The Pennsylvania State University

The Graduate School

Department of Biochemistry and Molecular Biology

ROLES OF LIPID RAFTS IN BOTULINUM NEUROTOXIN SEROTYPE A ACTIVITY AND DIFFERENTIATION OF NEUROBLASTOMA CELLS

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by

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Abstract

Botulinum neurotoxin serotype A (BoNT/A), one of seven serotypes of botulinum neurotoxin, is taken up by neurons of the peripheral nervous system. Within the neurons it catalyzes cleavage of the synaptosomal-associated protein having a mass of 25kDa, SNAP-25, thereby blocking neurotransmission. BoNT/A has been shown to interact with SV2, as well as gangliosides that are often found in lipid rafts. Lipid rafts are microdomains that can be found on the outer leaflet of the plasma membrane and are enriched in cholesterol and glycosphingolipids. To determine whether lipid rafts were needed for BoNT/A activity, those associated with the plasma membranes of murine N2a neuroblastoma cells were disrupted using either the cyclic oligomer methyl-β-cyclodextrin or the polyene filipin. Disruption of cholesterol containing lipid rafts by either reagent did not prevent the action of BoNT/A on murine neuroblastoma N2a cells, in fact activity was enhanced. While our results indicate that disruption of lipid rafts enhances BoNT/A activity, disruption of clathrin-dependent endocytosis appeared to be inhibitory.

In addition to disruption of lipid rafts enhancing BoNT/A activity, it also induced neuritogenesis of N2a cells. Therefore, studies were performed to determine the type of process formation induced by disruption of lipid rafts by either MβCD or filipin. Because ganglioside composition has been shown to change during neuronal differentiation, the question of whether process expression was accompanied by changes in ganglioside content or subcellular distribution was addressed. The results indicate that the processes formed were axonal in nature and their expression was accompanied by changes in both ganglioside content and subcellular localization.
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<td>Botulinum neurotoxin</td>
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<td>BoNT/A</td>
<td>Botulinum neurotoxin serotype A</td>
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<tr>
<td>CTx</td>
<td>Cholera toxin</td>
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<tr>
<td>CTxB</td>
<td>Binding subunit of cholera toxin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
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<td>DRM</td>
<td>Detergent resistant microdomain</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGFR</td>
<td>Epidermal growth factor</td>
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<td>ErbB2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
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<td>FITC</td>
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<tr>
<td>GPL</td>
<td>Glycerophospholipid</td>
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<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPDS</td>
<td>Lipoprotein deficient fetal calf serum</td>
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<td>MBCD</td>
<td>Methyl-β-cyclodextran</td>
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<td>MA</td>
<td>Mevalonic acid</td>
</tr>
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<td>MEV</td>
<td>Mevinolin</td>
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<tr>
<td>MDCK</td>
<td>Madine darby canine kidney cells</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>N2a</td>
<td>Neuro-2a murine neuroblastoma cells</td>
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<td>NBS</td>
<td>Newborn bovine serum</td>
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<td>NPP</td>
<td>Nuclear pore proteins</td>
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<td>PC12</td>
<td>Pheochromocytoma cells</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PPMP</td>
<td>d,l-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-propanol·HCl</td>
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<tr>
<td>PVDF</td>
<td>Polyvinyl difluoride</td>
</tr>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>SNAP-25</td>
<td>Synaptosomal associated protein of 25 kDa</td>
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<tr>
<td>SNARE</td>
<td>Soluble N-ethylemaleimide sensitive factor attachment receptor</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic vesicle protein 2</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline containing Tween-20</td>
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<tr>
<td>TeNT</td>
<td>Tetanus neurotoxin</td>
</tr>
<tr>
<td>TIFF</td>
<td>Triton-insoluble floating fraction</td>
</tr>
<tr>
<td>TIM</td>
<td>Triton-insoluble membranes</td>
</tr>
<tr>
<td>TNE</td>
<td>25mM Tris-HCl, 150mM NaCl, 5mM EDTA, pH 7.5</td>
</tr>
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<td>Tfr</td>
<td>Transferrin</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>Trk</td>
<td>Tyrosine kinase</td>
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Chapter 1

General Introduction to Lipid Rafts and Their Involvement in Pathogen Internalization and Signaling

Historical perspective of lipid rafts

The plasma membrane is crucial to life of a cell: it encloses it, defines its boundaries, and maintains a distinct difference between the extracellular and intracellular environments. It was 1925 when Gorter and Grendel published their finding that the plasma membrane of red blood cells was comprised of a lipid bilayer that was believed to be a rather symmetrical composition of lipids. Almost 50 years later in 1972, Singer and Nicolson proposed the fluid mosaic model which, while incorporating the finding that lipids were able to freely diffuse, did not dispel the belief that the bilayer was a relatively uniform, homogeneous mixture of lipids. For many years, a widely held view of the plasma membrane was that cholesterol functioned as a membrane stabilizer by evenly intermixing with other lipids to minimize any temperature induced changes in membrane fluidity. However, this model was later challenged by the discovery that cholesterol in plasma membranes was not evenly distributed (Harder and Simons, 1997).

Two landmark observations that initiated a flurry of research into the structure of the plasma membrane were the findings that 1) the apical and basolateral domain of epithelial cells had different protein and lipid compositions (Simons and van Meer, 1988)
and 2) GPI-anchored proteins were present in subdomains on the plasma membrane that were enriched in glycosphingolipids (GSLs, Brown and Rose, 1992). These findings led to the development of the “lipid raft” model in which microdomains enriched in sphingolipids and cholesterol are distinct from the rest of the plasma membrane (Simons and Ikonen, 1997). These findings have been supported by research in which lipid rafts (cholesterol dependent liquid-ordered phases) were reconstituted in model membranes. Reconstitution was followed by monitoring the partitioning of fluorescently labeled GPI-anchored proteins (such as Thy-1) and the ganglioside GM1 into liquid ordered phases (Dietrich et al., 2001). It was also noted that fluorescein-conjugated phospholipid analogs were excluded from the liquid ordered phase.

**Definition of lipid rafts**

Lipid rafts are specialized microdomains on cell membranes that are enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins. Operationally they are defined by their low density and resistance to solubilization with non-ionic detergents. The nomenclature used to describe these domains has changed over time; for example they have been labeled detergent resistance microdomains (DRM), Triton-insoluble membranes (TIM), and Triton-insoluble floating fraction (TIFF) with each name noting their detergent insoluble nature. Specialized microdomains in non-neuronal cells can not only be differentiated as lipid rafts but as caveolae, flask-like shaped invaginations containing the protein caveolin. In neurons, the existence of caveolae has remained controversial. While some studies report that neurons express
little to no caveolin and do not possess the morphological qualities of caveolae (Shyng et al., 1994; Fra et al., 1995; Gorodinsky and Harris, 1995), other reports dispute this (D’Orlando et al., 2007; Lentini et al., 2007). For the purpose of the studies described in this thesis, the term lipid rafts will refer to the specialized microdomains found in neuroblastoma N2a cells and will exclude caveolae.

**Isolation of lipid rafts**

The glycosphingolipids (GSLs), lipids that are enriched in lipid rafts, tend to have long, saturated, acyl chains that allow for a more ordered packing compared to that seen in adjacent areas of the membrane that have more phospholipids. The elevated concentration of cholesterol intercalated between the hydrocarbon side chains contributes to the reduced fluidity of lipid rafts compared to the rest of the plasma membrane. The combination of the more ordered packing of the lipids which results in increased van der Waals forces, and possible phase separation is the likely cause for the resistance of lipid rafts to solubilization by non-ionic detergents (Brown and London, 2000).

The traditional method for isolating lipid rafts relies on their characteristics of detergent insolubility and low density. In these methods cells are usually lysed in a cold buffer containing a non-ionic detergent (frequently Triton X-100), homogenized and then isolated by centrifugation on a discontinuous sucrose density gradient in which the lipid rafts “float” to the top, least dense fractions of the gradient (see Fig. 1-1). Purity of the preparation can be determined by monitoring the enrichment of lipid raft markers such as
the protein, flotillin, or the lipid, ganglioside GM1, as well as by exclusion of soluble markers such as the transferrin receptor (Bickel et al., 1997; Chamberlain et al., 2001). A major controversial point of this approach is the fact that isolation of lipid rafts must be done at a non-physiological temperature. This permits the question of whether the lipid raft fractions are truly indicative of native rafts, or merely an artifact of the method of isolation. To counter this argument, Brij-98 was used in the same manner but at the physiological temperature 37°C to isolate lipid rafts. It gave a low-density fraction that was similar in composition to those isolated using Triton X-100 (Drevot et al., 2002; Parton 1994). Many other detergents have been used to isolate lipid rafts such as NP-40, octylglucoside, CHAPS and Lubrol (Chamberlain, 2004). Detergent-free purification of lipid rafts can also be done using a sodium carbonate buffer at high pH, which separates proteins firmly attached to the membrane and less soluble from those that are peripherally associated and more soluble (Song et al., 1996).
Figure 1-1: Schematic for the isolation of lipid rafts using a non-ionic detergent and sucrose density centrifugation.

Lipid rafts can be isolated from cells lysed in cold, non-ionic detergent, applied to a discontinuous sucrose gradient and centrifuged. The less dense nature of lipids enriched in rafts in comparison to the more dense, soluble proteins causes the lipid rafts to “float” and separate from the soluble cell organelles.

Composition of lipid rafts

Using the aforementioned technique to isolate lipid rafts, several studies have attempted to determine constituents (both lipid and protein) of lipid rafts. In general, it has been shown that lipid rafts are enriched in cholesterol and glycosphingolipids, but are somewhat depleted in glycerophospholipids (GPLs) (Pike et al., 2002). Lipid raft fractions prepared from both model membranes and cell membranes yielded fractions...
enriched in cholesterol, sphingomyelin, and glycosphingolipids with a subset of GPI-anchored proteins (Ahmed et al., 1997; Brown and London, 1998; Fridriksson et al., 1999). More specifically, lipid rafts isolated from Madine-Darby Canine Kidney cells (MDCK) contained 32% cholesterol and 14% sphingomyelin, whereas the membrane from intact cells contained ~12% cholesterol and ~1% sphingomyelin (Brown and Rose, 1992). Additionally, the same study revealed that lipid rafts were enriched with about five-fold more glycosphingolipids than were present in membranes from intact cells. Figure 1-2 illustrates the general organization of lipids in rafts.

Protein composition of lipid rafts can vary as many proteins involved in signaling may move in and out of lipid rafts as part of a regulatory process. While proteomic studies of lipid rafts have yielded widely varying results, several proteins have been noted for their constitutive localization to lipid rafts. These include but are not limited to: flotillins, heterotrimeric G proteins, src kinases, MAP-kinase, protein C kinase and Thy-1.
Figure 1-2: Composition of lipid rafts.

Illustration of lipid rafts showing an enrichment of cholesterol (depicted here in yellow) and the straight, saturated hydrocarbon chains of glycosphingolipids. Multiple GPI-anchored proteins and tyrosine kinases are enriched in lipid rafts (Sharom and Lehto, 2002).

Size of lipid rafts

Due to the morphological character of caveolae, they can be readily seen by microscopy and their size determined (~100nm in diameter, Rothberg et al., 1992). However, lipid rafts cannot be directly visualized via microscopy and therefore size
determination has relied on several indirect measurements that have given varying results. Lateral diffusion of GPI-anchored proteins that are markers for lipid rafts indicated that lipid rafts were ~200nm in diameter (Simson, 1998). Single particle tracking of GPI-anchored proteins indicated they were much smaller with a diameter of ~26nm (Pralle et al., 2000). Fluorescence resonance energy transfer (FRET) has failed to identify lipid rafts in any of those size ranges, suggesting that lipid rafts are much smaller and are transient in their existence (Kenworthy et al., 1998; Kenworthy et al., 2000). Computer modeling has suggested lipid rafts may only be ~5nm (Yethiraj et al., 2007).

**Disruption of lipid rafts**

As cholesterol is viewed as the glue that holds lipid rafts together, studies aimed at understanding the functions of lipid rafts have used methods that alter cholesterol content in the plasma membrane to disrupt them. Extraction or sequestration of cholesterol from the outer leaflet of the plasma membrane, as well as the inhibition of *de novo* cholesterol biosynthesis, can be used to alter lipid rafts. Disruption can then be assayed by localization of lipid raft markers after their extraction and isolation by sucrose density centrifugation.

A number of compounds [e.g. methyl-β-cyclodextrin (MβCD) and filipin] that interact with cholesterol can be used to acutely alter its content in the plasma membrane. MβCD is a cyclic oligomer (Fig. 1-3) that complexes cholesterol in its hydrophobic core and extracts it from the outer leaflet of the plasma membrane (Ohtani et al., 1989;
Filipin (Fig 1-3), on the other hand, is a polyene antibiotic that binds to cholesterol in the outer leaflet and sequesters it into ultrastructural aggregates on the cell surface (Kitajima et al., 1976; Robinson and Karnovsky, 1980; McGookey et al., 1983; Bolard, 1986; Castanho et al., 1992). Both drugs have been shown to alter lipid raft formation (Keller and Simons, 1998).

Fig. 1-3

Figure 1-3: Structures of the cholesterol-binding agents (a) MβCD and (b) filipin.

