processing and glycosilation of N-linked glycoproteins. The Lec-2 cell line is characterized by a 90% reduction of sialated glycoconjugates, because the transport of sialic acid sugar donors into the Golgi is reduced dramatically(Rolsma et al. 1998).

The function of glycosphingolipids can also be analyzed with the use of GM95, which is a cell line deficient in glycosphingolipids, because it lacks the ceramide glucosyl-transferase(Smith, Lilie & Helenius 2003).

The rat C6 cell line is deficient in gangliosides. As stated before, these are sialylated structures, so it can be useful to take this cell line along in assays where gangliosides are thought to play a major role(Tsai et al. 2003).

2.4.2.3. Enzymatic removal of sialic acids

Neuraminidases have long been used to proof whether sialic acids on cellular structures are important for binding. Most neuraminidases take of the terminal sialic acids but cannot reach internal sialic acids. Involvement of terminal sialic acids can be analyzed with these neuraminidases. Figure 9 shows the action of most neuraminidases on gangliosides(Guo et al. 1999).
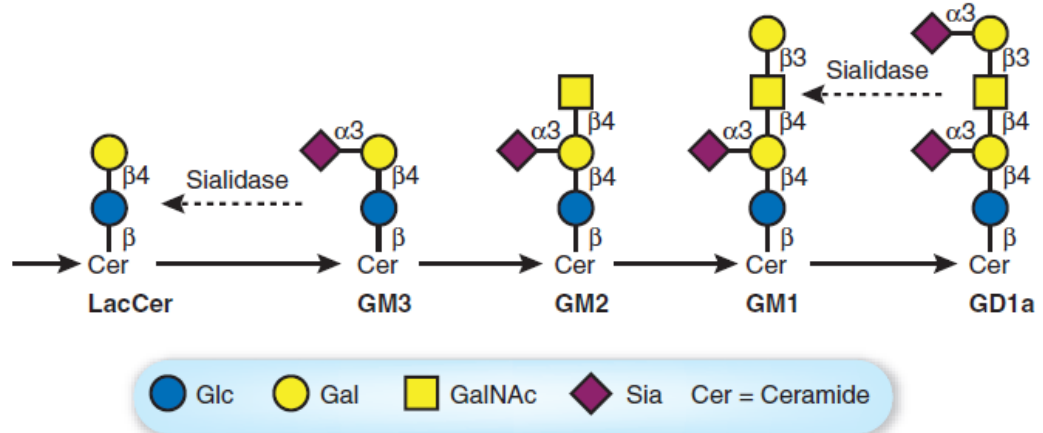
Figure 9 | Biosynthetic pathways of gangliosides and sialidase action(Banda, Kang & Varki 2009)

Table 1 | Useful techniques.

Technique		Application
Detection of sialic acids		The use of fluoresceinated lectins and histochemistry(Nicholls et al. 2007)
Alteration of sialic acids	Glucosyltransferase inhibitory drugs	PPMP, inhibits glycosyltransferase(Kovacs, Pinter & Csaba 2000)
		DGJ inhibits glycosyltransferase(Kasperzyk et al. 2004)
		DNJ inhibits glycosyltransferase(Kasperzyk et al. 2004)
	Mutant cell lines	Lec-1; deficient in *N*-linked glycoproteins(Rolsma et al. 1998)
		Lec-2; 90% reduction of sialated glycoconjugates(Rolsma et al. 1998)
		GM95; deficient in glycosphingolipids(Smith, Lilie & Helenius 2003).
		C6; deficient in gangliosides(Tsai et al. 2003).
	Enzymatic removal of sialic acids	Neuraminidases take of the terminal sialic acids but cannot reach internal sialic acids(Guo et al. 1999).

Table 1 | Summary of useful techniques to detect and alter sialic acids on cellular surface structures.

3. The different endocytic pathways

The non-cytoplasmic monolayer of the plasma membrane is the part of the cell through which it communicates with its external milieu. To be able to respond to the exterior in an appropriate way, the plasma membrane and all it is composed of is regulated in a very strict manner. Endocytosis is for example a way to regulate the expression of integral membrane receptors. Viruses employ various endocytic routes normally used by the cell to take up essential nutrients and macro-molecules, additionally these routes have a function in membrane recycling and signalling. The endocytic vesicles can be targeted to different intracellular compartments and transport between these intracellular compartments is also possibility, as depicted in figure 10.
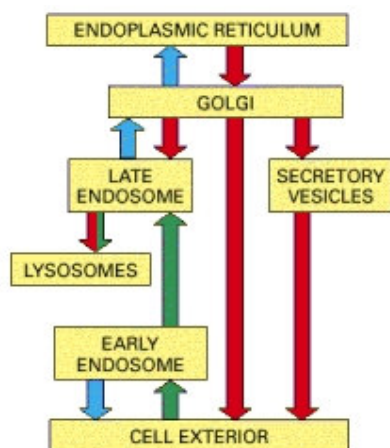
Figure 10 | Targeting of cargo to intracellular compartments(Alberts et al. 2002b)(Alberts et al. 2002a).

This chapter will discuss different ways of endocytosis. First, Clathrin Mediated Endoctosis (CME) will be discussed. This classical endocytic pathway makes use of Clathrin Coated Vesicles (CCVs). Subsequently, endocytosis dependent on caveolae is summarized. Both CME and caveolae mediated endocytosis are dependent on dynamin, a fission molecule that mediates the release of sealed vesicles from the membrane. Finally clathrin- and caveolae independent endocytic pathways will be discussed, which are additionally dynamin independent. It is important to state that this is not a complete overview of the endocytic pathways cells asset. The reason that merely these routes are discussed is that they all have been implicated in the entry of viruses that employ sialic acid containing structures as receptors.

3.1. Clathrin mediated endocytosis

The clathrin mediated endocytic route is often referred to as the 'classic endocytic pathway', because it is a route that is used for the internalization of many molecules. For example, CME is constitutively turned on for the recycling of the plasma membrane and cell surface receptors(Alberts et al. 2002b). CME is a way to transport receptors and their ligands in CCVs and it occurs at the plasma membrane. Additionally, CCVs transports cargo to and from various intracellular compartments(Doherty, McMahon 2009).

Initiation of this routes starts by the formation of Clathrin Coated Pits (CCPs). Adaptor Protein-2 (AP-2) plays a central role in the formation of the CCPs. AP-2 is recruited to the plasma membrane where it associates with clathrin triskelions at shallow invaginations of the plasma membrane to form CCPs. Additionally AP-2 recognizes endocytosis motifs in the cytoplasmic domain of the receptors which have to be internalized. It also occurs that an alternative adaptor is necessary for the recognition of specific receptors which do not directly interact with AP-2. AP-2 independent clathrin dependent endocytosis has also been reported and it has been suggested that alternative adaptors are responsible for the assembly of those CCPs, since they can interact with the clathrin triskelions and the plasma membrane and additionally recognize the endocytic signals(Benmerah, Lamaze 2007).
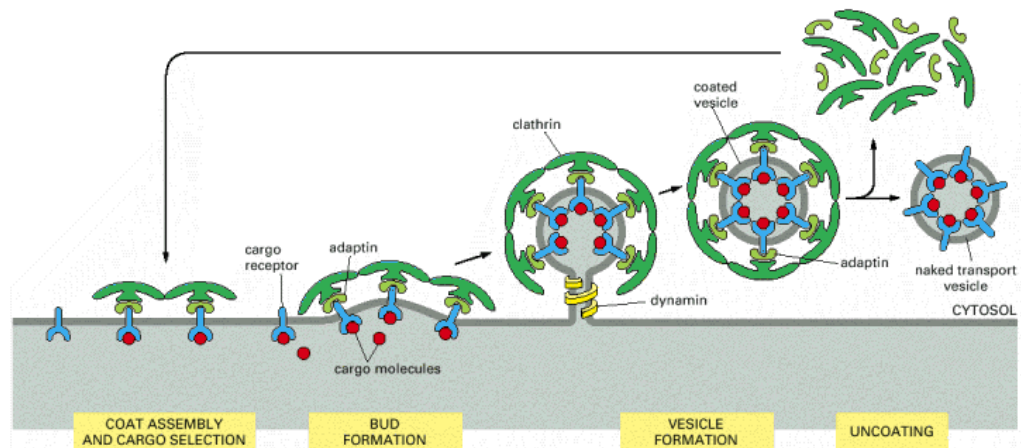
Figure 11 |The assembly and disassembly of the CCV(Alberts et al. 2002b)(Alberts et al. 2002a).

The assembly of the clathrin coat at the plasma membrane should finally result in the formation of a sealed vesicle. In order to achieve this, the association of dynamin proteins are necessary(Benmerah, Lamaze 2007). Dynamin is a large GTPase, which assembles around the neck of a vesicle(Doherty, McMahon 2009). In cooperation with other cellular proteins dynamin forms a ring and upon GTP hydrolysis facilitates the destabilization of the plasma membrane. Subsequently the non-cytoplasmic leaflets of the plasma membrane fuse, which results in the release of the sealed vesicle. The CCV rapidly looses its coat, once it is released from the plasma membrane, which is mediated via an uncoating ATPase. A schematic overview of this process can be found in figure 11(Doherty, McMahon 2009, Alberts et al. 2002b).

Once the CCV has lost its coat, the cargo can be targeted to different intracellular compartments, as depicted in figure 10. Most vesicles from the clathrin mediated pathway fuse with early- and late endosomes and can eventually end up in the trans-golgi network and the ER. As stated before, transport takes place between intracellular compartments, what can result in recycling of molecules back to the plasma membrane(Alberts et al. 2002b).

3.2. Clathrin independent endocytosis | Caveolae dependent endocytosis

While CME is the most common and one of the best studied endocytic routes, also clathrin-independent routes exist to accommodate internalization. An example of this route is caveolae dependent endocytosis. Caveolae are a sub domain of lipid rafts on the plasma membrane, therefore both caveolae and rafts often exploit similar routes(Nabi, Le 2003). The caveolar invaginations are rich in sphingolipids and cholesterol, similar to lipid rafts. For the formation of caveolae a threshold value of caveolin-1 (Cav-1) and cholesterol is essential(Lajoie, Nabi 2007). Cav-1 is a palmitoylated membrane protein (Doherty, McMahon 2009), what causes it to be membrane associated (Resh 2006). Additionally, Cav-1 binds cholesterol and fatty acids, this stabilizes the formation of oligomeres(Doherty, McMahon 2009). Its association with actin via the actin binding protein, filamin, restricts cav-1 movement at the cell surface. It is suggested that the actin cytoskeleton located close to the membrane is a regulator of the caveolae dependent endocytosis(Lajoie, Nabi 2007). Caveolae dependent endocytosis can be blocked with tyrosine kinase inhibitors, suggesting regulation of endocytosis by (one or several) specific kinases. It is known that Cav-1 is phosphorylated on a tyrosine residue by Src kinase, but the functional result of this phosphorylation in endocytosis is unclear(Lajoie, Nabi 2007). Caveolae dependent endocytosis is dynamin mediated(Oh, McIntosh & Schnitzer

1998, Henley, Cao & McNiven 1999), where it also functions as a fission protein to form sealed vesicles.

3.3. Clathrin- and caveolae independent endocytosis / Dynamin independent endocytosis

As discussed in the above section dynamin proteins are necessary for the fission of the clathrin coated vesicles and caveolae from the plasma membrane. However, dynamin independent endocytic routes have been reported. To illustrate this, the arf6 and GEEC-pathway will be discussed as examples of such a route.