Cells can obtain cholesterol two ways: by de novo synthesis and by uptake of low density lipoproteins (LDLs). Treatment of cells with mevinolin (MEV), a potent competitive inhibitor of HMG-CoA reductase that catalyses the rate-limiting step in cholesterol synthesis, prevents its de novo synthesis (Alberts et al., 1980). The main drawback to inhibiting cholesterol synthesis is that many downstream, essential
isoprenoids (such as dolichol, ubiquinone, and prenylated proteins) needed for cell survival are also inhibited. Therefore, supplementing cells with mevalonic acid (MA) allows for the formation of essential downstream components while having a minimal effect on cholesterol synthesis (Cole et al., 2005). Figure 1-4 provides a schematic of the cholesterol biosynthetic pathway and the downstream essential isoprenoids synthesized form mevalonate.

Fig. 1-4

![Diagram of the biosynthetic pathway of cholesterol.](image)

**Figure 1-4:** The biosynthetic pathway of cholesterol.

HMG-CoA reductase catalyzes the rate-limiting step in cholesterol synthesis. It can be inhibited by drugs such as mevinolin and simvastatin. Inhibition of this step prevents synthesis of essential downstream components (e.g. heme a, dolichol, ubiquinone) that are crucial for cell survival. Addition of mevalonate allows for synthesis of these products or compounds.
The second manner in which cells obtain cholesterol is through the receptor-mediated uptake of low density lipoproteins (LDLs) and the subsequent hydrolysis of their cholesterol esters to yield fatty acid and free cholesterol. This route can be eliminated by growing cells in medium lacking LDLs.

**General overview of lipid raft functions**

Lipid rafts have been implicated in a variety of cellular processes such as protein sorting, membrane trafficking, signal transduction, cell proliferation, differentiation, and apoptosis. The interaction of proteins with lipids found in rafts allows them to move in and out of lipid rafts, resulting in lipid rafts affecting multiple cell functions.

For numerous pathogens, lipid rafts have been implicated as “portals of entry”. The pathogens bind specific components within the raft and are subsequently internalized. Lipid rafts can facilitate binding by offering a concentrated platform of receptors for the pathogens to bind to. Of particular interest has been the binding and internalization of bacterial toxins (toxic peptides that are released by bacteria), such as *Vibrio cholerae* and *Clostridium tetani*. The finding that cholera toxin (CTx) bound with high affinity to ganglioside GM1, a lipid raft component, supported the hypothesis that lipid rafts were necessary for its binding and internalization (Van, 1974). Subsequent work indicated the hypothesis was correct when CTx: 1) was found in association with detergent-insoluble raft fractions isolated from the cell surface of hippocampal neurons (Shogomori and Futerman, 2001) and 2) when its internalization by CaCo-2 human
intestinal epithelial cells was inhibited by the disruption of lipid rafts by filipin (Orlandi and Fishman, 1998). These observations supported the idea that peptides could utilize lipid rafts for entry. Further support for this hypothesis was provided by studies of the cellular uptake of tetanus neurotoxin (TeNT). TeNT binds to the ganglioside GT1b (also found in lipid rafts, Prinetti et al., 2000; Vinson et al., 2003) and, upon internalization, catalyzes cleavage of the vesicle associated membrane protein, VAMP2 (Link et al., 1992). Disruption of lipid rafts by either filipin or MβCD inhibited TeNT catalyzed cleavage of VAMP2 in cultured mouse neurons and PC-12 cells (Munro et al., 2001; Herreros et al., 2001, respectively). Aside from bacterial toxins, other pathogens such as bacteria and viruses have been shown to exploit lipid rafts for entry into cells, Table 1-1.

Lipid rafts can also be considered platforms for cell signaling due to the fact that many proteins enriched in lipid rafts are involved in signaling pathways. This is evidenced by the number of receptor tyrosine kinases (RTKs) that are enriched in lipid rafts, such as epidermal growth factor receptor (EGFR, Mineo et al., 1996), platelet-derived growth factor receptor (PDGFR, Liu et al., 1996), insulin receptor (Yamamoto et al., 1998), and many Trks (Wu et al., 1997). Lipids can also modulate signal transduction, in particular gangliosides. GM1, GD1a, and GT1b all have modulatory effects on epidermal growth factor receptor (Li et al., 2001; Mirkin et al., 2002; Liu et al., 2004). The gangliosides GM1, GM2, GD1a and GT1b appear to inhibit the phosphorylation of platelet-derived growth factor receptor, which has a downstream effect on gene expression and cell cycle (Hynds et al., 1995; Farooqui et al., 1999).
Tab. 1-1

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<tr>
<th>Toxins</th>
<th>Ligands</th>
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<tr>
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<td>GM1</td>
</tr>
<tr>
<td>Tetanus neurotoxin</td>
<td>GT1b</td>
</tr>
<tr>
<td>Shiga toxin</td>
<td>Gb3</td>
</tr>
<tr>
<td>Campylobacter jejuni enterotoxin</td>
<td>GM1</td>
</tr>
</tbody>
</table>

**Bacteria**

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td>Asialo-GM1</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>Asialo-GM1 and -GM2</td>
</tr>
<tr>
<td>Chlamydia pneumonia</td>
<td>Asialo-GM1 and -GM2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Asialo-GM1 and -GM2</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>GM3</td>
</tr>
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</table>

**Viruses**

<p>| | |</p>
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<tbody>
<tr>
<td>Sendai virus</td>
<td>GD1a</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>GM3, GD1a</td>
</tr>
</tbody>
</table>

Table 1-1: Examples of pathogens that “hijack” lipid rafts and their glycosphingolipid ligands. Table adapted from Mañes et al., 2003 and Schengrund, 2003.
Chapter 2

Determination of whether intact lipid rafts are essential for binding and internalization of botulinum neurotoxin serotype A

Introduction: Botulism and Botulinum Neurotoxin

Botulinum neurotoxins (BoNT) are the most toxic substances known to man, with a single gram having the potential to kill more than 1 million people if successfully dispersed (Arnon et al., 2001). Despite its poisonous nature BoNTs have emerged as valuable therapeutic agent under the tradenames of Botox®, NeuroBloc® and Myobloc®, used for treatment of dystonia, strabismus, migraines and many hyperactive muscle disorders (Tsui et al., 1986; Jankovic and Brin, 1991; Blumenfeld, 2003; Thnat and Tan, 2003). The cosmetic industry has also capitalized on the therapeutic effects of Botox® to alleviate wrinkles, with the American Society of Plastic Surgery estimating that in 2002 alone, nearly one million Botox® injections were administered. Although BoNTs have found a niche in the medical and cosmetic fields and are used with increasing frequency, many questions remain regarding the mechanism of the interaction of BoNT with neurons.
Botulism

Botulinum neurotoxin (BoNT) is the causative agent of botulism, a disease that results in flaccid muscle paralysis leading to death if untreated. It is a disease that is rarely seen in the United States with an average of only 100 cases per year reported to the CDC, most of which are infant botulism. Nevertheless, the increasing usage of BoNT/A for medical purposes, as well as the risk associated with botulism as a bioterrorism agent, highlights the need for basic research and the development of vaccines and antitoxins.

Generally, botulism results from consumption of food contaminated with either the *C. botulinum* bacteria or the neurotoxin. In the case of the bacteria, it enters the gastrointestinal tract where it colonizes and releases the toxin. The developed stomach flora (bacteria) in healthy individuals is able to outcompete the *C. botulinum* bacteria before it can colonize, and subsequently one escapes developing botulism. However, for those who are immuno-compromised, in particular newborns with underdeveloped intestinal flora, exposure to the bacteria can prove deadly. In the United States, infant botulism is the leading type of botulism at ~75%, with food botulism at ~25%.

The symptoms of botulism are initially asymptomatic: blurred vision, difficulty focusing, dry mouth and muscle fatigue. These symptoms lead to a symmetrical, descending flaccid paralysis that leads to death if untreated. Even with an early diagnosis and treatment, paralysis can last for several months. One of the reasons for the enduring disease is the half-life of the enzymatic portion of the toxin, which for BoNT serotype A
(BoNT/A) is about three months (Tab. 2-1 shows the comparison of the half-life of BoNT/A with common enzymes).

Currently there is no treatment for the paralysis associated with botulism. Antitoxin is available that is effective when taken immediately after exposure to the toxin. Because the disease is initially asymptomatic, treatment with the toxin is typically delayed and therefore has diminished effectiveness. This emphasizes the need for basic research and the development of vaccines and antitoxins able to reverse the nerve damage associated with botulism and reduce/eliminate the lengthy recovery.

Tab. 2-1

Table 2-1: Comparison of half-life of BoNT/A with half-lives of common enzymes. Adapted from Table 30-10. Voet and Voet, 2nd Ed.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half-Life (h)</th>
</tr>
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<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>0.2</td>
</tr>
<tr>
<td>RNA polymerase I</td>
<td>1.3</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>5.0</td>
</tr>
<tr>
<td>Aldolase</td>
<td>118</td>
</tr>
<tr>
<td>GAPDH</td>
<td>130</td>
</tr>
<tr>
<td><strong>BoNT/A</strong></td>
<td><strong>2180</strong></td>
</tr>
</tbody>
</table>
Characteristics of botulinum neurotoxin serotype A

The *Clostridium botulinum* bacteria produce seven distinct serotypes of neurotoxin (A-G). BoNT/A is produced as a single polypeptide chain of ~150 kDa which is then post-translationally modified to produce a heavy (H) and a light (L) chain, linked together by a disulfide bond (see Fig. 2-1, Lacy and Stevens, 1999). The L chain is a zinc endoprotease that catalyzes cleavage of the synaptosomal-associated protein having a mass of 25kDa (SNAP-25). SNAP-25 is found in the presynaptic terminal of neurons where it functions as an essential component of the soluble NSF attachment receptor (SNARE) complex (see Fig. 2-2, Blasi et al., 1993). The H chain consists of two functional domains: the carboxy-terminal portion that facilitates binding and the amino terminal part that mediates cytoplasmic entry of the L chain (Lalli et al., 1999).
Figure 2-1: Ribbon diagram of BoNT/A.

The zinc atom (yellow/green) is coordinated in the catalytic domain (light blue). Toxin binding to a neuron occurs through the binding domain (dark green), and the translocation domain (gold) is suggested to form a channel through which the catalytic domain is transported from the endosome to the cytosol. The active site loops are labeled 50, 200, and 250 (Hanson and Stevens, 2000).
**Binding of BoNT/A to neurons**

To reach the cytosol where BoNT/A catalyzes cleavage of SNAP-25, the toxin must progress through a lengthy and complex sequence of events beginning with binding to the surface of the presynaptic membrane, followed by endocytosis and translocation into the cytosol where the light chain of the toxin interacts with and catalyzes cleavage of the SNARE protein SNAP-25. Figure 2-2 illustrates neurotransmission in healthy neurons as well as neurons exposed to the different serotypes of BoNT. To explain the interaction of the toxin with the neuron membrane, a double-receptor model was proposed over 20 years ago (Montecucco, 1986). This model theorized that BoNT/A bound with low affinity to gangliosides that were abundant in the peripheral nervous system. This ganglioside-BoNT/A complex then laterally searched for a protein receptor to which it bound with high affinity. Circular dichroism indicated that BoNT/A bound to ganglioside GT1b and underwent a conformational change, and surface plasmon resonance data fit a conformational change model (Yowler et al., 2004). The observed conformational change upon binding to GT1b was hypothesized to allow the neurotoxin to bind to a protein receptor by opening a second binding site. The proposed protein receptor for BoNT/A remained unidentified until 2006, when Dong et al (2006) found that BoNT/A binds to a portion of the synaptic vesicle protein SV2, a protein found on the luminal surface of synaptic vesicles. Three isoforms of SV2 have been identified: SV2A, SV2B, and SV2C; with SV2A and SV2B found in the brain (Sudhof, 2004). Hippocampal neurons from SV2A and SV2B knockout mice were protected from the activity of BoNT/A while restoration of expression of SV2 had the opposite effect.
Furthermore, mice that lacked SV2B displayed a reduced, though not complete, reduction in BoNT/A sensitivity (Dong et al., 2006).

Fig. 2-2

Figure 2-2: Neurotransmission in healthy and BoNT intoxicated cells.

In healthy neurons, synaptic vesicles carrying neurotransmitter fuse with the presynaptic membrane, in a SNARE complex dependent manner, and subsequently release neurotransmitters (A). In neurons exposed to BoNTs, cleavage of different SNARE proteins disrupts the fusion step with the result that no neurotransmitter is released at the neuromuscular junction (B), Arnon et al., 2001.
While the binding of BoNT/A to SV2 was only recently discovered, the binding of BoNT/A to gangliosides is well documented. Gangliosides, complex sialylated glycosphingolipids present in lipid rafts, have long been known to play a role in the binding and action of many pathogens (Krivan et al., 1988; Schengrund, 2003; Varki and Varki, 2007, see also Tab. 1-1). More specifically, the preincubation of BoNT/A with the gangliosides GT1b and GD1a attenuated toxicity in mice (Simpson and Rapport, 1971) and treatment with neuraminidase to cleave terminal sialic acid residues from carbohydrate moieties resulted in the loss of the ability of the gangliosides to inhibit toxicity (Simpson and Rapport, 1971). Studies of N2a cells depleted of gangliosides by being grown in the presence of d,l-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol·HCl (PPMP), an inhibitor of glucosyl ceramide synthase, indicated that BoNT/A catalyzed cleavage of SNAP-25 decreased as the concentration of PPMP increased. Replenishment of the cells with GT1b restored activity of the toxin (Yowler et al., 2002). Similarly, growth of the toxin-resistant, ganglioside deficient cell line SK-N-SH in the presence of GT1b resulted in its becoming susceptible to BoNT/A as monitored by cleavage of SNAP-25. Over the past twenty years, studies of BoNT/A have repeatedly shown the importance of gangliosides, particularly trisialoganglioside GT1b, for the action of the toxin on its intracellular target, SNAP-25 (Montecucco et al., 1988; Schengrund et al., 1991; Rummel et al., 2004). Similar results were obtained in studies of the action of BoNT/A in mice engineered to lack complex gangliosides (GM1, GD1a, GD1b, GT1a, GT1b and GQ1b; Kitamura et al., 1999). When challenged with BoNT/A, the knockout mice were resistant to the activity of the toxin, whereas wild type mice were not.
**BoNT/A internalization, translocation, and SNAP-25 cleavage**

After binding to the neuronal membrane, BoNT/A is internalized into endosomes by an unknown mechanism. For the toxin to be active in the cytosol, it is necessary that the disulfide bond between the heavy and the light chain be reduced as the catalytic cleft of the light chain is masked by the heavy chain. It is believed that acidification of the endosomes is responsible for breaking the disulfide bond that holds the heavy and light chains together. Once inside the endosome, it is theorized that the drop in pH facilitates translocation of the toxin into the cytosol, where its substrate SNAP-25 is located. This possibility was tested by pretreating neuromuscular junctions with drugs that prevent acidification of the endosome (chloroquine, methylamine, bafilomycin). Each was found to attenuate activity of the toxin (Simpson, 2004). While the exact mechanism for translocation of BoNT/A remains unknown, the current model hypothesizes that the toxin inserts part of itself into the endosome and forms a channel through which the light chain can pass into the cytosol.

Once inside the neurons, BoNT/A proceeds to catalyze the cleavage of SNAP-25. SNAP-25 is a member of the SNARE complex, an association of proteins (SNAP-25, syntaxin and synaptobrevin/VAMP-2) that facilitates the docking and fusion of synaptic vesicles. For successful neurotransmitter release, the SNARE complex is essential. Therefore, cleavage of any of the SNARE complex proteins results in severe blockade of neurotransmitter release (Schiavo and van der Goot, 2001).
Lipid rafts and BoNT/A

Because TeNT and BoNT/A are both produced from bacteria in the Clostridium family, they share a sequence homology of ~65% and a sequence identity of ~35% (Lacy and Stevens, 1999) and consequently TeNT has often served as a model for subsequent studies on BoNT/A. In the case of TeNT, disruption of lipid rafts via cholesterol depletion resulted in inhibition of its ability to catalyze hydrolysis of the vesicle associated membrane protein, VAMP2 (Herreros et al., 2001; Munro et al., 2001). While transcytosis of BoNT/A across T-84 human epithelial cells was shown to be dependent on clathrin-mediated transcytosis (Maksymowych and Simpson, 2004), the mechanism of its binding to and internalization by neurons was unknown. The similarities between TeNT and BoNT/A as seen in their sequence homology and their interaction with gangliosides (Eidels et al., 1983; Montecucco et al., 1988; Schengrund et al., 1991; Kitamura et al., 1999; Yowler et al., 2002; Rummel et al., 2004), coupled with the evidence that gangliosides are enriched in lipid rafts (Prinetti et al., 2000; Vinson et al., 2003) led to the hypothesis that the ability of BoNT/A to inhibit neurotransmission might be dependent upon lipid rafts. In this work, the question of whether intact cholesterol-containing lipid rafts are necessary for BoNT/A to reach its site of action within cholinergic, murine, neuroblastoma N2a cells was addressed.
Materials and Methods

Materials

BoNT/A was kindly provided by Dr. B. R. DasGupta (University of Wisconsin, Madison, WI). Mouse N2a (cholinergic murine neuroblastoma) cells were obtained from the ATCC (Manassas, VA). SNAP-25 mouse monoclonal antibody (SMI 81) was purchased from Sternberger Monoclonals Inc. (Lutherville, MD); synaptotagmin mouse monoclonal antibody and VAMP-2 monoclonal antibody from Synaptic Systems (Goettingen Germany); flotillin-1 mouse antibody from BD Transduction Laboratories (San Jose, CA) and HRP conjugated goat anti-mouse IgG, methyl-β-cyclodextrin (MβCD), mevinolin, mevalonic acid, and filipin were from Sigma (St. Louis, MO); and anti-actin mouse monoclonal antibody from Abcam (Cambridge, MA). The Vybrant® Lipid Raft Labeling Kit, Alexa Fluor® 594-Transferrin, and Hoechst Dye 33342 was purchased from Molecular Probes (Eugene, OR). Wako Cholesterol E assay kit was bought from Wako Bioproducts (Richmond, VA).

Cell culture

Cells were seeded into either 25cm² tissue culture flasks or onto coverslips in 60mm tissue culture dishes at a cell density of $5 \times 10^5$ or into 75cm² tissue culture flasks at a cell density of $1.5 \times 10^6$. Mouse N2a neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn bovine
serum (NBS) and grown at 37°C in an atmosphere of 5% CO₂, 95% air, and 90% humidity and the media changed after 48h. At 96h, cells used to maintain the culture were split 1:10 and seeded into new tissue culture flasks.

**Cholesterol depletion/sequestration**

Conditions to reduce or sequester membrane cholesterol while maintaining cell viability were as follows. Cells were seeded at a density of $5 \times 10^5$ cells per 25cm² tissue culture flask and allowed to grow for 72h. The almost confluent N2a cells were then incubated with either MβCD (1.25, 5, or 10mM) or filipin (3 or 6 µg/ml) for 1 h in medium containing 10% lipoprotein deficient serum (LPDS). The cells were washed three times in phosphate buffered saline (PBS) and then incubated in medium containing LPDS, 5µM mevinolin, and either 1µM or 0µM mevalonic acid for either 12 or 24 h. Control cells (cells not exposed to MβCD, filipin, mevinolin, or mevalonic acid) were incubated in medium containing LPDS. After rinsing the cells three times in PBS, they were harvested by rapping into PBS and recovered by centrifugation at 250 × g for 5 min. The cells were resuspended in PBS and aliquots were analyzed for cell viability by trypan blue exclusion.

**Lipid raft isolation**

Lipid rafts were isolated using the procedure of Popik et al. 2002. Cells were washed three times with PBS prior to harvesting by rapping into PBS. Cells were
recovered by centrifugation at 250 x g for 5 min and resuspended in 1 ml TNE (25mM Tris-HCl, 150mM NaCl, 5mM EDTA, pH 7.5) containing either 0.1, 0.5, or 1% Triton x-100 supplemented with a 1:100 dilution of Halt Protease Inhibitors\textsuperscript{TM} (Pierce, Rockford, IL). After incubation at 4ºC for 30 min, cells were centrifuged at 4ºC for 1 min at 1000 x g, and 1ml of the postnuclear supernatant added to an equal volume of 80% (w/v) sucrose in TNE. This was overlaid successively with 5ml of 30% sucrose and 3ml of 5% sucrose, both in TNE. After centrifugation at 4ºC for 19 h at 38,000 RPM in a Beckman SW41Ti rotor, 1 ml fractions were collected from the top of the gradient. Proteins present in equal volumes of each fraction were separated by SDS-PAGE, transferred to a PVDF membrane and exposed to antibodies directed against flotillin-1 or synaptotagmin. After exposure to a HRP-conjugated secondary antibody, blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate\textsuperscript{TM} and exposed in the SynGene Gene Gnome (Frederick, MD).

**Disruption of lipid rafts**

Disruption of lipid rafts by cholesterol depletion and or sequestration was assayed by raft isolation and sucrose density centrifugation. Cells were treated for optimal conditions of cholesterol depletion/sequestration as follows. **For cholesterol depletion:** Cells were treated with either DMEM-LPDS alone, with DMEM-LPDS containing 1.25 mM MβCD for 1h, or DMEM-LPDS containing 1.25 mM MβCD for 1h followed by either a 12h or 24h incubation in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid. **For cholesterol sequestration:** Cells were treated with either DMEM-
LPDS alone, DMEM-LPDS containing 3µg/ml filipin for 1h, or DMEM-LPDS containing 3µg/ml filipin for 1h followed by either a 12h or 24h incubation in medium containing 10% LPDS, 5µM mevinolin and 1µM mevalonic acid. Cells were harvested and resuspended in TNE containing 1% Triton x-100, and lipid rafts isolated and location of flotillin determined as described above (Lipid raft isolation).

**Total cholesterol content and visualization of N2a surface cholesterol after treatment with MβCD**

After depletion of cholesterol by treatment of N2a cells with MβCD as described (Disruption of lipid rafts), total cell concentration of cholesterol was determined using the colorimetric Wako Cholesterol E Assay kit. After cholesterol depletion, cells were harvested into PBS (an aliquot was taken and cells counted) and recovered by centrifugation at 250 × g for 5min. Cells were lysed with cold PBS containing 1% (v/v) Triton X-100 and a 1:100 dilution of HALT protease inhibitor. Lysates were incubated on ice for 30min. An aliquot was taken to determine protein concentration using a modified Lowry colorimetric assay. For cholesterol determination, an aliquot was incubated with cholesterol oxidase (as well as peroxidase) that permit the colorimetric detection of hydrogen peroxide produced upon oxidation of cholesterol. Absorbance was read in a microplate reader at 600nm.

To visualize cell surface cholesterol after depletion with MβCD, cholesterol was depleted from N2a cells on glass coverslips as described above (Disruption of lipid rafts). Twelve or 24 h later, cells were washed three times in PBS cells and fixed by a 30
min incubation with 4% paraformaldehyde on ice, rinsed with PBS, and stained with 125µg/ml filipin for 5min in the dark. Cells were washed three times in PBS to rinse off excess filipin. Images were acquired (objective 100x/1.4) on a Nikon Optiphot-2 epifluorescence microscope equipped with a Retiga Exi Q Imaging camera, using the acquisition software QED InVivo™. All images were obtained using the same length of exposure.

**Lipid raft depletion assay**

Cholesterol was depleted from N2a cells grown on glass coverslips using MβCD as described above. Twelve or 24 h later, cells were washed three times in PBS, fixed in 4% paraformaldehyde, and labeled with cholera toxin binding subunit (CT-B) conjugated with Alexa Fluor 488 according to directions provided with the Molecular probes Vybrant® Lipid Raft Labeling Kit. Slides were visualized at room temperature. Images were acquired (objective 100x/1.4) on a Nikon Optiphot-2 epifluorescence microscope equipped with a Retiga Exi Q Imaging camera, using the acquisition software QED InVivo™. All images were obtained using the same length of exposure.

**Lipid raft disruption and clathrin-mediated endocytosis**

To determine whether treatment of N2a cells with either MβCD or filipin had an effect on internalization mediated by clathrin-coated pits, the uptake of labeled transferrin, (a marker for clathrin mediated endocytosis) was determined. Cells on
coverslips were treated for 1h with either MβCD (1.25mM) or filipin (3µg/ml) in DMEM-LPDS. Control cells were incubated in DMEM-LPDS alone for 1h, and washed three times in PBS prior to exposure to 50µg/ml Alexa Fluor® 594-Transferrin in PBS for 20 min at 4°C. After washing three times in PBS to remove unbound transferrin, cells were incubated for 15 min at 37°C. They were then rinsed and fixed in 4% paraformaldehyde. Slides were mounted and visualized at room temperature. Images were acquired (objective 60x/1.4) on a Nikon Optiphot-2 epifluorescence microscope equipped with a Retiga Exi Q Imaging camera, using the acquisition software QED InVivo™.

**Determination of BoNT/A and TeNT activity**

The activity of both BoNT/A and TeNT on N2a cells was determined. N2a cells were grown for 72h in regular media, prior to exposure to either BoNT/A (6nM) or TeNT (10 and 20nM) for 12h. Cells were harvested, recovered by centrifugation, and lysed in RIPA buffer. Forty µg of protein were resolved on a 5% stacking/13% resolving SDS-PAGE gel and transferred to PVDF. The PVDF membrane was probed using either an anti-SNAP-25 mouse monoclonal antibody (1:5000 dilution) that recognized both SNAP-25 and the large fragment of SNAP-25 generated by the action of BoNT/A, or an anti-VAMP2 mouse monoclonal antibody (1:5000). After washing to remove unbound antibody, blots were exposed to a HRP-conjugated goat anti-mouse secondary antibody. After washing, blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome.
To determine the effect of disruption of lipid rafts on BoNT/A activity, lipid rafts were disrupted as described previously and 6nM BoNT/A added after the 1h exposure to either MβCD or filipin. Cells were harvested after exposure to BoNT/A for 1, 12, or 24h. Control cells were treated in the same manner but were not exposed to MβCD, filipin, mevalonic acid, or mevinolin. Proteins in samples containing 40µg of total protein were separated by SDS-PAGE (5% stacking and 13% resolving), transferred to a PVDF membrane and intact and cleaved SNAP-25 visualized as described. Band density was ascertained using SynGene GeneTools software. Statistics were performed using the paired t test, and data was considered statistically significant when p ≤ 0.05. Multiple exposures were taken of each blot to ensure linearity, and all bands used for analysis were exposed at less than saturated levels.