3.3.1. Arf6 pathway

The arf family of GTP binding proteins are membrane bound GTPases involved in membrane trafficking. The function of arf is to modify the lipid composition and recruit cytosolic proteins to the surface membrane in order to this. Arf6 is mainly located at the plasma membrane and involved in the internalization of integrins, Major Histocompatibility Complex class I (MHC I) and Glycosylphosphatidylinositol-anchored proteins (GPI-AP). 5-10 minutes after internalization the arf6 positive vesicles fuse with endosomes containing proteins internalized via the classical clathrin dependent pathway and later traffic to lysosomes to be degraded. Another possibility is the recycling back of the proteins to the plasma membrane(Donaldson 2005). Results have shown that in addition to its clathrin independency, the arf6 pathway is also dynamin independent. The arf6 pathway has been analyzed by comparing the internalization of the two membrane proteins Tac (transfected), which is internalized via this clathrin-independent pathway, to Tac-LL, which is internalized via the clathrin-dependent pathway. Together with Tac, a dominant negative mutant form of dynamin (dynK44A) was co-transfected, which has been shown to inhibit dynamin mediated endocytosis. Internalization of Tac was not impaired as compared to Tac-LL which did show impaired internalization. The membrane protein Tac was found in arf6 positive structures, which later fused with the intracellular compartments containing Tac-LL.

This shows that arf6 positive Tac internalization, in addition to it's clathrin independency, is not dynamin dependent(Naslavsky, Weigert & Donaldson 2003).

3.3.2. GEEC pathway

Another dynamin independent pathway is the GEEC pathway, which stands for GPI-AP-enriched Early Endosomal Compartments. The GEEC pathway is marked by the internalization of GPI-AP. In addition to its dynamin independency, this pathway is also clathrin- and caveolae independent and regulated by the small GTPase cdc42. Immediately after internalization GPI-AP are localized in GEECs. These intracellular compartments are distinct from the endosomes which are targeted by the CCVs. By the use of dynK44A it was shown that the internalization of GPI-AP proteins was not impaired as compared to the clathrin mediated endocytosed controls. The same result was observed when cells were depleted of K^+ to disturb the clathrin coats. This shows that the internalization is neither dynamin- nor clathrin mediated. In addition, there was no Cav-1 present in the peripheral GEECs, what shows that internalization is also not mediated through caveolae. Using Electron Microscopy (EM) it was observed that tubular shaped structures held the GPI-AP soon after internalization. These tubular and ring shaped structures are the likely precursors of GEECs. It was thought that internalization of fluid also occurred via this pathway. Indeed, Horse Radish Peroxidase (HRP) internalization, which is a fluid phase marker, also occured via these GEECs. This can be concluded, because when labelled GPI-AP and labelled HRP was visualized, co-localization was observed. By transfecting the cells with a dominant negative mutant of cdc42 (cdc42N17) a substantial reduction HRP was observed, indicative for a reduction of fluid uptake. Remarkably, no reduced internalization of the GPI-AP was observed, this indicates that cdc42 has an important regulatory function for this endocytic

pathway, but also indicates that if the GEEC pathway is disrupted, GPI-APs internalization can be compensated by endocytosis via a different pathway(Sabharanjak et al. 2002). The mechanism of fission of the dynamin independent vesicles remains a mystery until now, but is the focus of much research.

Here several cellular endocytic routes have been discussed; the classical clathrin mediated endocytosis, as well as the more novel caveolae dependent route was discussed. Additionally clathrin-/caveolae- and dynamin-independent routes were summarized. An overview of the different endocytic routes can be found in figure 12.
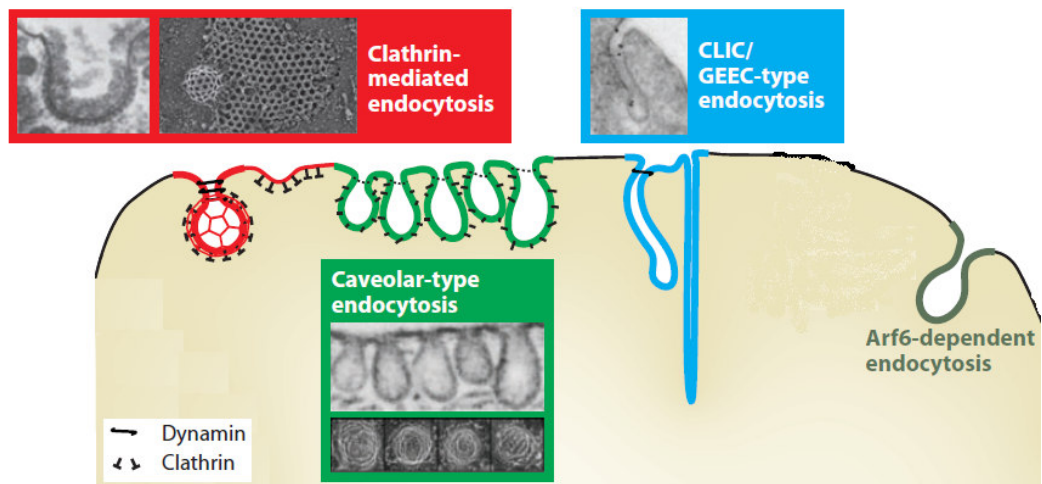
Figure 12 | An overview of the discussed endocytic routes. (Adapted from Doherty *et al.*)(Doherty, McMahon 2009)

3.4. Useful techniques

The endocytic pathways that are employed by the cell, discussed above, have been unravelled using various techniques. Up until now there are still empty spaces in our understanding of some mechanisms. In this section, different techniques will be discussed that can be helpful tools to fill up these empty spaces.

3.4.1. Electron Microscopy

One of the oldest techniques to look at membranes and its invaginations is via the electron microscope. Observation of electron-dense plasma membrane structures is indicative for clathrin coated vesicles. Figure 13 shows an example of an Arenavirus entering a cell via CME(Martinez, Cordo & Candurra 2007).
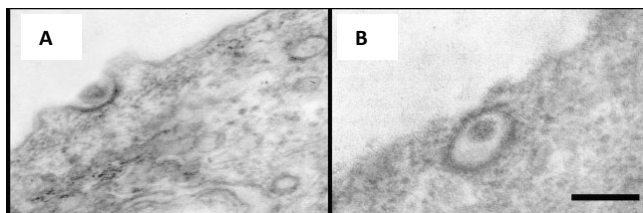
Figure 13 A | Shows the arena virus in a CCP **B |** Shows a sealed CCV. The bar is 200 nm(Martinez, Cordo & Candurra 2007).

3.4.2. Specific markers

Some molecules are specifically internalized via certain endocytic pathways. The treatment of cells with a specific agent can induce an increase or reduction of the net internalized molecule. This can give an indication about the up- or down regulation of that specific endocytic pathway.

The glycoprotein transferrin for example, is endocytosed with its transferrin receptor via CME. Consequently it is often used as a marker for CME. Reduction of transferrin after treatment with a specific agent, indicates that CME is blocked(Benmerah et al. 1999).

As discussed above, HRP can be used as a fluid phase marker(Sabharanjak et al. 2002).

3.4.3. Intervention of endocytosis with drugs

Several drugs are known to influence specific pathways, either by intervening with assemblage proteins, depletion of cholesterol or inhibiting fission of the vesicle.

The following drug is known to inhibit CME endocytosis:

- Treatment of the cells with chlorpromazine (CPZ) inhibits the assembly of coated pits at the cell surface, because it causes accumulation of AP-2 and clathrin on the endosomal membranes(Martinez, Cordo & Candurra 2007).

The following drugs are known to inhibit caveolae mediated endocytosis in a way:

- Cells can be treated with Phorbol 12-Myristate 13-Acetate (PMA), which is a phorbol ester. It activates protein kinase C and thereby disrupts caveolae and blocks their invaginations(Anderson, Chen & Norkin 1996).

- Cells can be treated with nystatin, which is a sterol binding drug. It removes cholesterol from the plasma membrane what disrupts caveolae(Anderson, Chen & Norkin 1996).

- Cells can be treated with methyl-β-cyclodextrin (MβCD), which is a sterol binding drug. It depletes cholesterol from the plasma membrane and impairs caveolae-mediated endocytosis(Martinez, Cordo & Candurra 2007).

- As nystatin and MβCD, filipin is a sterol binding drug and disrupts caveolae formation and function(Orlandi, Fishman 1998).

It has to be noted that nystatin, MβCD and filipin all are sterol binding drugs which are not very specific for inhibition of caveolae. When the concentration of these drugs is too high, also CME can be inhibited with these drugs, since this pathway is also cholesterol sensitive. Therefore, it is of great importance that the concentration of these drugs is controlled for their effect on CME.

Inhibition of dynamin dependent endocytosis:

- The treatment of cells with dynasore, which is a dynamin inhibitor, is a way to proof whether a cell uses dynamin for internalization(Sabharanjak et al. 2002).

3.4.4.   The use of dominant negative mutants

Eps15 (epidermal growth factor receptor pathway substrate 15) is a component that is constitutively associated with AP-2 in the clathrin coat complex. The use of Eps15Δ95/295, which is a dominant negative mutant of Eps15, can show whether a molecule is internalized via CME. Inhibition of internalization after transfection with the dominant negative mutant indicates that the molecule utilizes CME(Vela et al. 2007).

Caveolae mediated endocytosis is dependent on expression of caveolin-1. The transfection of cells with a dominant negative form of caveolin-1 (caveolin-1Y14F) can therefore tell you whether or not a molecule makes use of caveolae mediated endocytosis(Rojek et al. 2008).

This also works the other way around, when it is necessary to induce caveolae in a specific cell type, transfection of caveolin-1 is the tool to do so(Torgersen et al. 2001).

Dynamin functions as a fission protein to form sealed vesicles in clathrin- and caveolae mediated endocytosis. Inhibition of dynamin function can be accomplished by the expression of dynK44A, which is a dominant negative mutant of dynamin. Reduced internalization after expression of dynK44 is indicative for the use of a dynamin dependent pathway(Sabharanjak et al. 2002).

3.4.5.   Altering the intracellular physiological milieu of cells

When cells are incubated for 3 hours in an isotonic potassium (K^+) free buffer, cells can be depleted of K^+. This disturbs clathrin coats, and can therefore inhibit CME(Sabharanjak et al. 2002).

CME can also be inhibited by incubating cells with an acidification buffer ("KCI [140 mM],CaCi2 [2 mM], MgCl2 [1 mM], amiloride [1 mM], BSA [1%], and HEPES [20 mM], pH-adjusted to 7.0 with Tris") (Anderson, Chen & Norkin 1996).

3.4.6.   Other tools

One of the main building blocks for CME is clathrin. As discussed clathrin triskelions assocatiate met the plasma membrane to form clathrin coated pits. Three heavy chains, associated with a light chain are assembled in such a triskelion. With the use of small interfering RNA (siRNA), the heavy chain can be knocked down and the involvement of CME can be analyzed(Wang, Jiang 2009).

A very elegant way to analyze specific endocytic routes is the use of life cell fluorescence microscopy. Endocytic structures and molecules that are internalized can be visualized by tagging them with fluorescent markers. Interaction between the two players can be followed in real time. This has the advantage that a specific route can be analyzed without inhibiting specific pathways(Rust et al. 2004).

Table 2 | Useful techniques

Technique		Application
Elektron microscopy		Electron dense plasma membrane structures are indicative for CCV(Martinez, Cordo & Candurra 2007)
Specific markers		Transferrin for CME(Benmerah et al. 1999)
		HRP as a fluid phase marker(Sabharanjak et al. 2002)
Specific drugs		CPZ; accumulation of AP-2 and clathrin on endosomal membranes (Martinez, Cordo & Candurra 2007).
		PMA; activates protein kinase C and thereby disrupts caveolae and blocks their invaginations(Anderson, Chen & Norkin 1996).
		Dynasore; a dynamin inhibitor(Sabharanjak et al. 2002).
	Sterol binding drugs	Nystatin, MβCD, filipin; remove cholesterol from the plasma membrane what disrupts caveolae (Anderson, Chen & Norkin 1996).
Dominant negative mutants		Eps15Δ95/295; inhibits internalization via CME(Vela et al. 2007).
		Caveolin-1Y14F; inhibits internalization via caveolae mediated endocytosis(Rojek et al. 2008).
		DynK44A; inhibits dynamin mediated endocytosis(Sabharanjak et al. 2002).
Intracellular physiological milieu		K^+ depletion; disturbance of the clathrin coat(Sabharanjak et al. 2002).
		Acidification of cells; disturbs CME(Anderson, Chen & Norkin 1996).
Other tools		Knock down of clathrin heavy chain using siRNA inhibits

	CME(Wang, Jiang 2009). Life cell fluorescence microscopy specific routes can be analyzed without inhibiting other specific pathways(Rust et al. 2004).