**Disruption of clathrin-mediated endocytosis**

Two procedures were tested for their efficacy at disrupting clathrin mediated endocytosis. They were depletion of potassium from N2a cells (Hansen et al., 1993) and exposure to the drug amantadine (et al., 1982; Phonphok and Rosenthal, 1991; Van de Walle et al., 2001). For potassium depletion: cells on coverslips were incubated in potassium depletion buffer (0.14M NaCl, 20mM HEPES, 1mM CaCl₂, 1mM MgCl₂, glucose 1mg/ml, pH 7.4) for 30 min at 37°C, followed by hypotonic shock (potassium depletion buffer/ddH₂O 1:1) for 5 min at 37°C. The exposure to hypotonic shock allowed the cells to swell, altering the membrane so clathrin-mediated endocytosis could
not occur. Control cells were incubated in buffer containing potassium (0.14M NaCl, 20mM HEPES, 1mM CaCl₂, 1mM MgCl₂, glucose 1mg/ml, 10mM KCl, pH 7.4). For amantadine: cells on coverslips were incubated at 37°C in PBS containing 150µM amantadine for 1h. Control cells were incubated at the same temperature in PBS alone for 1h. To monitor disruption of clathrin-coated pits after treatment with either potassium depletion or amantadine, cells on coverslips were washed three times in PBS and then labeled with 50µg/ml Alexa Fluor® 594-Transferrin for 20 min at 4°C. After washing three times in PBS to remove unbound transferrin, cells were incubated for 15 min at 37°C. They were then rinsed and fixed in 4% paraformaldehyde. Slides were mounted and visualized at room temperature. Images were acquired (objective 60x/1.4) on a Nikon Optiphot-2 epifluorescence microscope equipped with a Retiga Exi Q Imaging camera, using the acquisition software QED InVivo™.

Effect of clathrin disruption on BoNT/A activity

Potassium-depleted N2a cells, prepared as above, were incubated with 12nM BoNT/A for 1 h at 37°C, washed three times with PBS, and then incubated in DMEM-NBS for 24 h. Cells were harvested and proteins in samples containing 40µg of total protein separated by SDS-PAGE (5% stacking and 13% resolving), transferred to a PVDF membrane and probed with the anti-SNAP-25 mouse monoclonal antibody (1:5000 dilution). After washing to remove unbound antibody, blots were exposed to a HRP-conjugated goat anti-mouse secondary antibody. After washing, blots were
developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome.

**Results**

A number of pathogens have been shown to require lipid rafts in order to bind to and be internalized by cells. More specifically, TeNT toxin needs intact lipid rafts to reach its intracellular target, VAMP-2. Given the similarities between TeNT and BoNT/A, we hypothesized that BoNT/A would also require intact lipid rafts. Because cholesterol plays an integral part in the cell membrane, altering the amount or distribution of cholesterol in the plasma membrane for extended periods of time could be injurious to the cell. However, previous experiments in our lab indicated that little SNAP-25 cleavage by BoNT/A is seen in less than 12h. Therefore, we sought to disrupt lipid rafts by acute cholesterol depletion or its sequestration, and to keep them disrupted by inhibiting cholesterol synthesis for a period of time by using concentrations of inhibitor low enough to maintain cell viability. To prevent the cells from obtaining cholesterol from LDLs in serum added to the medium, lipoprotein-deficient medium was used. Incubation of N2a cells in DMEM-LPDS had virtually no effect on cell viability compared to cells incubated in DMEM containing NBS (Fig. 2-3). To inhibit the cells from synthesizing cholesterol *de novo*, the HMG-CoA reductase inhibitor mevinolin was used at a concentration previously determined in this lab to be nontoxic. However as the inhibition of cholesterol synthesis also prevented the synthesis of essential isoprenoids, treatment with mevinolin alone for either 12 or 24h resulted in a decrease in cell viability.
This effect was reversed when cells were incubated with mevalonic acid plus mevinolin (Fig. 2-3). Mevalonic acid is a precursor for synthesis of the aforementioned isoprenoids.

Figure 2-3: Growth of N2a cells under various conditions

N2a cells were seeded into 25cm² tissue culture flasks at a density of $5 \times 10^5$ cells per flask and grown for 72h in NBS-containing medium. Medium was removed and replaced with either DMEM-NBS, DMEM-LPDS, or DMEM-LPDS containing 5µM mevinolin (+mev), or DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (+ma). At 12 and 24h time intervals, cells were harvested and viable cells counted in the presence of trypan blue. Values shown are the average of $n=3$. Error bars represent the standard error of the mean, * indicates a statistical significance (p<0.05).

Because disruption of lipid rafts by either MβCD or filipin could be toxic to N2a cells, various concentrations of both drugs were used and cell number determined after a one hour exposure (Fig. 2-4 A and B). In the case of MβCD (Fig. 2-4 A), 1.25mM proved to be least toxic to the cells. 5mM and 10mM resulted in decreased cell viability.
Two different concentrations of filipin were tested (Fig. 2-4 B). 3µg/ml has essentially no effect on cell number while 6µg/ml caused it to decrease.

Fig. 2-4

Figure 2-4: Cell number after a 1h exposure of N2a cells to either MβCD or filipin.

N2a cells were seeded into 25cm² tissue culture flasks at a density of 5 × 10⁵ cells per flask and grown in DMEM-NBS for 72h. In A, cells were treated with various concentrations of MβCD for 1h in DMEM-LPDS, while control cells were incubated in DMEM-LPDS. For B, cells were treated with various concentrations of filipin for 1h in DMEM-LPDS. Cells were harvested and viable cells counted in the presence of trypan blue. Values shown are the average of n=3. Error bars represent the standard error of the mean, * indicates a statistical significance (p<0.05).
To determine whether cells remained viable for a period of time after a 1h exposure to either MβCD or filipin, cells were incubated in DMEM-LPDS containing 1µM mevalonic acid and 5µM mevinolin, for either 12 or 24h (Fig. 2-5 A and B). At these concentrations, cells treated for cholesterol depletion (A) and cholesterol sequestration (B) remained viable and continued to proliferate at a rate comparable to that of control cells, which were incubated in DMEM-LPDS.

Fig. 2-5

![Figure 2-5](image)

Figure 2-5: Growth of cells after treatment to deplete or sequester cholesterol with MβCD or filipin, in conjunction with mevinolin and mevalonic acid.

N2a cells were seeded into 25cm² tissue culture flasks at a density of $5 \times 10^5$ cells per flask and grown DMEM-NBS for 72h. In A, medium was removed and replaced with DMEM-LPDS for either 1, 12 or 24h (C1, C12, C24) or treated for 1h with 1.25mM MβCD (T1), or treated for 1h with 1.25 MβCD followed by incubation in LPDS-containing Medium supplemented with 1µM mevalonic acid and 5µM mevinolin for either 12 or 24h (T12, T24). For B, cells were seeded as described above and then
medium was removed and replaced with DMEM-LPDS for either 1, 12 or 24h (C1, C12, C24) or treated for 1h with 3µg/ml filipin (T1), or treated for 1h with 3µg/ml filipin followed by incubation in LPDS-containing Medium supplemented with 1µM mevalonic acid and 5µM mevinolin for either 12 or 24h (T12, T24). Cells were harvested and viable, trypan blue negative cells counted. Values shown are the average of \( n=3 \). Error bars represent the standard error of the mean.

The detergent to lipid ratio affects solubility of lipid rafts (London and Brown, 2000; Edidin, 2003), and high detergent-to-lipid ratios can actually result in their solubilization (Chamberlain, 2004). For this reason, plus the fact that different cell types can have different lipid compositions, studies were done to determine which concentration of Triton X-100 in TNE would be most effective for the isolation of lipid rafts from N2a cells. Efficacy was determined by monitoring the gradient fractions in which the lipid raft marker flotillin (Bickel et al., 1997) was recovered. While the SNARE (soluble NSF attachment protein receptor) protein synaptotagmin has been shown to associate with lipid rafts as well as the soluble fractions isolated from rat brain (Gil et al., 2005) in PC12 cells it was found predominantly in the soluble fraction (Chamberlain et al., 2001; Cabrera-Poch et al., 2004). Based on the observations made in PC12 cells we determined whether synaptotagmin could be used as a soluble marker for N2a cells under conditions used to isolate lipid rafts. Analysis of Western blots indicated that use of a 0.1% concentration of Triton X-100 resulted in both synaptotagmin and flotillin being recovered in the buoyant, lipid raft fractions, as well as in the denser, soluble fractions (Fig. 2-6 A). Use of 0.5% Triton X-100 (Fig. 2-6 B), resulted in recovery of more synaptotagmin in the soluble fractions, and more flotillin in the buoyant raft fractions. TNE containing 1% Triton X-100 was found to give the best separation of lipid rafts as defined by flotillin distribution in buoyant raft fractions and by
synaptotagmin distribution in the soluble ones (Fig. 2-6 C). Therefore, 1% Triton X-100 in TNE was used for isolation of lipid rafts.

Figure 2-6: Effect of the concentration of Triton X-100 on the distribution of synaptotagmin and flotillin after centrifugation on a sucrose density gradient.

Cell lysates were extracted using either 0.1% (A), 0.5% (B), or 1% Triton X-100 in TNE (C). After sucrose density gradient centrifugation, proteins in aliquots of each fraction were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were probed using either a mouse anti-synaptotagmin or a mouse anti-flotillin monoclonal antibody, followed by a HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome. Fraction 1 = top of the sucrose gradient, fraction 10 = bottom of the sucrose gradient.
Cholesterol depletion disrupts lipid rafts, causing a shift of lipid raft markers from the buoyant, low density fractions on a sucrose gradient to the soluble, high density fractions. To reduce reformation of cholesterol-containing lipid rafts, the HMG-CoA reductase inhibitor mevinolin was added to the LPDS-containing medium after treatment of cells with MβCD. Mevinolin inhibits the rate-limiting step of cholesterol biosynthesis, inhibiting replenishment of plasma membrane cholesterol. However complete inhibition of HMG-CoA reductase leads to cell death. Therefore, a small amount of mevalonic acid (1µM), the product of HMG-CoA reductase activity, was added to the medium. Control cells treated in the same manner were not exposed to MβCD, mevinolin, or mevalonic acid. It can be seen that when lipid rafts were extracted from control cells, flotillin was found in the low density fractions of the gradient (Fig. 2-7, A). However after a 1h incubation of cells with MβCD prior to extraction of lipid rafts, the location of the protein shifted from the less dense fractions to the more dense ones (Fig. 2-7 B), indicating that lipid rafts were disrupted. After incubation of cholesterol depleted cells for 12h with mevinolin and mevalonic acid, flotillin was still recovered in the more dense, soluble fractions (Fig. 2-7 C), whereas after 24 h it was recovered in the less dense lipid raft fractions (Fig. 2-7 D). Therefore, while lipid rafts were disrupted for at least 12h they reformed between 12 and 24h.
Figure 2-7: Localization of the lipid raft marker flotillin after MβCD-induced disruption of lipid rafts and sucrose density gradient centrifugation.

Cells were treated with either DMEM-LPDS (A), with MβCD for 1h (B), or MβCD for 1h followed by either a 12 h (C) or 24 h (D) incubation in DMEM-LPDS, 5µM mevinolin and 1µM mevalonic acid. Cell extracts were prepared in 1% Triton X-100 in TNE. Lipid rafts were isolated by sucrose density gradient centrifugation. Protein distribution in fractions was detected by Western blot analysis. Blots were probed with an anti-flotillin antibody followed by a HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome. Fraction 1 = top of the sucrose gradient, fraction 10 = bottom of the sucrose gradient. Figures are from four different Western blots.
Filipin is an antibiotic that binds to cholesterol forming ultrastructural aggregates (Kitajima et al., 1976; Robinson, et al., 1980; McGookey et al., 1983; Bolard, 1986; Castanho et al., 1992) resulting in disruption of lipid rafts (Schnitzer et al., 1994). Cells were treated with filipin and then incubated for either 12 or 24 h in DMEM-LPDS containing mevinolin and mevalonic acid. Control cells, treated in the same manner, were not exposed to filipin, mevinolin, or mevalonic acid. It can be seen that rafts were intact in control cells since the lipid raft marker, flotillin, was recovered primarily in the buoyant fractions (Fig. 2-8, A). One hour after incubation with filipin, flotillin was recovered in the denser fractions (Fig. 2-8 B). At 12 and 24 h after exposure to filipin, the lipid raft marker was located almost completely in the denser fractions (Fig. 2-8 C & D; lanes 9,10) indicating that the lipid rafts remained disrupted.
Figure 2-8: Localization of the lipid raft marker flotillin after filipin-induced disruption of lipid rafts and sucrose density gradient centrifugation.

Cells were treated with either DMEM-LPDS (A), with filipin for 1h (B), or filipin for 1h followed by either a 12 h (C) or 24 h (D) incubation in DMEM-LPDS, 5µM mevinolin and 1µM mevalonic acid. Cell extracts were prepared in 1% Triton X-100 in TNE, and lipid rafts isolated by sucrose density gradient centrifugation. Protein distribution in fractions was detected by Western blot analysis. Blots were probed with an anti-flotillin antibody followed by a HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome. Fraction 1 = top of the sucrose gradient, fraction 10 = bottom of the sucrose gradient. Figures are from four different Western blots.
To determine whether inhibition of cholesterol synthesis by mevinolin and the addition of mevalonic acid affected the integrity of lipid rafts in the absence of MβCD or filipin, cells were treated with DMEM-LPDS containing both mevinolin and mevalonic acid for either 12 or 24h (Fig. 2-9, B and C, respectively), while control cells were incubated in DMEM-LPDS (Fig. 2-9 A). Lipid rafts were then isolated and fractions probed for the raft marker flotillin. Flotillin was present in the less dense lipid raft fractions, indicating that treatment with mevinolin and mevalonic acid alone does not result in lipid raft disruption.

Fig. 2-9

![Figure 2-9](image_url)

**Figure 2-9: Effects of mevinolin and mevalonic acid on lipid rafts.**

Cells were treated with either DMEM-LPDS (A), or incubated in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid for either 12 or 24h (B and C, respectively). Cell extracts were prepared in 1% Triton X-100 in TNE, and lipid rafts isolated by sucrose density gradient centrifugation. Protein distribution in fractions was detected by Western blot analysis. Blots were probed with an anti-flotillin antibody followed by a HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome.
The B subunit of cholera toxin (CTxB), pentavalently binds to the oligosaccharide portion of five molecules of the monosialoganglioside GM1. The affinity of CTxB binding to GM1 is significantly greater when multiple oligosaccharides of GM1 are present (Thompson et al., 1997). When lipid rafts were disrupted by cholesterol depletion the ganglioside GM1 was no longer recovered in the less dense lipid raft fractions but in the more dense gradient fractions (Popik et al., 2002), indicating that the gangliosides were no longer concentrated in lipid microdomains. Therefore the binding of fluorescently-labeled CTxB to GM1 can be used to visualize lipid rafts due to the enrichment of GM1 in them. Use of MβCD to deplete cholesterol was found to alter association of CTxB with N2a cell membranes (Fig. 2-10). It can be seen that exposure of cells to 1.25mM MβCD for one h markedly diminished fluorescence (Fig. 2-10 B), indicating the loss of GM1 clusters needed for CTxB binding. Twelve hours later a marked reduction in fluorescence was still seen (Fig. 2-10 C), however, after 24 h (Fig. 2-10 D), fluorescence reappeared, indicating re-establishment of lipid rafts.
Figure 2-10: Fluorescent CTxB staining of the lipid raft marker ganglioside GM1.

N2a cells were incubated in DMEM-LPDS containing 1.25 mM MβCD for 1 h (B) followed by incubation DMEM-LPDS containing 5 μM mevinolin and 1 μM mevalonic acid for either 12 h (C) or 24 h (D). Control cells (A) were incubated in DMEM-LPDS.
Total cell cholesterol content of control cells and cells depleted of cholesterol by MβCD were assayed using the Wako Cholesterol E assay kit and cholesterol content of control cells was normalized to 100%.

Fig. 2-11

N2a cells have less cholesterol 1, 12, and 24h after treatment with MβCD. N2a cells were incubated in DMEM-LPDS containing 1.25mM MβCD for 1 h followed by incubation in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid for either 12 h or 24 h. Control cells (ctrl) were incubated in DMEM-LPDS.

Filipin binds to cholesterol on the outer leaflet of the plasma membrane, and fluoresces similarly to FITC. Therefore for cells treated with MβCD, cell surface cholesterol was visualized using filipin. A reduction in cholesterol content was indicated by a decrease in fluorescence of MβCD-treated cells compared to controls (Fig. 2-12). It can be seen that exposure of cells to 1.25mM MβCD for one h resulted in somewhat diminished fluorescence (Fig. 2-12 B) indicating loss of cholesterol on the cell surface.
Twelve hours later a marked reduction in fluorescence was still seen (Fig. 2-12 C), however, after 24 h (Fig. 2-12 D), fluorescence reappeared, indicating re-establishment of lipid rafts.

**Fig. 2-12**

Figure 2-12: Filipin stained N2a cells have less cholesterol 1 and 12h after treatment.

N2a cells were incubated in DMEM-LPDS containing 1.25mM MβCD for 1 h (B) followed by incubation in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid for either 12 h (C) or 24 h (D). Control cells (A) were incubated in DMEM-LPDS.
As N2a cells contain the substrate for TeNT (VAMP-2, Fig. 2-13, lane 1), the ability of TeNT to enter N2a cells and act on its substrate after 12h of exposure to toxin was tested. Different concentrations of TeNT were used to determine the amount needed to obtain significant cleavage of VAMP-2. Western blot analysis indicated that no discernible cleavage of VAMP-2 occurred when cells were exposed for 12h at 37°C to either 10 or 20nM TeNT (Fig. 2-14 lanes 2 and 3, respectively). Therefore, failure of TeNT to catalyze cleavage of VAMP-2 could not be used as an indicator for disruption of lipid rafts.

Fig. 2-13

Figure 2-13: Western blot analysis of SNAP-25 and VAMP.

Proteins in an aliquot of cell lysate containing 40µg of protein from N2a cells cultured in DMEM-NBS. Proteins were separated by SDS-PAGE under reducing conditions on a 5% stacking/13% running gel and transferred to PVDF. The blot was probed with anti-SNAP-25 or an anti-VAMP antibody followed by HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome.
Figure 2-14: Incubation of N2a cells with TeNT did not result in cleavage of VAMP-2.

Proteins in an aliquot of cell lysate containing 20µg of protein from N2a cells cultured in either DMEM-NBS (1) or DMEM-NBS plus 10nM or 20nM TeNT (2 and 3, respectively) for 12h. Proteins were separated by SDS-PAGE under reducing conditions on a 5% stacking/13% running gel and transferred to PVDF. The blot was probed with an anti-VAMP2 antibody followed by HRP-conjugated goat anti-mouse antibody. Actin, used as a loading control, was probed with an anti-actin antibody followed by HRP-conjugated goat anti-mouse antibody. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposed in the SynGene Gene Gnome.

Effect of lipid raft disruption on the ability of BoNT/A to act on SNAP-25 in N2a cells

To determine the role of lipid rafts on the activity of BoNT/A, lipid rafts were disrupted and the ability of BoNT/A to cleave SNAP-25 ascertained. MβCD or filipin was used to acutely deplete cholesterol, prior to a 12 or 24 h incubation of the cells with mevinolin, mevalonic acid, and BoNT/A. Only intact SNAP-25 was present in control cells (no BoNT/A, MβCD, filipin, mevinolin or mevalonic acid), Fig. 2-15 lanes 3,6. Incubation of control cells with BoNT/A for 12 h resulted in cleavage of 11± 6% of the SNAP-25 (n=3), while similar treatment of MβCD-treated cells resulted in cleavage of
50±13% of SNAP-25 (n=3), a value significantly greater (p < 0.05) than that for control cells (Fig. 2-15, compare lanes 1,2). These results were mirrored in cells that were treated with filipin to disrupt the lipid rafts (Fig. 2-16). In two separate experiments a 12 h exposure of control cells to BoNT/A resulted in cleavage of 18 ± 11% of the SNAP-25 (Fig. 2-16, lane 2), while in three experiments BoNT/A catalyzed the cleavage of 61 ± 6% of the SNAP-25 present in filipin-treated cells (lane 3). Again the increase in activity was statistically significant (p < 0.05). At 24 h, regardless of lipid raft integrity, SNAP-25 cleavage was still more in the filipin-treated cells than controls (compare Fig. 2-15, lanes 4 to 5; Fig. 2-16, lanes 5 to 6).
Figure 2-15: The effect of MβCD disruption of lipid rafts on BoNT/A catalyzed cleavage of SNAP-25.

Treated cells were incubated for 1h with 1.25mM MβCD, washed, and then incubated for either 12 (1A) or 24 h (1B) in DMEM-LPDS containing 5µM mevinolin, 1µM mevalonic acid, and 6nM BoNT/A. Cells were harvested in RIPA buffer. Forty micrograms of protein in each whole cell extract were separated by SDS-PAGE. Blots were probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Lanes 3 and 6 represent untreated, control cells (-/-), lanes 1 and 4 show SNAP-25 in untreated cells incubated with BoNT/A (-/+), lanes 2 and 5, SNAP-25 from MβCD treated cells that were exposed to BoNT/A (+/+). (2) Values shown give the average % of SNAP-25 cleavage by BoNT/A acting on control (ctrl) and cholesterol-depleted (chol. depleted) cells for 12h. Bars indicate the standard error of the mean (n=3).
Figure 2-16: The effect of filipin disruption of lipid rafts on BoNT/A induced cleavage of SNAP-25.

Treated cells were incubated for 1h with 3µg/ml filipin, washed, and then incubated for either 12 (IA) or 24 h (IB) in DMEM-LPDS containing 5µM mevinolin, 1µM mevalonic acid, and 6nM BoNT/A. Cells were harvested in RIPA buffer. Forty micrograms of protein in each whole cell extract were separated by SDS-PAGE. Blots were probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Lanes 1 and 4 represent untreated, control cells (-/-), lanes 2 and 5 show SNAP-25 in untreated cells incubated with BoNT/A (-/+), lanes 3 and 6, SNAP-25 from MβCD treated cells that were exposed to BoNT/A (+/+). (2) Values shown give the average % of SNAP-25 cleavage by BoNT/A acting on control (ctrl) and cholesterol-depleted (chol. depleted) cells for 12h. Bars indicate the standard error of the mean; ctrl (n=2), chol. depleted (n=3).
Clathrin mediated endocytosis can be disrupted by either amantadine, a cationic amphiphilic drug (Schlegel et al., 1982; Phonphok and Rosenthal, 1991; Van de Walle et al., 2001), or by the effect of potassium disruption and hypotonic shock (Hansen et al., 1993). To visualize disruption of clathrin mediated endocytosis, clathrin-dependent internalization of transferrin was ascertained after cells were treated with either amantadine or potassium depletion buffer followed by hypotonic shock. In regards to amantadine, it can be seen that transferrin was successfully internalized by control cells and also appears to be internalized by cells treated with amantadine, Fig. 2-17. Therefore, the concentration of amantadine used was ineffective at disrupting clathrin mediated endocytosis. In contrast, cells treated with potassium depletion buffer followed by hypotonic shock did not take up transferrin readily, as most of it remained on the plasma membrane. Again, it can be seen that the transferrin was successfully internalized by control cells incubated in buffer containing potassium, Fig. 2-18.
Figure 2-17: Effect of amantadine on uptake of the clathrin-dependent marker transferrin in N2 cells.

N2a cells were seeded onto coverslips in 60mm tissue culture dishes at a density of $5 \times 10^5$ cells/dish and grown for 72h in NBS. Medium was removed and replaced with DMEM-NBS (ctrl) or DMEM-NBS supplemented with 150µM amantadine. One h later, cells were stained with tfr as described (Disruption of clathrin-mediated endocytosis).
Figure 2-18: Effect of potassium depletion on uptake of the clathrin-dependent marker transferrin by N2a cells.

N2a cells were seeded onto coverslips in 60mm tissue culture dishes at a density of $5 \times 10^5$ cells/dish and grown for 72h in NBS. Cells were incubated in buffer containing potassium (A-C) or in potassium depletion buffer for 30 min at 37 °C, followed by hypotonic shock for 5 min at 37 °C (D-E). Panels (B) and (E) represent Hoechst staining of nuclei in control and clathrin-disrupted cells, respectively. Panels (C) and (F) represent merged images of A and B and D and E, respectively.
To determine whether clathrin disruption prevented BoNT/A from reaching its intracellular target, cells were treated with potassium depletion buffer, followed by hypotonic shock. Because clathrin disruption by potassium depletion cannot be maintained for extended periods of time, the cells were exposed to BoNT/A for only 1 h, rinsed, and incubated in medium for 24 h to allow bound toxin to be internalized and act on SNAP-25. When clathrin-disrupted cells were incubated in the presence of BoNT/A, no detectable level of SNAP-25 cleavage was observed. While activity was low, BoNT/A was able to act on SNAP-25 present in control cells (Fig. 2-19, compare lanes 3 and 4). The amount of SNAP-25 cleavage in these cells was significantly less than seen in control cells exposed to medium containing BoNT/A for 24 h (Fig. 2-15 lane 4, Fig. 2-16 lane 5). The difference presumably reflects the difference in the length of time each was exposed to BoNT/A.
Figure 2-19: Effect of potassium depletion/hypotonic shock disruption of clathrin on BoNT/A-induced cleavage of SNAP-25.