Table 2 | Summary of useful techniques to analyze endocytic routes

Sialylated structures as receptors for viruses and bacterial toxins

4.1. Viruses

Previously, sialic acid containing receptors were discussed as well as the different endocytic routes that can be taken to enter a cell. In this section the dependence of sialic acid of several viruses will be reviewed. This will be followed by the discussion of the functional receptor of the virus, some viruses use attachment- and entry-receptors and whenever applicable these will be treated as two separate subjects. Finally the different routes that can be exploited by the virus will be reviewed.

All viruses, except Arenavirus and the bacterial toxin discussed here have in common that they use sialic acids as attachment or entry receptors and are necessary structures to enter the cell. In the case of Arenavirus it is thought that this is also true, however direct scientific proof for this is lacking.

4.1.1. Viruses | Simian Virus 40

Simian Virus 40 (SV40) is part of the family of polyomaviridae, which are non-enveloped DNA viruses. Cultured cells can be transformed by SV40 and the virus can induce tumors in animals. The icosahedral capsid is composed of 72 pentamers of the structural VP1 protein, which bind to the cellular receptor gangliosides GM1(Flint et al. 2004, Tsai et al. 2003). Upon infection of SV40, cells express the large T antigen (TAg). Specific tumor suppressors and cell cycle regulatory proteins are bound and manipulated by TAg. It therefore functions as an oncoprotein that is capable of transforming cells. Additionally, the expression of this protein can function as a marker for infection by SV40(Ali, DeCaprio 2001).

4.1.1.1. Sialic acid dependency

Often assays in which agglutination of red blood cells are done to test sialic acid dependency. SV40 did not agglutinate red blood cells(Neu, Stehle & Atwood 2009) and it was therefore thought that SV40 was not sialic acid dependent. However, SV40 attachment is exclusively mediated via the sialic acid containing gangliosides GM1(Tsai et al. 2003, Neu, Stehle & Atwood 2009). Detailed analysis with isothermal titration calimetry was performed which determined the affinity of the binding site of SV40 to GM1. This analysis of the VP1/GM1 binding revealed that binding to sialic acid is the major contact point. 60% of the contact area between VP1 and GM1 can be accounted for interaction between the sialic acid of GM1 and VP1. The narrow specificity of SV40 for GM1 results in attachment of the virus via the binding of exclusively this sialic acid containing structure(Neu, Stehle & Atwood 2009, Neu et al. 2008). So the combination of the exclusive binding to the sialylated gangliosides GM1 and sialic acid being the major contact point in this interaction makes me believe that binding of SV40 is sialic acid dependent.

4.1.1.2. Receptor

It has been clear for a long time that GM1 is the cellular receptor for SV40. First several binding- and inhibition assays for polyomaviridae in general were done, subsequently SV40 particles were used to find the receptor specific for this virus.

It was stated before that SV40 is a member of the polyomaviridae. All members contain VP1 protein in their capsid layer. Identification of receptors for the polyomaviridae was therefore analyzed with the use of polyoma virus-like particles (PyVLP). In this method, VP1 protein is synthesized by *Escherichia coli* and these proteins will then assemble into PyVLP. Incubation of GM95 cells, which lack the ceramide glucosyltransferase enzyme, with GM1, GD1a and GT1b induced internalization of the PyVLP into these cells. Additionally, a binding assay was

performed in which the PyVLP were allowed to bind to 3T6 fibroblast cells. Binding of PyVLP was partially inhibited by co-incubation with cholera toxin. This bacterial toxin is known to bind to GM1. The above discussed results show that these gangliosides are possible receptors for the polyomaviridae family(Smith, Lilie & Helenius 2003).

Naturally, identification of the specific receptor of SV40 was accomplished using SV40 viral particles. Recently, analysis of the results of a sucrose flotation assay has shown that the sialic acid containing ganglioside GM1 is the cellular receptor for SV40. Vesicles constructed of phospholipids and specific gangliosides were tested on their interaction with SV40. This test takes advantages of the intrinsic characteristic of viruses to sink to the bottom of a tube when centrifuged. Binding of the virus to components of the constructed vesicle causes it to be found in the top fractions of the sucrose. These sucrose flotation assays were performed when the liposomic vesicles were spiked either, with GM1, GM2, GM3, GD1a, GD1b and GT1b. It was only possible to immunoblot all fractions of the sucrose when the liposomic vesicles were spiked with GM1 ganglioside, what indicates binding to GM1. Additionally, researchers used a rat C6 cell line, which is deficient in gangliosides. Cells were pre-incubated with various gangliosides as with the sucrose flotation assay, but only C6 cells pre-incubated with GM1 expressed SV40 TAg, which shows that GM1 is the only ganglioside responsible for infection of cells (Tsai et al. 2003). This was confirmed by Neu *et al.* who performed a glycan array screening where the VP1 protein of SV40 exclusively bound to the carbohydrate part of GM1, although several carbohydrate structures were present during the screen(Neu et al. 2008). Therefore, it is clear that GM1 is the functional receptor for SV40.

4.1.1.3.   Endocytic route

Already in 1989 it was shown that most SV40 particles enter the cell through uncoated vesicles, while only a few enter it through the CME pathway(Kartenbeck, Stukenbrok & Helenius 1989).

This was later confirmed by Anderson *et al.* who investigated the involvement of caveolae on SV40 entry. Cells were treated with PMA, this phorbol ester disrupts caveolae and blocks their invaginations. TAg expression was reduced with 70% after treatment of the cells compared to non-treated controls. Treatment of cells with nystatin also inhibited SV40 infectious entry. Nystatin removes cholesterol from the plasma membrane, in the right concentrations it thereby disrupting caveolae without affecting CME. The above findings suggest that caveolae are necessary for SV40 entry. To exclude involvement of CME, the cytosol of CV-1 cells was acidified. This had no effect on SV40 infectious entry, but did, as expected, effectively reduce transferrin internalization, showing that CME was inhibited. This shows that SV40 entry into cells occurs via caveolae mediated endocytosis, which is a major entry route for this virus(Anderson, Chen & Norkin 1996). This was also confirmed by Pelkmans *et al.* which performed a very elegant study using video-enhanced, live microscopy in CV-1 cells. With the use of Texas Red-X Labeled SV40 virions (TRX-SV40) it was shown that this co-localized with GFP labelled Cav-1 (GFP-Cav-1) into caveolae-like structures. When BODIPY-FL-labelled transferrin was incubated in combination with TRX-SV40, these two markers did not end up in the same organelles. Additionally SV40 containing organelles could not be stained for EEA1, a protein which is specific for early edosomes. These findings confirm again that SV40 is endocytosed in a caveolae dependent manner(Pelkmans, Kartenbeck & Helenius 2001).

As stated before, Anderson *et al.* showed a 70% reduction of infection after treatment with PMA. This means that SV40 can compensate for this inhibition by inducing internalization via other endocytic mechanisms. It is possible that CME endocytosis then does play a role, since SV40 particles have been observed in CCV(Kartenbeck, Stukenbrok & Helenius 1989). Further investigation of this subject would be an interesting focus of research.

4.1.2. Viruses | Rota virus

Rota viruses are the leading cause of infectious, severe dehydrating diarrhoea among infants. Specific cell and tissue tropism characterizes rota virus infection, since it mainly infects villus tip cells of the small intestine(Mendez et al. 1999). The rota virus is a member of the reoviridae, which is a family of non-enveloped double stranded RNA viruses. The segmented genome is enclosed by three protein layers, of which the outermost layer contains VP4, a spike like protein responsible for the binding of the target cells(Arias et al. 2002). Infectivity of the virus is activated when the VP4 protein is cleaved into VP8 and VP5, by proteases in the small intestine. It has been described that VP8 mediates attachment via sialic acids on the cell surface, while integrin recognition domains have been found on VP5(Haselhorst et al. 2009). Until now, much effort has been put into the identification of the functional receptor(s) for Rota virus, but no conformity has been reached. Originally rota virus strains were divided into two groups; neuraminidase-sensitive and -insensitive strains, which is based on their ability to infect cells after treatment with neuraminidase(Guo et al. 1999), but the division into these groups has become subject of discussion and will be discussed below.

The current opinion on the infective route of Rota virus is that they attach to sialic acids containing structures on cellular surfaces. However, these do not necessarily have to be terminating sugars, as was thought until now. They can also be internal sialic acids, as is the case in GM1 and GM2. Subsequently, molecules like integrins mediate entry of the virus, after which the viral particle is transported deeper into the cell's interior(Mendez et al. 1999, Arias et al. 2002) via a mechanism that is not completely understood yet. All of the above steps in the infective route will be discussed below.

4.1.2.1. Sialic acid dependency

As stated before, originally rota virus strains were divided into neuraminidase-sensitive and -insensitive strains and it was thought that sialic acid binding was not necessary for the neuraminidase-insensitive strains. Most used neuraminidases cleave of the terminating sialic acid of the sugar chain, and after treatment the neuraminidase-insensitive strains can still bind target cells. Human Rota viruses have typically been classified as neuraminidase-insensitive(Guo et al. 1999). What is interesting however, Méndez *et al.* presented results of neuraminidase insensitive rota virus strains that were able to infect neuraminidase treated MA104 cells, and in addition the were able to agglutinate erythrocytes. These results show that the virus strains did not lose their ability to bind to sialic acids, but that binding to terminal sialic acids was simply not necessary for infection of MA104 cells(Mendez, Arias & Lopez 1993). The role of sialic acid as a determinant for infection by rota virus has therefore been further investigated.

It has been discussed earlier that sialic acids are terminating sugars characteristically found on glycosphingolipids, N-glycans and O-glycans(Varki et al. 2009). In the study of Guo *et al.* the ganglioside content of MA104 cells was examined; cells contained mainly gangliosides GM1 and GM3 and the neutral glycosphingolipid LacCer. Subsequently the effect of neuraminidase on ganglioside contents was analyzed. Results showed that, after treatment with neuraminidase, the GM3 content decreased significantly, LacCer increased correspondingly. Strikingly, GM1 content also increased slightly, this means that neuraminidase treatment did not affect GM1 content. When we take a close look at the neuraminidase/sialidase action on the discussed gangliosides, as depicted in figure 14, it can be seen that hydrolysis of the terminal sialic acid residue of GM3 yields LacCer. Neuraminidase treatment of GD1a yields GM1, a ganglioside without a terminal sialic acid residue and thus insensitive to treatment with ost neuraminidases. Treatment of MA104 cells resulted in the expression of gangliosides with no terminating sialic acid residues, but infection of the MA104 cells by the human rota virus strain was not inhibited after

neuraminidase treatment, what indicates that it used GM1, with it's internal sialic acid, as a receptor(Guo et al. 1999).

Additionally, Delorme *et al.* showed that three different rota virus strains, 2 neuraminidase-sensitive (simian; SA11- and bovine; NCDV) and 1 neuraminidase-insensitive (bovine; UK) strain bound gangliosides. All three strains were shown to bind GM2 and GD1a. However, only UK recognized NeuAc-GM3 and GM1, whereas both SA11 and NCDV recognized NeuGc-GM3(Delorme et al. 2001).