Treated cells were incubated for 30 min with potassium depleted buffer, 5 min with hypotonic buffer, washed, and then incubated for 1 h in the presence (4) or absence (2) of 12 nM BoNT/A. Control cells were incubated in buffer containing potassium, followed by a 1 h incubation in the presence (3) or absence (1) of 12 nM BoNT/A. After the 1 h incubation with or without BoNT/A, cells were incubated for 24 h in DMEM-NBS. Cells were harvested in RIPA buffer. Forty micrograms of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on a 5% stacking, 13% running gel. Following transfer to a PVDF membrane, blots were probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate™ and exposure in the SynGene Gene Genome.

To evaluate the possibility that lipid raft disruption by either cholesterol depletion or cholesterol sequestration might affect clathrin mediated endocytosis, N2a cells were treated with either MβCD or filipin for one hour. Control cells were treated with DMEM-LPDS. The uptake of fluorescently labeled transferrin was then assayed (Fig. 2-20). Fluorescence of treated cells (Fig. 2-20 B and C) was similar to that of controls (Fig. 2-20 A) indicating that disruption of lipid rafts had no apparent effect on transferrin internalization.
Figure 2-20: Effect of disruption of lipid rafts on transferrin uptake by N2a cells.

N2a cells were seeded onto coverslips in 60mm tissue culture dishes at a density of $5 \times 10^5$ cells/dish and grown for 72h in NBS. Cells were then incubated in DMEM-LPDS (A) or in DMEM-LPDS containing either 1.25 MβCD or 3 µg/ml filipin for 1 h (B and C, respectively). Cells were stained with tfr as described (Disruption of clathrin-mediated endocytosis).
Discussion

Forty-six years after botulinum toxin was labeled as the “most poisonous poison” (Lamanna, 1959), the molecules and mechanisms required for the binding and internalization of BoNT/A are still being identified. Due to the extreme potency of BoNT/A, it is considered a potential biowarfare agent (Greenfield et al., 2002; Ting and Freiman, 2004). Yet despite its threat as a biological agent, BoNT/A is increasingly used in the clinical setting to treat a variety of disorders. Therefore, a more thorough understanding of how BoNT/A actually enters the cell and reaches its SNARE substrate could facilitate development of inhibitors that would restrict its deadly effects, or of compounds to improve its efficacy as a therapeutic agent. In this work, the internalization pathways of lipid rafts and clathrin-dependent endocytosis in N2a cells were disrupted by depletion of cholesterol or potassium, respectively. While MβCD can be used to disrupt lipid rafts, the extraction of cholesterol from the plasma membranes of cells can potentially have multiple cellular effects. To confirm that any changes in BoNT/A activity on MβCD-treated cells were due to raft disruption and not an ancillary cellular effect, replicate experiments were done using filipin. MβCD and filipin acutely alter cholesterol distribution within the plasma membrane, MβCD by complexing with it and extracting the cholesterol from the membrane (Neufeld et al., 1996); filipin by forming ultrastructural aggregates with it (Kitajima et al., 1976; Robinson, et al., 1980; McGookey et al., 1983; Bolard, 1986; Castanho et al., 1992). Both drugs have been shown to alter lipid raft formation (Keller and Simons, 1998). Previous results from our laboratory indicated that the effect of BoNT/A on N2a cells could be effectively
measured when cells were exposed to BoNT/A for several hours. Therefore to prevent the cells from synthesizing cholesterol de novo which might result in reformation of the lipid rafts, the HMG-CoA reductase inhibitor mevinolin was added to DMEM-LPDS in which N2a cells were maintained. Lipoprotein deficient serum was used to eliminate cellular uptake of cholesterol-containing lipoproteins. Because many precursors of cholesterol synthesis are utilized for the synthesis of other cell components (such as dolichol, ubiquinone, and prenylated proteins) essential for cell survival, mevalonic acid (MA) was added to the medium to obtain a balance between maintenance of disrupted lipid rafts and cell viability. Inhibition of HMG-CoA reductase in conjunction with mevalonic acid has been shown to result in minimal cholesterol synthesis, while allowing the cells to synthesize essential isoprenoids (Cole et al., 2005). Treatment with MβCD resulted in a reduction of total cholesterol. Because of filipin’s intrinsic property of fluorescing in the range of FITC, filipin was used to visualize cholesterol on the plasma membrane after depletion with MβCD. The observation of a decrease in filipin fluorescence 1, 12 and 24h after cholesterol depletion confirmed the decrease in total cellular cholesterol. The cumulative effect of treating N2a cells for 1 h with either MβCD or filipin, followed by incubation in DMEM-LPDS supplemented with mevinolin and mevalonic acid, was disruption of cholesterol-dependent lipid rafts for at least 12 h. The results obtained by allowing fluorescently-labeled cholera toxin B subunit to bind cell surface GM1 provided a visual confirmation that rafts remained disrupted by MβCD for 12 h.
Tetanus toxin binds to the ganglioside GT1b (Fishman, 1982) which is found in lipid rafts (Prinetti et al., 2000; Vinson et al., 2003). We decided to monitor the ability of TeNT to catalyze cleavage of VAMP-2 to confirm disruption of lipid rafts based on the observation (Herreros et al. 2001) that cholesterol depletion blocked the intracellular activity of TeNT. It was anticipated that if TeNT was not able to enter cholesterol depleted cells, then little to no cleavage of VAMP-2 would be observed, while significant cleavage would be seen in control cells exposed to TeNT. The results indicated that concentrations of TeNT comparable to those found to catalyze cleavage in PC12 cells (Kasai et al., 1999) had no effect on VAMP-2 in N2a cells. Kitamura et al. (2005) found that when knockout mice lacking b-series gangliosides (GD3, GD2, GD1b, GT1b and GQ1b) were challenged with TeNT it had little to no affect. This led them to conclude that b-series gangliosides are essential for toxin activity. HPTLC analysis of gangliosides present in N2a cells indicated that little, if any b-series gangliosides were present (Yowler at al., 2002). This could account for the resistance of N2a cells to TeNT.

The finding that treatment of N2a cells with either MβCD or filipin did not protect the cells from the action of BoNT/A indicates that cholesterol-dependent lipid rafts are not essential for the toxin to reach its intracellular target. This result was not expected since tetanus toxin, also a clostridial neurotoxin, was found to require lipid rafts in order to act upon VAMP-2 in its target cells (Herreros et al., 2001; Munro et al., 2001). It is of interest that BoNT/A contains only one ganglioside binding site its the heavy chain (Rummel et al., 2004), unlike the lipid raft binding toxins TeNT or CTX that contain two and five, respectively (Bakry et al., 1991; Merritt et al., 1994). As lipid rafts
are commonly thought of as concentrated platforms of receptors for binding and signaling, it is possible that pathogens lacking multiple binding sites may not benefit from the clustering of receptors.

Perhaps even more interesting was the observation that BoNT/A activity actually increased when lipid rafts were disrupted. Despite the reformation of lipid rafts seen 24h after treatment with MβCD, SNAP-25 cleavage was still greater in MβCD treated cells than controls. This result can be accounted for by an increased amount of BoNT/A reaching its intracellular target while lipid rafts were disrupted. Once the BoNT/A is internalized, reformation of lipid rafts would not be expected to affect its ability to cleave SNAP-25, as the toxin would already be inside the cell where it could continue to act. The increase in BoNT/A activity observed when lipid rafts were disrupted for 12 h is similar to results obtained in studies of the effect of filipin-induced disruption of lipid rafts on the activity of diphtheria toxin (Orlandi and Fishman, 1998). Diphtheria toxin was found to require a clathrin-dependent pathway for internalization (Moya et al., 1985; Orlandi and Fishman, 1998; Beaumelle, 1992). Recently, the transcytosis of BoNT/A across T-84 human epithelial cells was shown to be dependent on clathrin (Maksymowych and Simpson, 2004). Potassium depletion in combination with hypotonic shock has been shown to disrupt the formation of clathrin-coated pits and inhibit clathrin-mediated endocytosis (Carpentier et al., 1989). To determine whether BoNT/A is internalized by N2a cells via a clathrin-dependent mechanism, cells were depleted of intracellular potassium and subjected to hypotonic shock. Inhibition of clathrin-dependent endocytosis resulted in no discernible cleavage of SNAP-25, while a
limited amount of cleavage was noted with control cells. The small amount of cleavage seen with control cells probably reflects the one h exposure to BoNT/A because: 1) in previous studies (Schengrund et al., 1991) it was shown that a 2 h incubation was needed for optimal adherence of BoNT/A to GT1b immobilized on plastic, 2) subsequent studies indicated that a 2 h incubation of BoNT/A with GT1b was required in order to see a change in BoNT/A conformation using circular dichroism (Yowler et al., 2004), 3) results of in vivo studies in which BoNT/A was injected into the gastrocnemius muscle and the appearance of cleaved SNAP-25 in nerve endings innervating the muscle was monitored over time indicated that no cleavage was seen in less than 24h (Jurasinski et al., 2001). Evidence indicates that SV2, the protein recently identified as the high affinity receptor for BoNT/A, is not localized to lipid rafts because 1) SV2 in rat brain synaptic vesicles was found to be in detergent soluble complexes (Bennett et al., 1992), and 2) when lipid rafts were isolated from rat brain synaptosomes, almost all of the SV2 is was recovered in the more dense, soluble fractions after sucrose density gradient centrifugation (Gil et al., 2005). 3) Cholesterol depletion of hippocampal neurons did not affect the synaptic location of fluorescently labeled SV2 indicating that it was not sensitive to lipid raft disruption (Fortin et al., 2004), furthermore synaptic vesicles, in which SV2 is located, are recycled by clathrin mediated endocytosis (Brodin et al., 2000; Slepnev and De Camili, 2000; Jarousse and Kelly, 2001). Therefore, failure to detect cleaved SNAP-25 in clathrin-disrupted N2a cells supports the conclusion that uptake of BoNT/A after it binds to SV2 is probably clathrin-dependent.
There are conflicting reports that treatment of cells with cholesterol depleting drugs such as MβCD to disrupt lipid rafts also interferes with clathrin mediated endocytosis. For example, treatment of HEp-2 cells with various cyclodextrins, including 10mM MβCD for one hour, resulted in reduction of the internalization of clathrin-dependent $^{125}\text{I}$-labeled transferrin (Rodal et al., 1999). However, spinal cord cells assessed for $^{125}\text{I}$-labeled transferrin uptake after treatment with 4.5 mM MβCD for one hour showed no significant difference from untreated, control cells (Herreros et al., 2001). It is possible that the discrepancy in results reflects the different concentrations of MβCD used, and potentially varying degrees of cholesterol extracted. Here we report that treatment of N2a cells with either 1.25mM MβCD or 3µg/ml filipin did not appear to prevent internalization of fluorescently labeled transferrin, suggesting that cholesterol depletion or sequestration did not affect clathrin mediated endocytosis.

The increase in BoNT/A activity on cells with disrupted lipid rafts was an unexpected result. Because SV2 is a component of synaptic vesicles, fusion of these vesicles results in association of SV2 with the plasma membrane during exocytosis and exposure of luminal portions of synaptic vesicle proteins on its outer surface. Previous models regarding regulation of neuronal exocytosis indicated that lipid rafts were essential. However, this concept has been challenged by the hypothesis that rafts may be negative regulators of exocytosis (Salaun et al., 2005). The disruption of lipid rafts and possible increase in exocytosis may increase the amount of SV2 on the plasma membrane, thereby enhancing uptake of BoNT/A and resulting in increased cleavage of SNAP-25. Because cholesterol is a major component of lipid rafts, its depletion may also
cause an increase in lateral mobility within the outer surface of the plasma membrane thereby allowing BoNT/A greater access to SV2. Another possible explanation for the increase in BoNT/A activity is that SNAP-25 is more accessible to BoNT/A when lipid rafts are disrupted. Evidence shows that disruption of lipid rafts by cholesterol depletion can alter the location of SNAP-25 (Chamberlain et al. 2001; Xia et al. 2004; Gil et al. 2005). It was reported that treatment of cells with either the cholesterol sequestering drug saponin or MβCD resulted in SNAP-25 moving from the less dense, lipid raft location upon sucrose density gradient centrifugation to the denser, soluble fractions. It is possible that the change of environment for SNAP-25 makes it more susceptible to the action of BoNT/A. However, it is not known whether the change in SNAP-25 localization results in an increased physiological window in which SNAP-25 is in a proper configuration for cleavage by BoNT/A.

The requirement of gangliosides for BoNT/A to act on cell-associated SNAP-25, the localization of gangliosides in lipid rafts, and the involvement of lipid rafts in the binding of tetanus (another clostridial toxin of similar sequence homology to BoNT/A), led to the hypothesis that internalization of BoNT/A by N2a cells, as monitored by SNAP-25 cleavage, was dependent upon lipid rafts. Instead of inhibiting the action of BoNT/A on N2a cells, depletion of lipid rafts actually enhanced its activity as monitored by SNAP-25 cleavage. Based on the forgoing discussion, the increased activity may be due to either increased exocytosis by synaptic vesicles providing more SV2 on the plasma membrane, an increase in lateral mobility enhancing the interaction of the BoNT/A-ganglioside complex with SV2, or both. If lipid raft disruption does enhance
uptake of the SV2-BoNT/A-ganglioside complex in a clathrin-dependent manner, it would be expected to result in uptake of more toxin and therefore enhanced SNAP-25 cleavage.
Chapter 3

Lipid raft disruption promotes axonogenesis in N2a neuroblastoma cells

Introduction

Increasing evidence indicates that lipid rafts play a crucial role in cellular signaling. While much attention has been given to signaling proteins, within the past 20 years or so lipids have been shown to do more than provide the bilayer needed for membrane structure (Hakomori, 1990; Sonnino et al., 2007). With the identification of lipid rafts (Simons and Ikonen, 1997), interest in the biological functions of lipids has grown. Gangliosides, sialic acid containing glycosphingolipids, have been found localized to lipid rafts and have been shown to play a role in numerous cell events (Hagmann and Fishman, 1982; Parton, 1994; Vinson et al., 2003).

Biological roles of gangliosides

Gangliosides are a large family of glycosphingolipids found predominantly on the cell surface and anchored in the external leaflet of the lipid bilayer by a ceramide moiety (see Fig. 3-1 for a schematic of ganglioside structure). They are synthesized by two different pathways designated “a” (GM2, GM1a, GD1a) and “b” (GD3, GD2, GD1b, GT1b, GQ1b). The cerebroside (Glc-Cer) portion of gangliosides is synthesized on the ER and further glycosylation occurs in the Golgi. Interest in the biological roles of
gangliosides has been stimulated not only by the number of different gangliosides identified based on differences in their carbohydrate composition (over 70 species identified, Stults et al., 1988) but because they are found in highest concentration in the brain (Rapport, 1981), where they have been shown to function in intracellular signaling (Hakomori et al., 1998), cell-cell recognition (Sheikh et al., 1999), and cell proliferation and differentiation (Prinetti et al., 1999; Hirschberg et al., 1996; Walkley et al., 2000; Kwak et al., 2006).

Numerous studies have implicated gangliosides in neuronal differentiation and development as their composition changes during development of the nervous system. For example, it was observed that when neuroblastoma cells were induced to differentiate by exposure to retinoic acid the differentiation was accompanied by an increase in total cell ganglioside content (Li and Ladisch, 1992; Rebhan et al., 1994). Alteration of GM1 by exposure of neuroblastoma cells to either anti-GM1 antibodies, exogenous GM1, or cholera toxin B (which binds to GM1) has been shown to induce neuritogenesis (Roisen et al., 1981; Carlson et al., 1994). Synaptogenesis, the formation of nascent synapses, is accompanied by a strong up-regulation in expression of a-series gangliosides (Ledeen et al., 1984; Hirschberg et al., 1996).

The need for gangliosides in order to have normal neural development was underscored by the discovery of the underlying cause of an infantile-onset symptomatic epilepsy syndrome (Simpson et al., 2004). The syndrome is caused by a loss-of-function mutation in GM3 synthase. Lack of GM3 synthase activity results in failure to synthesize
GM3 and any of the gangliosides for which it is a precursor (e.g. GM1, GD1a, GD1b, and GT1b, major ganglioside components found in brain). Failure to synthesize the gangliosides was found to result in developmental stagnation, regression and death. Loss of ganglioside synthesis would be expected to have multiple cellular consequences because of the effect it would have on formation of lipid rafts. Through their association with lipid rafts, gangliosides can elicit numerous responses in cells by their interaction with growth factors. For example, the growth factor tyrosine kinase, ErbB2, was shown to colocalize in lipid rafts with GM3 (Sottocornola et al., 2006) and when ganglioside synthesis was inhibited the ErbB2 moved out of the rafts. GM1 has been shown to bind to Trk (high-affinity tyrosine kinase-type receptor for nerve growth factor (Mutoh et al., 1995) and can enhance expression of the synaptic membrane dopamine transporter as well as the vesicular monoamine transporter 2 (Goettl et al., 2003).
Figure 3-1: General structure of major gangliosides found in the mature, central nervous system.

The sugar backbone consists of glucose, galactose, N-acetyl galactosamine, and galactose to which are linked one to three sialic acid residues.
Gangliosides can also play a biological role in the regulation of calcium. GM1 has been found on the nuclear membrane in association with a sodium-calcium exchanger and has been implicated in regulation of nuclear Ca\(^{2+}\) homeostasis during neuronal differentiation (Wu et al., 1995; Xie et al., 2002). GM1 on the endoplasmic reticulum (ER) also affects release of Ca\(^{2+}\) during activation of the ER stress response system (Tessitore et al., 2004; d’Azzo et al., 2006).

GM1, GD1a, and GT1b, gangliosides that account for approximately two-thirds of those in human brain gray matter (Tettamanti et al., 1973) are, along with cholesterol, abundant in lipid rafts (Brown and London, 2000; Simons and Toomre, 2000). It is believed that the interactions between cholesterol and glycosphingolipids act as the glue holding lipid rafts together. If true, it explains why disruption of lipid rafts can be accomplished by altering their cholesterol content. Previous studies in this lab of the effect of lipid raft disruption by either the cyclic oligomer MβCD or the polyene antibiotic filipin on uptake of botulinum neurotoxin by neuroblastoma cells (Petro et al., 2006) indicated that the disruption induced morphological changes in the cells. The work described on the following pages was done to determine the type of process formation induced by filipin and MβCD disruption of lipid rafts as well as whether those changes were accompanied by changes in ganglioside composition or subcellular distribution. The results indicate that the processes formed were axonal in nature and that their expression was accompanied by changes in both ganglioside content and subcellular localization.
Materials and methods

Materials

EZ-Run™ Protein Gel Solution (BP7710) and Millipore Immobilon-P PVDF membrane were obtained from Fisher Scientific (Pittsburgh, PA); mouse monoclonal anti-nuclear pore complex proteins (NPP, MAb414) from Covance (Berkeley, CA); mouse monoclonal anti-GD1a antibody (370706-1) from Seikagaku (Falmouth, MA); mouse monoclonal neurofilament antibody (ab7795) from Abcam (Cambridge, MA); Hoechst dye (33342), and cholera toxin B-subunit Alexa Fluor® conjugated 594 (C-34777), and goat anti-mouse IgG-conjugated Alexa Fluor® 594 (A21135) from Molecular Probes (Eugene, OR); goat anti-mouse IgG conjugated Hilyte™ 488 from Anaspec (San Jose, CA); mouse monoclonal anti-microtubule associated protein (MAP) 2 (2A+2B) antibody (M2320), mevinolin, mevalonic acid, filipin, and poly-L-lysine (P4707) were from Sigma (St. Louis, MO) and cholera toxin binding-subunit (CTxB) conjugated to horseradish peroxidase (105) was obtained from List Biological Laboratories (Campbell, CA). Halt Protease Inhibitors™ were purchased from Pierce (Rockford, IL).
Cell culture

Mouse N2a neuroblastoma cells were seeded at a density of $1.5 \times 10^6$ cells per 75cm$^2$ tissue culture flask and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum (NBS) at 37 °C in an atmosphere of 5% CO$_2$, 95% air, and 90% humidity.

Lipid raft disruption

Lipid rafts were disrupted using filipin and MβCD as previously described. Briefly, cells were seeded as above, allowed to grow for 72h, and then treated with either 1.25mM MβCD or 3μg/ml filipin for 1h at 37°C in DMEM containing 10% lipoprotein deficient serum (LPDS), followed by either a 12 or 24h incubation at 37°C in DMEM-LPDS containing 5μM mevinolin and 1μM mevalonic acid. After 24h, cells to be studied for reversibility of the effects of disruption of cholesterol containing lipid rafts were fed DMEM containing 10% NBS. Control cells were treated analogously but were not exposed to filipin, MβCD, mevinolin or mevalonic acid.

Immunofluorescence

$5 \times 10^5$ N2a cells were seeded onto coverslips in 60mm tissue culture dishes and cultured for 72h prior to disruption of lipid rafts as described above. Control cells were treated in the same manner but were not exposed to filipin, mevinolin, or mevalonic acid.
At various time-points after disruption of lipid rafts, coverslips were washed 3X with phosphate buffered saline (PBS), fixed for 20min at 4°C in 4% paraformaldehyde in PBS, and then permeabilized by exposure to 0.1% Triton X-100 in a mixture of PBS and fetal bovine serum (FBS, 1:1, v/v) for 1h at room temperature. Nonspecific binding sites were blocked by incubating the permeabilized cells in PBS-FBS for 1h at room temperature. After washing 3X in PBS, cells were exposed to primary antibodies in PBS-FBS for 1h at room temperature and unbound antibody removed by washing prior to exposure to labeled secondary antibody. More specifically: for isolated nuclei: anti-NPP antibody (1:1000) followed by goat anti-mouse HiLyte 488 conjugated antibody (1:1000), followed by counterstaining for GM1 using CtxB-594 (1µg/ml). Nucleic acid was stained using Hoechst stain (1µg/ml) for 5min; for neurite type: anti-MAP antibody (2µg/ml) or anti-NFH antibody (1:1000) followed by goat anti-mouse IgG-594 (2µg/ml); and for ganglioside staining: anti-GD1a antibody (1:250) followed by goat anti-mouse IgG-594. CTxB-594 (1µg/ml) was used to visualize GM1. After washing, coverslips were mounted on slides using glycerol/PBS and fluorescent microscopy was performed using a Nikon Eclipse TE2000 microscope equipped with a Photometrics CoolSNAP camera and NIS-Elements AR imaging software. Images were captured using either a 60× or 60× objective lens with 1.5 magnification.
Ganglioside analysis

Gangliosides were analyzed by two methods. *By SDS-PAGE:* Cells were grown and lipid rafts disrupted as described above. At various time-points after lipid raft disruption, cells were harvested into PBS and lysed in 500µl of RIPA buffer (50mM Tris HCl containing 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH8) supplemented with a 1:100 dilution of Halt Protease Inhibitors™. Twenty µg of cell protein were separated using 10% EZ-Run™ Protein Gel Solution SDS-PAGE, transferred to a PVDF membrane and exposed to either anti-GD1a antibody (1:250) followed by goat anti-mouse IgG-HRP (1:2500) or to CTxB-HRP (2µg/ml). Blots were developed using SuperSignal West Femto or Pico Maximum Sensitivity Substrate™ and visualized using the SynGene Gene Gnome. Densitometric analysis was performed using Biorad Discovery series™ Quantity One 1D analysis software. *By immunoblot:* At various times after disruption of lipid rafts, cells were harvested into PBS and recovered by centrifugation at 250 × g for 5 min. The cells were resuspended in 1ml of H2O, and cellular lipids were extracted by the addition of 10 volumes of methanol:chloroform 2:1 (v/v). The mixture was stirred at room temperature overnight. Cell debris was removed by centrifugation at 250 × g for 10 min and the supernatant was dried under N2 in a tared test tube. Lipids were dissolved in chloroform:methanol (2:1 v/v) and 25µg was spotted in dot blot fashion onto a PVDF membrane. The PVDF membrane was exposed to either anti-GD1a antibody (1:250) followed by goat anti-mouse IgG-HRP (1:2500) or to CTxB-HRP (2µg/ml). Blots were developed using SuperSignal West Femto or Pico Maximum Sensitivity Substrate™ and visualized using the SynGene Gene Gnome. Densitometric
analysis was performed using Biorad Discovery series™ Quantity One 1D analysis software.

**Isolation and purification of nuclei**

Nuclei were isolated using a modification of the procedure of Wu et al., (1995). At different times after exposure to filipin or MβCD, cells were harvested into cold PBS and recovered by centrifugation at 250 × g for 5min. All subsequent procedures were done on ice. The cells were lysed in 20mM Tris HCl [pH 7.5], containing 1mM MgCl, and 1mM DTT (TMD), supplemented with 0.1% Triton X-100 and incubated on ice for 30min. Nuclei were recovered by centrifugation (800 × g for 10min). The pellet was then homogenized in 2.0M sucrose in TMD and centrifuged at 100,000 × g for 20min. The nuclear pellet was taken up and homogenized in 0.32M sucrose in TMD, overlaid on 2.2M sucrose in TMD and centrifuged as before. The recovered nuclear pellet was washed in TMD, fixed in 4% paraformaldehyde in PBS for 20min at 4°C, and allowed to adhere to poly-L-lysine coated glass slides for 1h at room temperature. After washing with PBS, nuclei were stained as described above.
Results

To investigate the role played by lipid rafts in induction of neurite formation, N2a cells were treated with either filipin or MβCD in DMEM-LPDS to sequester or extract cholesterol, followed by treatment with DMEM-LPDS containing mevinolin to inhibit cholesterol synthesis and mevalonic acid to allow for formation of essential isoprenoid precursors. Disruption of lipid rafts in this manner induced neurite formation that was clearly visible 12 and 24h after exposure to both filipin and MβCD (Figs. 3-2 and 3-3). Neurites were essentially absent on control cells (cells not exposed to filipin or MβCD). When cells were allowed to recover for 24h in DMEM-NBS, they appeared to retract their neurites and to return to a morphology similar to that of control cells. It can be seen in the case of filipin treated cells, (Fig. 3-2) fluorescence due to filipin remained associated with the cells even after the 24h recovery period.
Figure 3-2: Effect of filipin-treatment on the morphology of N2a cells.