In conclusion, Delorme *et al.* showed that the neuraminidase-insensitive strain UK recognized GM1 and GM2 and Méndez *et al.* showed that neuraminidase-insensitive rota virus strains were still able to agglutinate red blood cells strongly suggests that sialic acid is a necessity for rota virus binding, but it does not necessarily has to be a terminating sialic acid.
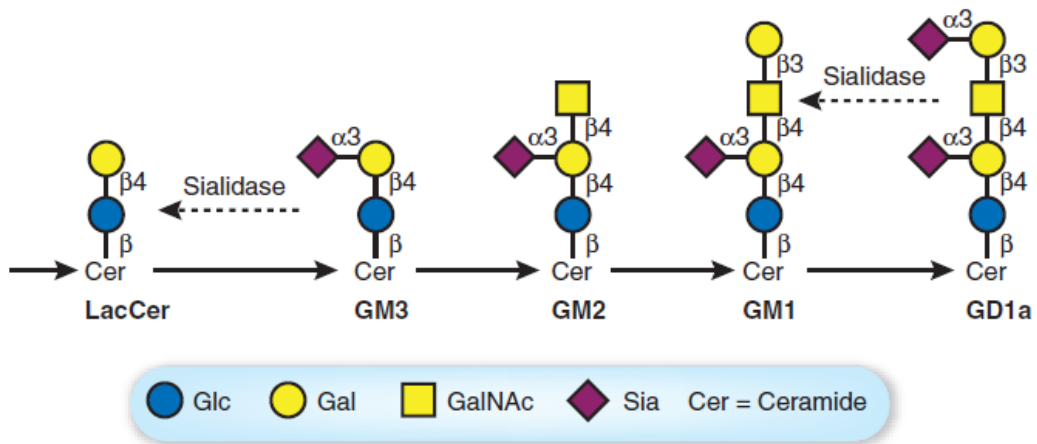
Figure 14 | Biosynthetic pathways of gangliosides and sialidase action(Banda, Kang & Variki 2009)

This was confirmed by Haselhorst *et al.* who used Nuclea Magnetic Resonance (NMR) to show the interaction between the VP8 protein of neuraminidase-sensitive and -insensitive strains to terminal- and internal sialic acid residues of gangliosides. It was shown that the neuraminidase-sensitive strain bound both to the terminal and the internal sialic acid of GD1a, so the binding epitope contained the whole upper part of the molecule, encircled in figure 15. For the neuraminidase-insensitive strain the binding epitope is encircled in figure 16. What is interesting is that neither the terminal galactose or the glucose residue are in close contact with the VP8 protein, but that the internal sialic acid is evident for VP8/GM1 complex formation(Haselhorst et al. 2009). This confirms the binding of internal sialic acid residues by VP8 protein of Rota virus.
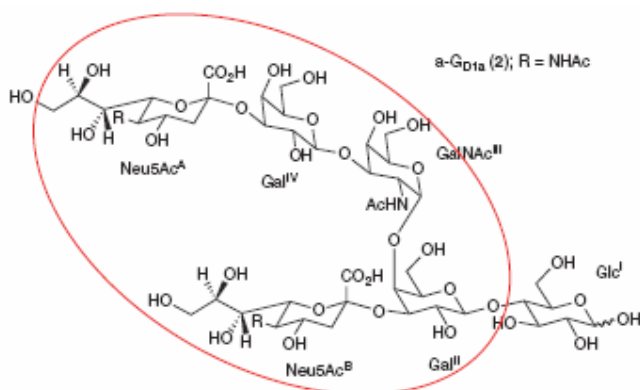
Fig 15 | The binding epitope of neuraminidase-sensitive rota virus to GD1a(Haselhorst et al. 2009).
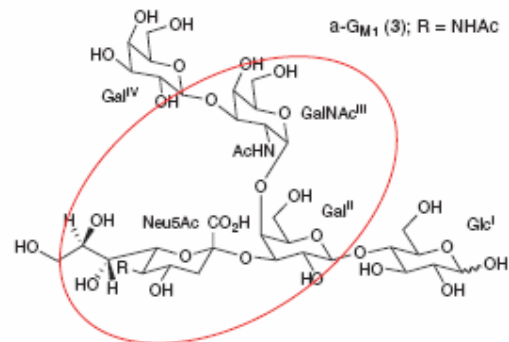
Fig 16 | The binding epitope of neuraminidase-insensitive rota virus to GM1(Haselhorst et al. 2009).

4.1.2.2.   *Attachment receptor*

As discussed above, infection by rota virus strains is dependent on the binding to sialic acids. These sialic acids are characteristically found on glycosphingolipids, N-glycans and O-glycans(Varki et al. 2009). Therefore involvement of these sialic acid containing structures in rota virus infection have been analyzed. Unidentified glycoproteins have been implicated as attachment receptors for the Rota virus, as well as certain gangliosides, recognized by the VP8 protein. Additionally the VP5 protein contains an integrin recognition domain(Haselhorst et al. 2009) and it is thought that entry of rota virus is a multistep process in which attachment of the virus is mediated through sialic acids and subsequently interaction with integrins is necessary for entry of the virus. In order to do so, proteases in the small intestine (where the virus eventually induces diarrhoea) cleaves the VP4 protein, of the outer protein layer, into VP5 and VP8. These products subsequently recognize their ligands(Dowling et al. 2000). The above mentioned players for viral entry will be discussed in the following section.

In the work of Bass *et al.* membranes of murine intestinal cells were isolated and used for binding assays to the Rhesus Rota Virus (RRV). The results showed that RRV particles could bind to proteins of 300 and 330 kDa, additionally it was shown that these proteins were to a greater degree expressed by villus tip cells then by other crypt cells. Treatment of the proteins with glycosidases, proteinases and neuraminidases showed that these were complex glycoproteins with terminal sialic acid residues. No binding of RRV was observed after treatment with the neuraminidase(Bass, Mackow & Greenberg 1991). Characterization of the proteins unfortunately has not been performed yet, which of course is of great interest. My

advice to the researchers is therefore to purify these proteins and analyze them in a mass spectrometer.

In addition to glycoproteins, also glycolipids have been put forward as receptors for rota virus. Both GM3 and GM1 have been put forward as attachment receptors for rota virus. The fact that two different structures are designated as attachment receptors is most likely the result of the use of different cell lines and different rota virus strains and it means that more then one type of gangliosides can function as a receptor.

A porcine rotavirus strain (OSU) was shown to bind to GM3 ganglioside, preferentially NeuGcGM3. In this study Lec-2 cells were used to test whether the presence of sialic acids was necessary for infectivity of the OSU strain. The Lec-2 cell line is characterized by a 90% reduction of sialated glycoconjugates, because the transport of sialic acid sugar donors into the Golgi is reduced dramatically. Binding to Lec-2 cells was 30 times less as compared to binding to control cells and binding could be reconstituted with the addition of NeuGcGM3. To further confirm the sialic acid dependence of the OSU strain, MA104 cells were treated with neuraminidase, what dramatically decreased infectivity of OSU. This means that the OSU strain is a neuraminidase-sensitive strain. Subsequently, infectivity of neuraminidase treated cells could be partly restored after incubation with NeuGcGM3. An interesting detail is that the amount of GM3 on the cell surface declines as the age of the pigs increases. This can explain the susceptibility of young piglets to rota virus infection. This age dependent pattern of sensitivity to rota virus infection is comparable to human, where mainly infants are at risk for infection(Rolsma et al. 1998).

The study of Guo *et al.* used two human rota viruses, KUN and MO and a feline rotavirus, FRV64. MA104 cells were treated with neuraminidase and this resulted in the strong inhibition of FRV64 infectivity (89% inhibition as compared to untreated cells) while the neuraminidase treatment did not have an affect on the infectivity of the two human strains. Earlier, the ganglioside content of MA104 cells before and after treatment with neuraminidase was discussed. GM1 content was slightly increased after neuraminidase treatment and infectivity of the two human strains was not inhibited. This suggested that GM1 could be the receptor for human rota virus. GM1 is the known receptor for Cholera Toxin, as will be discussed later. To clarify that GM1 is the receptor for the human virus KUN and MO strains Cholera Toxin B subunit was used to block GM1 on the plasma membrane of the MA104 cells. This resulted in a significantly reduced infectivity of the two strains, indicative for the fact that ganglioside GM1 could be the receptor for the human rota viruses(Guo et al. 1999).

All together, the above discussed information show that Rota virus attachment to cells is dependent on sialic acid containing structures. For the neuraminidase-sensitive strains the presence of a terminal sialic acid is necessary, while neuraminidase-insensitive strains can bind to internal sialic acids.

4.1.2.3. Entry receptor

As mentioned earlier, it is proposed that after binding to sialylated structures on the cell surface, Rota virus exploit integrins as entry receptors in order to be internalized into the cell(Arias et al. 2002). Not so long ago, it was found that the VP5 protein contained an integrin binding domain(Haselhorst et al. 2009). Since many other viruses, like coxsackievirus, pappilomavirus and adenovirus, are known to use integrins as entry receptors(Guerrero et al. 2000a) it was then hypothesized that rotavirus could possibly use this integrin binding domain for entry into target cells. Guerrero *et al.* provided evidence for the existence of various proteins of different molecular weights (110, 75, 57, 45 and 30 kDa) to be cellular receptors for rotavirus, of which the largest was later on identified as the β_3 integrin subunit. These proteins were extracted from the plasma membrane of MA104 cells, which can be infected by the rota virus strains used in this study (neuraminidase sensitive and insensitive strains). As lipid rafts are often part of endocytic routes exploited by viruses, it was suggested that the proteins that

bound to the rota virus possibly resided in lipid rafts. To address this issue, cholesterol was depleted by treating MA104 cells with MβCD, this resulted in inhibition of infection, but binding of the virus was not affected. This indicates that binding and entry of the virus is mediated through 2 different molecules(Guerrero et al. 2000b). Subsequently, Guerrero *et al.* identified the largest protein (110 kDa) as the human β_3 integrin subunit. Analysis of attachment assays showed that inhibition of $\alpha_v\beta_3$ did not influence rota virus binding to the target cells. However, analysis of infectivity assays showed that both antibodies and $\alpha_v\beta_3$ ligands inhibited infectivity of rota virus. This shows that rota virus uses integrins as a post-attachment receptor(Guerrero et al. 2000a).

The discussed information has resulted in the development of a model (as depicted in figure 17) in which both neuraminidase sensitive and -insensitive rota virus strains make use of a multistep model in which the functional receptor is a complex of molecules. This complex presumably contains gangliosides as attachment receptors, integrins and possibly other proteins assembled on a lipid raft as entry receptors, after which the virus is transported deeper into the cell's interior(Mendez et al. 1999, Arias et al. 2002).
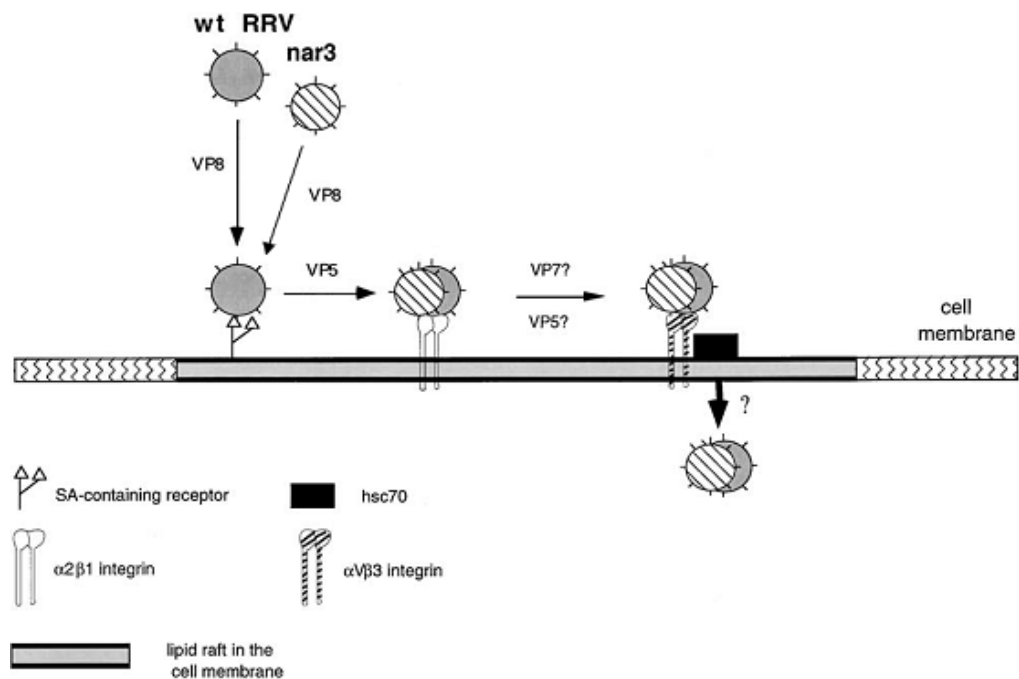
Figure 17 | Proposed model of rota virus binding (adapted from(Arias et al. 2002)).

4.1.2.4.   Endocytic routes

The internalization of the virus after binding to its attachment and entry receptors is a matter that is still subject to a lot of research. Some studies have reported Rota virus particles in CCP, CCV and endosomes, which is an indication for CME. Penetration of viruses internalized via this route is mainly mediated via a decrease in pH. However, it has been shown that rota virus entry is not sensitive to a low pH, but the internalization has been proposed to be a model dependent on calcium (Ca^{2+}) concentration, where permealization of the transcriptionally active (double layered) virion is mediated via VP5(Dowling et al. 2000, Ruiz, Cohen & Michelangeli 2000).

The extracellular Ca^{2+} concentration stabilizes the virus particle, and it's three protein layers are then nicely encapsulate the viral genome(Ruiz, Cohen & Michelangeli 2000)(). Prior to

attachment, proteases in the small intestine (where the virus eventually induces diarrhoea) cleave the VP4 protein into VP5 and VP8(Dowling et al. 2000). As discussed, VP8 attaches to sialidated structures on the cell surface, after which VP5 recognizes integrins. This interaction probably induces internalization of the virus particle via CME, since rota virus particles in CCP and CCV have been reported. Once internalized, the cytosolic Ca^{2+} concentration causes solubilization of the outer protein layer(Ruiz et al. 1997). Penetration of the virus into the cytoplasm is possibly mediated via the VP5 protein, which contains a hydrophobic loop that forms small pores when inserted in the glycolipid layer(Dowling et al. 2000). Once the endosomal membrane is permeable, the double layer virus particle is released into the cytosol. The double layer virus particle is transcriptionally active and initiates virus replication in the cytosol. The proposed model is depicted in figure 18(Ruiz, Cohen & Michelangeli 2000).
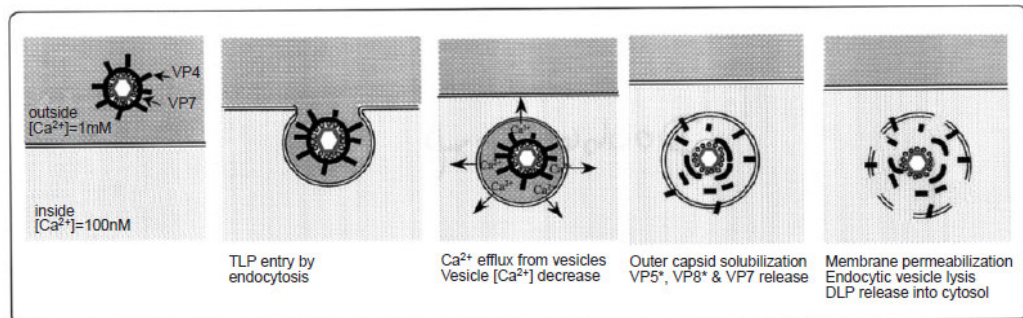
Figure 18 | Rota virus internalization. TLP: triple layer particle, DLP: double layer particle(Ruiz, Cohen & Michelangeli 2000).

4.1.3. *Viruses | Arena virus*

The family of *Arenaviridae* consist of a large group of viruses, which can be divided into two main subgroups; the New World and the Old World Arenaviruses. Arenaviruses in general cause a persistent a-symptomatic infection in their natural hosts, rodents. The cause of an infection in humans is often via rodents. The enveloped viruses have a bi-segmented negative stranded RNA genome. The large segment encodes the RNA-dependent RNA polymerase and the RING finger protein Z. The small segment encodes for the viral glycoprotein precursor (GPC) and the nucleoprotein (NP). GPC is cleaved posttranslationally into GP1 and GP2 which together form the spike proteins on the viral surface. GP1 mediates the attachment to the host cell. New World and Old World arenaviruses use different cellular receptors and also different entry routes, therefore two different viruses will function as examples to explain the viral entry of the two subgroups(Rojek, Kunz 2008)(). It is thought that only the Old World Arenaviruses bind to sialic acids prior to infection, therefore New World Arenaviruses will be discussed very briefly and the Old World Arenaviruses in more detail.

4.1.3.1. New World Arenaviruses | Junín virus

Junín virus (JUNV) is a New World arenavirus that causes Argentine hemorrhagic fever, which is an endemo-epidemic disease affecting the population of Argentina and has a high mortality rates. Arenaviruses are a serious threat for public health and it is a big worry that this specific virus might be used as an agent in bioterrorism(Martinez, Cordo & Candurra 2008).

4.1.3.1.1. Receptor

Radoshitzky and co-workers demonstrated that transferrin receptor 1 (TfR1) is the receptor for New World arenaviruses. CHO cells transfected with TfR1 were markedly more susceptible to infection with JUNV then non-transfected cells and addition of TfR1 antibody inhibited infection significantly. Results were similar for other New World Arenaviruses, like Machupo- and Guanarito virus, indicating that this is a receptor used by most New World arenaviruses(Radoshitzky et al. 2007). The transferrin receptor is a glycoprotein which consists of two subunits attached to each other by a disulfide bridge. It contains an *O*-linked polysaccharide chain that is build up of at least one sialic acid, dependent on the cell type the TfR comes from(Do, Enns & Cummings 1990). However, up until now, there is no scientific data that implicates the involvement of sialic acid when binding to this receptor.

4.1.3.1.2. Endocytic routes

It is now generally accepted that the New World Arenaviruses enter the cell via CME(Rojek, Kunz 2008, Martinez, Cordo & Candurra 2008, Kunz 2009). The study of Martinez *et al.* analyzes this pathway thoroughly, in which the expression of the NP was used as a readout for infection with JUNV. When cells were treated with chlorpromazine (CPZ), which inhibits the assembly of coated pits at the cell surface, it was shown that infection by JUNV was effectively reduced when compared to control cells. As a positive control the internalization of transferrin was examined, which was inhibited after treatment with CPZ. This shows that JUNV enters the cell through CME. This was confirmed by the use of electron microscopy where JUNV virions were only observed in electron-dense plasma membrane structures.

Additionally, cells were treated with nystatin (NS). This agent bind sterol from the plasma membrane and this treatment a-specifically disrupts caveolae and therefore inhibitis caveolae mediated endocytosis. After cells were treated, maximum 40% of the cells were infected. These results indicate that the virus can possibly enter the cell via this pathway and it is

probably used as a compensatory mechanism when CME is blocked(Martinez, Cordo & Candurra 2007).

4.1.3.2. Old World Arenaviruses | Lassa virus

Hemorrhagic fever caused by the Old World Arenavirus, the Lassa virus (LASV) results in significant mortality each year in the endemic areas in West Afrika. Therapeutic options are now restricted to antiviral agents with little effect and supportive care(Cao et al. 1998). It is therefore important to know more about the Old World Arena viruses.

4.1.3.2.1. Receptor

The primary cellular receptor of most Old World arenaviruses, including LASV is α-dystroglycan (α-DG)(Cao et al. 1998). Cao *et al.* observed binding of several Old World Arenaviruses to purified α-DG. Furthermore, the addition of soluble α-DG inhibited infection of cells, which are normally sensitive to infection, in a dose dependent manner(Cao et al. 1998). Translation of dystroglycan mRNA yields the polypeptide of the glycoprotein dystroglycan, which is posttranscriptionally processed by cleavage into an α- and a β-subunit. α-dystroglycan is an extracellular protein and contains three *N*-glycosylation sites. In a physiological situation α-DG interacts with high affinity to the extracellular matrix. A non-covalent binding connects α-DG with β-DG, which in turn intracellularly binds to the adaptor protein dystrophin that connects the cytoskeleton to dystroglycan. It is therefore thought that the whole complex acts as a transmembrane linker between the intracellular cytoskeleton and the extracellular matrix(McDearmon, Combs & Ervasti 2003, Barresi, Campbell 2006, Chiba et al. 1997). A schematical representation of both the physiological function of dystroglycan and the terminally sialylated core oligosaccharide can be found in figure 19(Kunz 2009, Barresi, Campbell 2006).
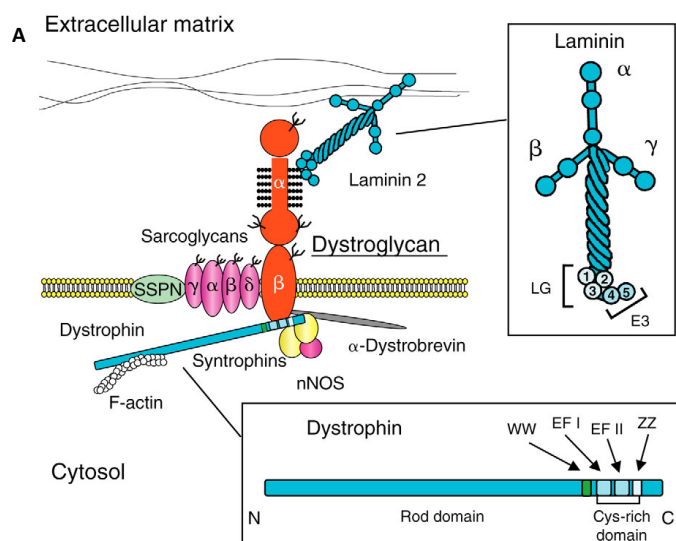
Figure 19 | the dystroglycan complex and its physiological function(Barresi, Campbell 2006)

4.1.3.2.2. Involvement of sialic acids

The core oligosaccharide of α-DG is heavily sialylated(McDearmon, Combs & Ervasti 2003, Barresi, Campbell 2006, Chiba et al. 1997). Chiba *et al.* showed that the sialic acid content of Crude Bovine Peripheral Nerve Membrane contained both NeuAc and NeuGc in a 4:1 ratio. These sialic acids were exclusively linked to the C-3 position of the galactose residue. Recently, results have shown that the sialic acids on α-DG play a major role in the binding to laminin. The addition of sialic acid to the incubation medium, or the treatment of α-DG with neuraminidases greatly diminished the binding of α-DG to laminin. However, there was a difference in inhibition capacity between sialic acids connected to a different neutral core structure. This indicates that not only the anionic charge, but also the structure of the neutral sugar is of importance(Chiba et al. 1997).

No binding assays have been performed in which direct evidence for the dependency of binding to α-DG via sialic acid by Old World Arenaviruses have been shown. However, the heavily sialylated structure of the core protein and binding α-DG to laminin via its sialic acids(Chiba et al. 1997) suggest that also in the binding of Old World Arenaviruses to α-DG, sialic acids might play a major role.

4.1.3.2.3. Endocytic routes

It seems that LASV and another Old World Arenavirus, LCMV are endocytosed via one specific pathway. Rojek et al have extensively studied this route. They demonstrated that both viruses are endocytosed via a clathrin, caveolae and dynamin independent pathway.

To circumvent biosafety restrictions, they took use of a recombinant LCMV, which expressed the GP of LASV (rLCMV-LASVGP). Additionally, this recombinant form was chosen to prevent the use of a LASV pseudotype, because artefacts are often a concomitant problem with these types of viruses. Hek293 cells were transfected with either wild type or dominant negative mutants of EPS15 (EPS15Δ95/295), which specifically interferes with clathrin coated pits assembly, dynamin (dynK44A), which block dynamin dependent endocytosis, and caveolin (caveolin-1Y14F) which is known to inhibit caveolae dependent endocytosis. Transfection of the dominant negative mutants of dynamin and caveolin-1 did not significantly inhibit infection with recombinant LCMV-LASVGP, but transfection with the dominant negative mutant of EPS15 reduced infectivity significantly, although only just. This was compared to the infection by JUNV of the same HEK293 cells transfected with the same dominant negative mutants. In that case only transfection of the caveolin-1 dominant negative mutant did not have affect, as was expected, since JUNV enters the cell via CME, as discussed above(Rojek et al. 2008).

These results indicate that the main endocytic pathway of LASV is clathrin-, caveolin- and dynamin independent, but CME plays a minor role in infection with Old World Arenaviruses. This was confirmed by Vela *et al.* who also observed reduced infectivity of another Old World arenavirus (Pichindé virus) after transfection of Vero cells with the dominant negative mutant of EPS15, but not when the caveolin-1 gene was silenced(Vela et al. 2007).

To conclude, Old World arenaviruses mainly use clathrin-, caveolae- and dynamin-independent endocytosis and possibly switch to CME as a compensatory mechanism.

4.2. Bacterial toxins

Of all the bacteria that exist, relatively few can cause disease. Virulence genes encode for proteins that contribute to cause a certain pathological condition. A toxin is an example of a protein encoded by such virulence genes(Alberts et al. 2002b). Various bacterial toxins belong to the AB5 toxins group which consist of 2 subunits. One moiety, the B subunit, binds to the cellular receptors, whereas the other, the A subunit, contains the enzymatic activity and enters the cytosol where it performs its action. Cholera Toxin (CT) is an example of such an AB5 toxin and will be discussed below. The AB5 toxins are retrogradilly transported to the Golgi and the Endoplasmatic Reticulum (ER), eventually, after cleavage, the A subunit is translocated to the cytosol and exert its action. CT is cleaved by *Vibrio cholerae* itself. The structure of Cholera Toxin can be found in figure 20(Sandvig, van Deurs 2002).
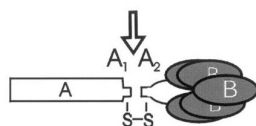
Figure 20 | Structure of Cholera Toxin (Sandvig, van Deurs 2002).

4.2.1. Bacterial toxins | Cholera Toxin

One of the most interesting and best studied bacterial toxins is the cholera toxin (CT). CT is secreted by *Vibrio cholerae* and causes massive diarrhoea. It is generally accepted that the pentameric B subunit of CT binds to the ganglioside GM1, a glycosphingolipid found enriched in lipid rafts and caveolae(Chinnapen et al. 2007). GM1 is also located in CCP, although in a lower concentration compared to caveolae(Torgersen et al. 2001). The binding of the regulatory subunit B merely has the function to transport the catalytic A subunit, which is linked to the B subunit, into the ER of the host cell. There the A chain is unfolded and translocated back into the cytoplasm. Via a cascade of events the concentration of intracellular cAMP is increased what causes secretion of salt and water. This eventually results in the massive diarrhoea(Chinnapen et al. 2007, Massol et al. 2004).

4.2.1.1.   Sialic acid dependency

The dependency of CT to sialic acid was first investigated by agglutination assays of sheep erythrocytes loaded with different gangliosides. Agglutination of the red blood cells loaded specifically with GM1 could partially be rescued by incubation with sialic acid. This is indicative for the competitive binding of the subunit of CT(Richards et al. 1979). Schengrund *et al.* additionally indirectly showed that sialic acid is necessary for binding of CT. They performed binding assays with CT, which were inhibited with various derivatives of GM1. Only in the case of the asialo-GM1, no inhibition of binding was observed, what results in the necessity of sialic acid for a successful competition(Schengrund, Ringler 1989).

4.2.1.2.   Receptor binding

GM1 as the sole receptor for CT has been established for a long time. Discovery of GM1 as the receptor for CT can be traced back to 1976, when Moss *et al.* showed that NCTC2071 cells were susceptible to CT infection only after incubating them with GM1(Moss et al. 1976). More recent studies have looked at the involvement of other gangliosides in the infection of CT. Critchley *et al.* for example, used Balb/c 3T3 cells, which contain quantitative the most GM3 and GD1a, but also a distinct amount of GM1. Lipid extracts of these Balb/c 3T3 cells were chromatographed, subsequently ^{125}I labelled CT was allowed to bind to this extract. There was no labelling of GM3 or GD1a, but only of GM1. Interestingly, neuraminidase

treatment of the cells increased binding of CT(Critchley et al. 1982), what can indicate that CT is able to hydrolyze GD1a to yield its receptor GM1. This is confirmed by Galen *et al.* who analyzed CT-neuraminidase function and saw that the neuraminidase removes the sialic acid from higher order gangliosides, what reveals the CT receptor GM1(Galen et al. 1992). This interesting property of CT can contribute to the pathogenesis of the bacterial toxin, since it can modulate cell surface structures in order to reveal more of its own receptors.

4.2.1.3.   Endocytic routes

There are many endocytic pathways via which CT can enter the cell. The redundancy of routes is remarkable and illustrates how 'clever' pathogens can be to infect their host, but the importance of each individual route is still subject of debate. It appears that CT can enter the target cells via CME and caveolae mediated endocytosis. Additionally, a clathrin and caveolae independent mechanism is employed, the GEEC pathway. The following section will first discuss the involvement of CME and caveolae mediated endocytosis, then the clathrin and caveolae independent pathway will be discussed.

To disrupt CME, cells (in this case CaCo-2 cells) can be treated with chlorpromazine (CPZ). It reduces the number of CCP associated receptors on the cell surface, by causing accumulation of AP-2 and clathrin in endosomal compartments. Treatment of the cells with CPZ did not seem to influence internalization significantly(Orlandi, Fishman 1998). This was confirmed by Kirkham *et al.*, who analyzed CT internalization in MEF cells, which expressed a dominant negative mutant form of EPS15. The dominant negative mutant was able to inhibit transferrin uptake by 90%, while CT uptake was only inhibited 40%(Kirkham et al. 2005).

The above results indicate the following: when CME does not completely inhibit CT internalization, there must be another route via which CT enters the cell. Therefore the involvement of caveolae mediated endocytosis was analyzed. Orlandi *et al.* analyzed caveolae mediated endocytosis by treating cells with filipin(Orlandi, Fishman 1998), which is a sterol binding drug and therefore bind to cholesterol and forms complexes in the plasma membrane. In the right concentration, it thereby disrupts caveolae and caveolae-like structures, without affecting CME(Kovbasnjuk, Edidin & Donowitz 2001). To quantify the degree of internalization, the immunoreactivity of cell surface CT was analyzed. Results showed that after treatment of cells with filipin, internalization of CT was not completely blocked, but reduced with ~45%. This confirms the hypothesis stated before, that CT internalization can occur via more then one pathway(Orlandi, Fishman 1998). There are microdomains in the plasma membrane that have the same content as caveolae, but lack cav-1. These microdomains are called Detergend-Insoluble Glycosphingolipid (DIG) rich domains and consist of cholesterol, glycolipids and sphyngomyelin. Cav-1 is necessary to form caveolae. It was investigated whether or not caveolae are necessary for internalization of CT. It was shown that the expression of cav-1 is not necessary for CT internalization. Infection of Jurkat T lymphoma cells (which do not express cav-1)(Orlandi, Fishman 1998) and caveolin [-/-] MEF cells was still possible(Kirkham et al. 2005). Furthermore, treatment of Jurkat cells with filipin readily blocked action of CT(Orlandi, Fishman 1998), this was also the case for the caveolin [-/-] MEF cells, when treated with cyclodextrin, another sterol binding drug(Kirkham et al. 2005). This confirms that the expression of solely DIGs is sufficient for cells to be sensitive to CT(Orlandi, Fishman 1998).

It was once more confirmed that CT can be internalized via clathrin and caveolae independent routes with the use of the dominant negative mutant of dynamin, dynK44A. Both these pathways are dependent of dynamin and should be inhibited when dynK44A is expressed. However, the amount of CT in the Golgi was comparable between dynK44A MEF cells compared to wild type MEF cells. This indicates that a major part of CT was internalized via a dynamin independent pathway(Kirkham et al. 2005). This dynamin independency was also analyzed in HeLa K44A cells. These cells express mutant dynamin in an inducible manner. This means that the amount of CT internalization after induction of the mutant can be

attributed to caveolae- and clathrin independent endocytosis. Additionally, HeLa cells express few caveolae on the plasma membrane, so this suggests that the reduction of CT internalization can be attributed to the inhibition of dynamin dependent CME. Indeed, after induction of mutant dynamin, HeLa K44A cells showed a 30-50% reduction in CT internalization as compared to control cells. These results show that CT is internalized in HeLa K44A cells via a dynamin dependent CME, but since this is not a 100% blocked, also clathrin- and caveolae independent routes can be used to internalize CT(Torgersen et al. 2001).

As discussed before, the existence of clathrin- and caveolae-independent pathways has been reported. Earlier the arf6 and the GEEC pathway have been discussed. Both of these pathways belong to the possible clathrin and caveolae independent routes. Therefore, a dominant negative mutant of Arf6 was expressed in caveolin$^{-/-}$ MEF cells. The amount of CT internalized in these cells was comparable to wild type MEF cells. This indicates that CT is not internalized via the Arf6 pathway.

Ultrastructural analysis revealed that CT containing structures had a tubular, ring like morphology. Additionally it was found that only 15% of these structures were derived from CME and that ~50% of the GPI-AP positive structures also contained CT. When these results are compared with the pathway that Sabharanjak *et al.* has described it is likely to assume that CT takes advantages of this same GEEC pathway, which is regulated by cdc42. Unfortunately, the role of cdc42 has not been investigated in the study of Kirkham *et al*(Kirkham et al. 2005)*.*

The above discussed results show the redundancy of endocytic routes of CT. It can be internalized via CME, caveolae mediated endocytosis, but also via a clathrin and caveolae independent pathway, which is most probably the GEEC pathway.

5. Influenza A | Its receptors and entry routes

The Influenza virus causes annual outbreaks in humans each year. Due to the impact that Influenza has on the society, extensive research has been done to elucidate the biology of influenza viruses. If you only look at the pandemics that emerged from 1918 on (figure 21), the importance of this research is stressed(Suzuki 2005).
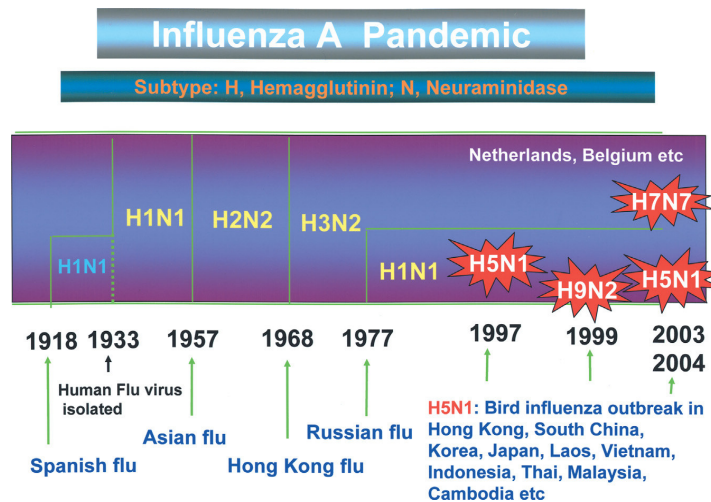
Figure 21 | Influenza A pandemic outbreaks (Suzuki 2005)

Influenza A is part of the family of *Orthomyxoviridae*, of which all members have a negative stranded, segmented RNA genome, which is protected by a viral envelope(Bouvier, Palese 2008). The virus is the causative agent of the common flu, but can also cause more severe diseases like, lower respiratory tract infection, encephalitis and pneumonia. Influenza A contains eight RNA segments, which encode for ten polypeptides including the surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA), which form the spikes that mediate in attachment to the cell via sialic acids (HA) and removal of sialic acids (NA) so infectious progeny viruses can be released. The 15 HA and 9 NA types that exist are used to categorize the different Influenza A strains. The Influenza A strains that infect humans can be directly or indirectly derived from its natural hosts; gulls, shorebirds and waterfowls, but can also infect other mammals like swine and equine(Baigent, McCauley 2003). Indirect infection occurs when the virus is reassorted in an intermediate host, like swine, and then infects humans(Bouvier, Palese 2008).

The recurrence of the seasonal Influenza A outbreaks are the result of antigenic drift. The amino acid sequence of the HA spike of the virus is subjected to immunologic pressure via antibodies and therefore minor changes occur. This results in a new infection of the susceptible host, who is no longer able to neutralize the virus(Bouvier, Palese 2008). Most pandemics that occur are the result of antigenic shift, in which the segmented genome is reassorted because a virus strain takes on a segment of another strain(Baigent, McCauley 2003).

In 1918, the first big influenza pandemic emerged, which is known as the "Spanish Flu". There has been debate for a long time whether or not this virus was directly or indirectly derived from birds, as the virus contained both mammalian and bird characteristics. First it was thought that the infection of humans happened through swine as an intermediate hosts(Bush 2004), but

nowadays it is becoming more and more accepted that the Spanish Flu is an human adapted avian influenza(Taubenberger et al. 2005) which is a result from antigenic drift. Hence, not everyone is convinced since some researchers find the gene phylogenies, on which this direct transmission theory is based, not satisfying(Gibbs, Gibbs 2006). The 1918 influenza generated a virus to which humans were susceptible but immunologically naïve and infected almost one third of the entire population(Taubenberger et al. 2005).

As stated before HA mediates attachment of the virus to the cell, this occurs via sialic acids bound to galactose(Baigent, McCauley 2003). Internalization of the virus occurs via several pathways, like CME and clathrin and caveolae independent endocytosis. In the following section the dependence of the virus on sialic acid will be discussed as well as the attachment and entry receptors. Finally the endocytic routes that can be employed by the virus will be reviewed.

5.1. Sialic acid dependence

In earlier sections I stated that sialic acids are found on structures like glycoproteins and glycolipids. The common linkages of sialic acid to galactose is the α-2,3- or α-2,6-linkage(Bouvier, Palese 2008).

Several studies have shown the necessity of sialic acid for infection with the Influenza A virus. Bergelson *et al.* for example, showed that Influenza A virus could not bind to EAC cells after treatment with neuraminidase. Influenza A was able to bind these cells without this treatment(Bergelson et al. 1982). Additionally, Suzuki *et al.* showed that erythrocytes were agglutinated after addition of Influenza A virus and abolishment of this agglutination was observed after treatment with neuraminidase(Suzuki, Matsunaga & Matsumoto 1985).

Recognition of sialic acid is mediated via the HA protein, which must be cleaved for the virus to retrieve infectivity. The cleavage results in the HA1 and HA2 protein; Ha2 protein is suggested to mediate fusion of the viral envelope and HA1 takes care of the receptor recognition(Bouvier, Palese 2008).

Avian influenza recognizes the α-2,3-sialic acid, which is predominantly found on the gut epithelium of the duck. Human influenza recognizes α-2,6-sialic acid, which is found on the respiratory epithelium of human. α-2,3-sialic acid can also be found on the human epithelium in the lower respiratory tract, this results in the fact that humans can also be infected with the avian influenza. However, since the lower respiratory tract is less accessible to the virus, infections with avian influenza occur less often(Nicholls et al. 2007, Bouvier, Palese 2008). Research has resulted in the observation that children have a greater expression of α-2,3-sialic acid then adults, which makes them more susceptible for avian influenza(Nicholls et al. 2007). As stated earlier, sialic acid can be modified in various ways. The molecules that make up sialic acid can receive additional substitutions at the hydroxylgroup on their carbon-atoms, like *O*-methyl, *O*-sulfate, *O*-acetyl and phosphate groups. At least 40 molecular species of sialic acid can be found in nature and this variation in sialic acids can all result in differential recognition by influenza A virus strains(Suzuki 2005).

5.2. Attachment receptors

As discussed before sialic acid can be found on various surfaces, including glycoproteins and glycolipids. It has been shown that sialic acids are crucial in Influenza A infection, but it Is thought that also more distal parts of the binding epitope mediate binding by Influenza A virus. For this matter, the involvement of gangliosides and glycoproteins will be discussed. This is very interesting as both structures seem capable to bind Influenza A virus.

Several researchers have documented the ability of influenza A to recognize and bind to gangliosides. Hidari *et al.* for example showed that the recognition-pattern of influenza for three different gangliosides was also dependent on the length of the carbohydrate chain. Both duck- and human influenza strains bound preferentially to gangliosides with a longer

carbohydrate structure(Hidari et al. 2007). Additionally Miller-Podraza *et al.* showed that human influenza A virus preferentially bound to gangliosides with 10 or more sugars, whereas the avian influenza bound to a wide range of gangliosides(Miller-Podraza et al. 2000). Also Stevens *et al.* showed similar results when they performed a glycan micro-array in which human influenza A viruses preferentially bound to sialylated structures with a long carbohydrate chain. Several times they observed specific prerequisites for binding, like the appearance of GlcNac or an additional negative charge in the carbon chain(Stevens et al. 2006).

Glycoproteins are also structures that contain sialic acids. Therefore, it was hypothesized that glycoproteins might also serve as receptors for Influenza A. Researchers have shown that indeed, in addition to glycolipids, also glycoproteins can serve as receptors for the Influenza A virus. A glycosphingolipid deficient cell line (GM95) was successfully infected with influenza A. The parental cell lines, MEB4 and B16, which do contain glycosphingolipids were infected with a similar efficiency(Ablan et al. 2001). Years later, this was confirmed by Matrosovich *et al.* who performed similar experiments. Only he grew the GM95 cells in serum free medium, to circumvent influenza A virus infection via exogenous gangliosides. Also they saw similar infection of GM95 and its parental cell lines(Matrosovich et al. 2006).

With the use of Lec-1 cells it was shown that not merely the presence of sialic acid is enough for influenza to use a structure as a functional receptor, but that N-linkage of sialic acid is involved in this. Lec-1 cells are deficient in receptor sialo-*N*-glycans and could not be infected by the influenza A virus, although binding was not abolished. Transfecting Lec-1 cells with GnT1, N-acetylglucosaminyltransferase I, fully restored influenza virus A infection, so it can be concluded that *N*-linked glycoproteins are necessary for successful infection with influenza A virus. This study used several different strains of the Influenza A virus, so the results can be extrapolated to other Influenza strains quite well(Chu, Whittaker 2004).

In conclusion, the whole scope of prerequisites on cellular attachment receptors for influenza A virus are not all crystallized yet. It seems that both gangliosides and sialylated glycoproteins are sufficient for Influenza A binding to the cell, but that there is a preference for long carbohydrate chains.

5.3. Entry receptors

The study of Chu *et al.,* which is discussed above, showed that the lack of sialylated glycoproteins did not abolish Influenza virus A binding, but did effect infectivity of the virus. Thus, merely the expression of gangliosides was shown to be insufficient for infection. In this study, researchers performed an internalization experiment to see whether or not the virus could be internalized. Therefore, cells were infected with biotinylated Influenza A virus and this could be detected on the surface of CHO and Lec-1 cells on ice when treated with straptavidin, which visualizes the biotin-label. Additionally the bound virus was almost completely undetectable when cells were treated with cell-impermeable reducing agent TCEP, which cleaves of the biotin groups. Subsequently, the internalization of the virus was induced at 37∘C. In CHO cells, this protected the virus from cleavage with TCEP and the particles could be visualized in the endosomes of the CHO cells. However, no internalization of the virus could be observed in Lec-1 cells(Chu, Whittaker 2004). This could indicate that although both gangliosides and sialidated glycoproteins are sufficient for attachment of the virus, only sialidated glycoproteins can serve as entry receptors.

5.4. Endocytic routes taken by Influenza A

The internalization of the virus after binding to its attachment and entry receptors is a very interesting subject which has been studied extensively. It is now clear that influenza can infect cells via at least two different endocytic routes that can be employed in parallel, these are CME and clathrin- and caveolae-independent endocytosis. The involvement of caveolae

mediated endocytosis is also discussed, as it contributes in a minor way to the internalization of Influenza A.

The study of Rust *et al.* used live fluorescent microscopy to analyze different endocytic pathways exploited by influenza in real time, without inhibiting specific pathways. For this matter, specific endocytic structures of BS-C-1 cells were transfected with a fluorescent marker. Additionally the influenza virus was fluorescently tagged, with DiD. When cells were infected with Influenza A, 65% of the viruses had associated with a clathrin-coated structure. It was observed that only 6% of the viruses bound to pre-existing CCPs, the other 96% induced CCP formation via binding(Rust et al. 2004). The study of Wang *et al.* confirmed the involvement of CME in Influenza A infection. Here, A549 cells were infected with influenza A virus H5N1 and treated with CPZ, which causes clathrin to assemble on endosomal membranes. Infection of A549 cells could be prevented when cells were treated with CPZ. To support these results, the heavy chain of clathrin was knocked down using siRNA in A549 cells and then infected with influenza A virus H5N1. The knock down of the clathrin heavy chain significantly enhanced cell viability as compared to control cells(Wang, Jiang 2009). Finally, Sieckarski *et al.* transfected cells with a dominant negative form of Eps15 (Eps15Δ95/295) to also analyze involvement of CME. After transfection of Eps15Δ95/295, infectivity of the cells was decreased, but not completely blocked(Sieczkarski, Whittaker 2002). This confirms once more that Influenza A can enter the cell via CME, but that also additional pathways exist.

Rust *et al.* showed that of all internalized viruses, 35% did not associate with a clathrin-coated structure, what suggest that they were internalized via a clathrin independent pathway. It was observed that only 5% of the internalized viruses co-localized with caveolae. Additionally, in cells treated with filipin, still 5% of the viruses was seen in caveolae like structures, what indicates that the clathrin independent endocytic pathway is also lipid raft- and caveolin-independent(Rust et al. 2004). Wang *et al.* have also analyzed the involvement of caveolae. This was done by comparing influenza A virus H5N1 infection to Cholera Toxin infection in A549 cells. Cholera Toxin (CT) is known to preferably enter the cell via caveolae mediated endocytosis and is often used as a marker for this route. In this study it therefore also functions as a positive control. Treating the cells with nystatin or filipin, which are sterol binding drugs, did inhibit CT uptake, however treatment with these drugs could not prevent infection of these cells with influenza A virus H5N1. This also suggests that caveolae mediated endocytosis does not play a major role in internalization of influenza A virus H5N1 infection(Wang, Jiang 2009). This is in correspondence with the study of Rust *et al.* in which only 5% of the influenza A viruses was co-localized to caveolae. In addition to this, Sieckarski *et al.* transfected cells with dominant negative forms of cav-1 and observed no reduced infectivity as compared to non-transfected cells(Sieczkarski, Whittaker 2002).

The results of Rust *et al.* described above show that the major part of influenza A viruses is internalized via CME, a substantial part is endocytosed via a clathrin- and caveolae independent pathway and caveolae dependent endocytosis contributes only minor to internalization of the Influenza A virus. The clathrin- and caveolae-independent endocytosis is not induced to compensate when CME is inhibited, but can function in parallel of CME(Rust et al. 2004). Additionally, Sieckarski and co-workers also demonstrated a clathrin- and caveolin independent pathway which is exploited by influenza A. For this matter they inhibited CME by transfecting cells with Eps15Δ95/295 and additionally treat the transfected cells with MβCD to inhibit caveolae mediated endocytosis. When both caveolae- and clathrin mediated endocytosis was inhibited, influenza A virus was still able to infect cells(Sieczkarski, Whittaker 2002).

Although multiple groups have confirmed the existence of a clathrin- and caveolae independent endocytic route, characterization of it has not been completed. It would be of great interest to know how this pathway yields sealed vesicles, which molecules mediate the

transport of the vesicles and if there is a specific receptor that initiates this internalization pathway.

6. Discussion | Cellular receptors and endocytic routes

In this work I analyzed how various viruses use sialylated cellular receptors as attachment and entry receptors. Subsequently I reviewed the (sometimes multiple) ways viruses could be endocytosed into their target cells. A summary of the discussed results can be found in Table 3.

6.1. Sialic acid dependence

It is evident that, except for Arenaviruses, in all cases sialic acids play a major role in infection by the various viruses. Glycoproteins and glycolipids are the typical structures on which sialic acids can be found. Sialic acids can be modified in many ways, which can influence recognition by viruses. For a long time neuraminidases have been used to determine whether a virus was depending on sialic acids for infection or not. Most neuraminidases only cleave of the terminal sialic acids. When viruses were still able to infect cells after treatment with neuraminidase, it was concluded that there was no dependence on sialic acids. However, recently Haselhorst *et al.* showed that sialic acids on internal braches are the determining factors for rota virus infection(Haselhorst et al. 2009). These internal sialic acids are not cleaved of by the action of most regularly used neuraminidases and thus for a long time it was thought that rota viruses did not need sialic acids for infection. There are only a few neuraminidases that have the unique property to cleave these internal sialic acids, like the one from Arthrobacter ureafaciens(Iwamori et al. 1997). I think it is therefore advisable for following research to use both regular neuraminidases and the one from A. ureafaciens to be really thorough in the analysis of sialic acid dependence.

In this review I focused on viruses that, for infection, are sialic acid dependent and, as said before, sialic acids can be modified in many different ways. The molecules that make up sialic acid can receive additional substitutions at the hydroxylgroup on their carbon-atoms, like *O*-methyl, *O*-sulfate, *O*-acetyl and phosphate groups this results in at least 40 molecular species of sialic acid that can be found in nature(Suzuki 2005). In this review I did not analyze the difference that these subtle changes can make in terms of recognition by the virus. It is plausible to think that these substitutions can influence infectivity of the viruses, by altering attachment or internalization. However, to be able to say something about this, it should be supported by scientific research.

6.2. Functional receptors

Table 3 shows that some of the viruses and bacterial toxins use the same sialylated structures as cellular receptors. For example, GM1 is used by Cholera Toxin and by SV40. However, the viral receptors that recognize these structures are not the same. This raises the question if the earlier mentioned subtle alterations of sialic acids, cause the recognition of the same sialylated structure by different viral receptors.

It is interesting to observe that some viruses use separate structures for the two steps of attachment and entry. Rota viruses only use sialylated structures as attachment receptors, but for entry an integrin is used, which is an asialo structure. However, Influenza A seems to use sialylated structures for both attachment and entry for its multistep internalization process. Why Rota viruses and Influenza A viruses use different structures for attachment and entry is not clear, but it would be very interesting to analyze which beneficial effect for infection results from this.

It seems to me that a multistep internalization model narrows the scope of target cells that can possibly be infected by these viruses. However, the two viruses that use an attachment and entry receptor (Rota virus and Influenza A virus) have quite a redundancy of receptors for this purpose, so it might not be the case. It seems there is not such a great specificity, but only the presence of sialic acid is sufficient, since the viruses both employ various glycoproteins and gangliosides.

6.3. Endocytic pathways

It is striking to see that the Influenza A virus and Cholera Toxin can use more then one endocytic pathway for internalization. Cholera toxin uses CME and caveolae mediated endocytosis, which are dynamin dependent, and clathrin-, caveolae- and dynamin independent endocytosis, referred to as the GEEC pathway. Influenza A can be internalized via CME, via a clathrin- and caveolae independent endocytosis and a minor part of the virus is internalized via caveolae mediated endocytosis. In contrast, SV40 is only capable to bind to GM1 and is mainly internalized via caveolae. The redundancy of endocytic pathways Influenza A and Cholera Toxin can use is notable. I can think of multiple reasons why a virus would do this; First, not every endocytic route yields a successful infection. In the case of Influenza A virus it has been shown that multiple routes can function in parallel of each other, and it seems that the virus spreads its chances to be sure infection of the target cell takes place. Second, sometimes target cells try to intervene when viruses are internalized into the cell. To be able to circumvent this intervention it is important that viruses can enter the cell in multiple ways.

In the case of Cholera Toxin, it is not known whether it employs the different pathways in parallel of each other. Therefore, it would be interesting to make use of the live cell imaging as used by Rust *et al.*(Rust et al. 2004) to address this redundancy. Analysis of the life cell imagining can tell us whether or not Cholera Toxin can make use of these pathways in parallel, or that the different pathways are only used as a compensatory mechanism when the main pathway is blocked.

6.4. Linking functional receptors to specific endocytic pathways

An interesting hypothesis is that specific functional receptors can be linked to specific endocytic pathways. However, when we take a close look at Table 3, it is hard to do this. Yet, caveolin-1, which is an essential component of caveolae, has been shown to bind to GM1(Fra et al. 1995), additionally it is known that caveolae are enriched in glycosphingolipids, like GM1(Lajoie, Nabi 2007). Knowing this, it is tempting to say that probably viruses that bind to GM1 as a cellular receptor tend to take the caveolae mediated pathway than viruses that bind to another cellular receptor. It is needless to say that for this matter, more research is necessary.

6.5. Influenza A

The importance of sialic acid in Influenza A infection has been an established fact for a long time now. But in the jungle of scientific information it is not easy to find articles that affirm each other with regard to the underlying cellular structure of Influenza A.

Back in the days it was thought that sialic acid was the only important prerequisite for recognition, nowadays, the situation has become far more complex. The interesting part of recognition of cells by the influenza A virus is that the affinity of HA for sialic acid is quite low. However, multiple HA proteins have the opportunity to bind to sialic acids, what results in a high avidity of the virus for the cell(Flint et al. 2004). It was shown that gangliosides cannot induce infection, although containing sialic acids. Gangliosides are therefore thought to function as attachment receptors. Subsequently, glycoproteins are thought to function as entry receptors for the virus(Chu, Whittaker 2004). Hence, it is important to note that this study of Chu *et al.* was only performed in CHO cells. It is important to confirm this in other cell lines. Thus subsequent assays should be performed in which different GnT1$^{-/-}$ cell lines should be used.

The redundancy of the cellular surface receptors is possibly the strength of the Influenza A virus. Its ability to use multiple structures as attachment and entry receptors results in the fact that the virus has a lot of chances to infect its target cells. The immunological pressure Influenza A virus is subject to, leads to minor changes in HA each year. This was earlier

discussed as antigenic drift. This probably also contributes to the fact that Influenza A is able to bind to multiple sialylated structures.

Influenza A is internalized via CME. It has also been shown that Influenza A can infect its target cell via a clathrin- and caveolae independent endocytic pathway(Wang, Jiang 2009, Rust et al. 2004, Sieczkarski, Whittaker 2002). Characterization of this pathway has not been performed yet, but it is of great interest to do this. The possible involvement of GEECs and arf6 is a way to start unravelling this mystery.

Furthermore it is important to note that most of the results are based on the use of different Influenza A virus strains. Additionally, different cell lines were used. It is important to remember that the variable results can be an effect of this. The diversity of different strains makes generalized conclusions hard to draw and to believe. Generalized conclusions about a specific Influenza A strain can only be drawn, when merely one specific virus strain is used, the cell lines are varied and then still the results are the same.

I want to conclude by saying that viral infections with regard to sialic acids is a very intriguing field of research. In this review many facets were discussed, as the general characteristics of sialic acids and the structures to which it can be attached. Additionally, the sialic acid dependence of the viruses was discussed and the many ways viruses can enter their target cells. The many questions that are still open with regard to these subjects are waiting to be answered with additional assays and experiments.

Tabel 3 | Summary of the discussed results

Virus/Toxin	Cellular receptor		Ways of endocytosis	Dependence on dynamin
	Attachment receptor	Entry receptor		
Cholera Toxin	GM1		CME	Yes
			Caveolae mediated endocytosis	Yes
			Clathrin- and caveolae independent endocytosis (GEEC)	No
SV40	GM1		Caveolae mediated endocytosis	n.d.
Rota virus	Various glycoproteins (not characterized) and various gangliosides (GM3, Gm2 and GD1a)	Integrins	Calcium dependent CME.	n.d.
Arenavirus (New World)	TfR1		CME	n.d.
			Caveolae mediated endocytosis	n.d.
Arenavirus (Old World)	α-DG		Clathrin- and caveolae independent endocytosis	No
			Possible entry via CME	n.d.
Influenza A virus	Various glycoproteins and glycolipids	Various glycoproteins	CME	n.d.
			Clathrin- caveolae independent pathway	n.d.

			Minor use of caveolae	n.d.

Tabel 3 | CME: Clathrin mediated endocytosis, N.D.: not determined in used article.

Abbreviation list

^{125}I	Radio-active labelled Iodium
AP-2	Assembly Polypeptide-2
CAD	Cathionic Amphiphillic Drug
Ca^{2+}	Calcium
Cav-1	Caveolin-1
CCP	Clathrin Coated Pit
CCV	Clathrin Coated Vesicle
CHO	Chinese Hamster Ovary
CME	Clathrin Mediated Endocytosis
CPZ	ChlorPromaZine
CRD	Carbohydrate Recognition Domain
CT	Cholera Toxin
DGJ	DeoxyGalactonoJirimycin
DIG	Detergent-Insoluble Glycosphingolipid rich domain
DLP	Double Layered Particle
DNJ	DeoxyNoJirimycin
EGF	Epidermal Growth Factor
EPS15	Epidermal growth factor receptor pathway substrate 15
ER	Endoplasmatic Reticulum
GalCer	Galactolipids
GBP	Glycan Binding Protein
GEEC	GPI-AP Early Endosomal Compartment
GFP-Cav-1	GFP labeled Caveolin-1
GlcCer	Glucolipids
GnT1	N-acetylglucosaminyltransferase I
GPC	Glycoprotein PreCursor
GPI-AP	Glycosyl Pphosphatidyl Inositol-Anchored Proteins
HA	Hemagglutinin
HLCC	HeLa Cell Clones
HRP	Horse Radish Peroxidase
HUS	Haemolytic Uremic Syndrome
JUNV	Junín Virus
K^+	Potassium
LASV	Lassa Virus
MAG	Myelin Associated Glycoprotein
MβCD	Methyl-β-Cyclodextrin
MHCI	Major HistoCompatibility Complex type I
NA	Neuraminidase
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
NP	NucleoProtein
NS	Nystatin
PyVLP	Polyoma Virus Like Particles
PMA	Phorbal 12-myristate-13-acetate
PPMP	1-phenyl-2-hexadecanoyl-amino-3-morpholino-1-propanol
RRV	Rhesus Rota Virus
siRNA	Small Interfering RNA
Stx	Shiga ToXin
SV40	Simian Virus 40
TAg	Large T antigen
TfR1	Transferrin Receptor 1
TLP	Triple Layered Particle
TRX-SV40	Texas Red-X labeled SV40 virions

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