Control cells were incubated in DMEM-LPDS for either 1, 12, or 24h (a-c), medium was then removed and replaced with DMEM containing 10%NBS and cells allowed to grow for an additional 24h (d). Filipin-treated cells were incubated for 1h in DMEM-LPDS containing 3µg/ml of filipin (e), and for an additional 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (f,g). Medium was then removed and replaced with DMEM containing 10%NBS and cells allowed to grow for an additional 24h (h). Cell-associated filipin is shown for filipin-treated cells in the fluorescence micrographs (i-l). Arrows indicate processes. Scale bar represents 90µm, 60X objective lens.
Figure 3-3: Effect of MβCD-treatment on the morphology of N2a cells.

Control cells were incubated in DMEM-LPDS for either 12 or 24h (a,b), medium was then removed and replaced with DMEM containing 10% NBS and cells allowed to grow for an additional 24h (c). MβCD-treated cells were incubated for 1h in DMEM-LPDS containing 1.25mM of MβCD and for an additional 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (d,e). Medium was then removed and replaced with DMEM containing 10% NBS and cells allowed to grow for an additional 24h (f). Arrows indicate processes. Scale bar represents 100µm, 60X objective lens.
Neurite outgrowth induced by either filipin or MβCD disruption of lipid rafts on N2a cells was characterized by monitoring cellular distribution of NF-H and MAP2_{A+B}. In differentiated cells, NF-H is known to be associated with axons (Shea et al., 1998), and MAP2_{A+B} with dendrites (Bernhardt and Matus, 1984). Fluorescent microscopy of N2a cells immunostained with anti-MAP2_{A+B} antibody followed by goat anti-mouse IgG-594 indicated that a diffuse staining of the cell body was seen on control and treated cells (Fig. 3-4, a-f). In contrast, processes expressed by filipin-treated cells exhibited intense fluorescence when immunostained with anti-NF-H antibody and goat anti-mouse IgG-594 (Fig. 3-5, d+e). Similar processes were not observed on control cells (Fig. 3-5, a-b) and they decreased when filipin-treated cells were allowed to recover for 24h in normal media (Fig. 3-5, f). Similar results were obtained with MβCD-treated cells. MAP2_{A+B} was present in the cell body with very little on neurites (Fig. 3-6, e), while NF-H staining was observed on the extended neurites (Fig. 3-6, d and f). These results indicate that disruption of lipid rafts with either filipin or MβCD induces N2a cells to undergo axonogenesis.
Figure 3-4: Immunostaining for expression of the dendritic marker MAP2$_{(A+B)}$ by filipin-treated cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 3µg/ml filipin for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (24/24h, c,f). MAP2$_{(A+B)}$ was detected using anti-MAP2$_{(A+B)}$ antibody and goat anti-mouse IgG Alexa fluor 594 as the secondary antibody. Scale bar represents 100µm, 60X objective lens.
Figure 3-5: Immunostaining for expression of the axonal marker NF-H by filipin-treated cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 3µg/ml filipin for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (24/24h, c,f). NF-H was detected using anti-NF-H antibody and goat anti-mouse IgG Alexa fluor 594 as the secondary antibody. Scale bar represents 100µm, 60X objective lens.
Figure 3-6: Immunostaining for expression of the dendritic marker MAP2(A+B) and the axon marker NF-H by MβCD-treated cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS for 12h (control, a,b) or DMEM-LPDS containing 1.25mM MβCD for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, c and d, e and f). Cells shown in g and h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10% NBS. MAP2(A+B) was detected using anti-MAP2(A+B) antibody and goat anti-mouse IgG Alexa fluor 594 as the secondary antibody. Scale bar represents 100µm, 60X objective lens.
The production of neurites following disruption of lipid rafts by either filipin or MβCD was associated with an increase in GD1α and GM1 staining relative to control cells (Figs. 3-7, 3-8, 3-9 and 3-10). Filipin treated cells showed an increase in GD1α immunofluorescence on the plasma membrane of both cell bodies and neurites, whereas control cells exhibited less overall fluorescence and it was more diffuse. The absence of neurite staining on control cells (compare Fig. 3-7, a and b with d and e) agrees with observations made using phase contrast microscopy (Fig. 3-2) indicating control cells did not extend neurites. Staining of GM1 by the binding subunit of cholera toxin also appeared to be more intense on cells treated with filipin (Fig. 3-8, d-f) in comparison to control cells (Fig. 3-8, a-b). Again, these results were mirrored in cells treated with MβCD to disrupt lipid rafts. Figure 4-9 shows that GD1α immunofluorescence on MβCD treated cells is greater than that of controls (compare a-b to d-e). There also appeared to be an increase in GM1 fluorescence on treated versus control cells (Fig. 3-10, compare a-b to d-e). Overall, regardless of whether the cells were treated with filipin or MβCD there appeared to be an increase in expression of both GD1α and GM1.
Figure 3-7: Immunofluorescent photomicrographs showing GD1a on filipin treated N2a cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 3µg/ml filipin for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (c,f). GD1a was detected using an anti-GD1a-specific antibody and a fluorescently tagged secondary antibody. Scale bar represents 50µm, 60X lens 1.5 mag.
Figure 3-8: Fluorescent photomicrographs showing the ganglioside GM1 on filipin treated N2a cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 3µg/ml filipin for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (c,f). GM1 was detected using fluorescently labeled CTxB. Scale bar represents 50µm, 60X lens 1.5 mag.
Figure 3-9: Immunofluorescent photomicrographs showing GD1a on MβCD treated N2a cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 1.25mM MβCD for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (c,f). GD1a was detected using an anti-GD1a-specific antibody and a fluorescently tagged secondary antibody. Scale bar represents 100µm, 60X lens.
Figure 3-10: Fluorescent photomicrographs showing the ganglioside GM1 on MβCD treated N2a cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, a,b) or DMEM-LPDS containing 1.25mM MβCD for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (treated, d,e). Cells labeled 24/24h were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (c,f). GM1 was detected using fluorescently labeled CTxB. Scale bar represents 50µm, 60X lens 1.5 mag.
The apparent increase in expression of GD1a and GM1 induced by lipid raft disruption was confirmed by the increase seen in each when blots of whole cell extracts separated by SDS-PAGE or dot blots were probed using either the anti-GD1a antibody and anti-mouse IgG-HRP or CTxB-HRP (Fig. 3-11, A and B). It can be seen that 12 and 24h after filipin treatment, there was an increase in both the GM1- and GD1a- to actin ratios relative to controls. An increase in GM1 and GD1a was also seen after the 24h recovery period in DMEM-NBS (compare Fig. 3-11 A and B, lanes 5 and 6). GM1 levels in cells treated with MβCD were determined after SDS-PAGE by exposing the blot to HRP-conjugated CTxB. It can be seen that MβCD-treated cells had significantly more GM1 than controls (Fig. 3-12). Dot blot analysis indicated that expression of GD1a was also greater in MβCD-treated cells than controls (Fig. 3-13).
Figure 3-11: Effect of filipin disruption of membrane rafts on the cellular concentration of GM1 and GD1a.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (control, lanes 1,3) or DMEM-LPDS containing 3μg/ml filipin for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5μM mevinolin and 1μM mevalonic acid (treated, lanes 2,4). Cells studied for reversibility of the effects of lipid raft disruption were treated as described for 24h and then allowed to grow for an additional 24h in DMEM containing 10%NBS (control, lane 5; treated, lane 6). Cells were harvested in RIPA buffer and 20μg of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on 10% running gel. Following transfer to a PVDF membrane, blots were probed using either an anti-actin monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody or HRP-conjugated CTxB to recognize GM1. GD1a was detected using an anti-GD1a monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate and exposure in the SynGene Gene Gnome. g/a: ganglioside to actin ratio.
Figure 3-12: The effect of MβCD disruption of lipid rafts on the cellular concentration of GM1

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS (ctrl) or DMEM-LPDS containing 1.25mM MβCD for 1h followed by incubation for 12h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid (chol. depleted). Cells were harvested in RIPA buffer and 20µg of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on a 10% running gel. Following transfer to a PVDF membrane, blots were probed using HRP-conjugated CTxB to recognize GM1. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate and exposure in the SynGene Gene Gnome.
Figure 3-13: Dot blot analysis of gangliosides isolated from MβCD treated cells.

Cells were seeded and allowed to grow for 72h. Medium was removed and cells were grown in the presence of either DMEM-LPDS for 12h (ctrl) or DMEM-LPDS containing 1.25mM MβCD for 1h followed by incubation for 12 or 24h in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid. Cells were harvested and gangliosides isolated using methanol:chloroform 2:1 (v/v). Cell debris was removed by centrifugation, and the supernatant was dried under N₂. Lipids were dissolved in chloroform:methanol (2:1 v/v) and 25 µg was spotted in dot blot fashion on a PVDF membrane and probed using HRP-conjugated CTxB to recognize GM1. GD1a was detected using an anti-GD1a monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate and exposure in the SynGene Gene Gnome.
Twenty-four h after disruption of lipid rafts, GM1 appeared to encircle isolated nuclei from filipin treated cells (Fig. 3-14 d,e) while nuclei isolated from undifferentiated, control cells had little to no GM1 colocalized with the nuclear pore protein complex (NPP, Fig. 3-14 a,b). Because filipin fluorescence is similar to that of FITC, labeling of treated cells was limited to Hoechst staining of the nuclei (blue) and CTxB-594 (red for GM1) in order to eliminate the possibility of cross-talk with potential membrane contaminants. The lack of staining by CTxB 12h after filipin treatment (Fig. 3-14 c) indicates that it takes more than 12h for the cells to respond in this manner; the continued expression of nuclear GM1 after 24h of recovery in DMEM-NBS (Fig. 3-14) indicates that the response is relatively long-lasting. MβCD-treated cells behaved in much the same way (Fig. 13-15, b-d), with the exception being that GM1 surrounding the nucleus could be seen 12h after disruption of lipid rafts (Fig. 3-15, e). Like filipin treated cells, the response was long-lasting as GM1 was still seen surrounding the nucleus after 24h recovery in regular media.
Figure 3-14: Effect of filipin-treatment on the appearance of GM1 on the nuclear membrane.

Control cells were incubated in DMEM-LPDS for either 12 or 24h (a, b) Filipin-treated cells were incubated for 1h in DMEM-LPDS containing 3µg/ml of filipin and for an additional 12 (c) or 24h (d,e) in DMEM-LPDS containing 5µM mevinolin and 1µM mevalonic acid. Medium was then removed and replaced with DMEM containing 10%NBS and cells allowed to grow for an additional 24h (f). Nuclei were isolated and stained with Hoechst, nuclear-associated GM1 was stained using fluorescently labeled CTxB, and control cells were additionally labeled using an anti-NPP monoclonal antibody followed by a fluorescently tagged secondary antibody. Arrows highlight areas positive for GM1.
Figure 3-15: Effect of MβCD-treatment on the appearance of GM1 on the nuclear membrane.

Control cells were incubated in DMEM-LPDS for 12h (a). MβCD-treated cells were incubated for 1h in DMEM-LPDS containing 1.25mM of MβCD and for an additional 12 (b) or 24h (c) in DMEM-LPDS containing 5μM mevinolin and 1μM mevalonic acid. Medium was then removed and replaced with DMEM containing 10% NBS and cells allowed to grow for an additional 24h (d). Nuclei were isolated and stained with Hoechst, nuclear-associated GM1 was stained using fluorescently labeled CTxB.
Discussion

Lipid rafts on N2a cells were disrupted using filipin, a polyene antibiotic that binds to cholesterol in the outer leaflet of the plasma membrane (Bittman et al., 1983), sequestering it into ultrastructural domains (Kitajima et al., 1976); or MβCD, a cyclic oligomer that binds to and extracts cholesterol from the plasma membrane (Neufeld et al., 1996). Both have been shown to disrupt lipid rafts (Schnitzer at al., 1994). As previously reported (Petro et al., 2006) growth of filipin and MβCD-treated cells in DMEM-LPDS containing the HMGCoA reductase inhibitor mevinolin and the isoprenoid precursor mevalonic acid resulted in maintenance of cell viability and disruption of lipid rafts for at least 24 and 12h, respectively. The results described indicate that disruption of lipid rafts induced neurite extension that was readily apparent 12 and 24h after exposure to filipin or MβCD. Neurites were axonal in nature as indicated by the presence on them of the axonal marker NF-H and absence of the dendritic marker MAP2(\(A+B\)). Expression of neurites was accompanied by an increase in total GM1 and GD1a in treated cells relative to their concentrations in control cells. GM1 was found to surround nuclei purified from both filipin and MβCD treated cells, while no GD1a was observed on the nuclear membrane.

Western analysis and dot blot assays were used to determine whether changes in ganglioside concentration were induced by disruption of lipid rafts. Previously, methods such as these were not used as high-affinity antibodies that specifically recognized major brain gangliosides, especially GM1, GD1a and GT1b, were not available (Kawashima et
The difficulty in preparing them was thought to be due to the conservation of brain gangliosides across mammalian and avian species. However, as understanding of ganglioside metabolism increased, genetic manipulations advanced the production of high-affinity mouse monoclonal antibodies derived from mice genetically engineered to lack complex gangliosides (Kotani et al., 1992, Ozawa et al., 1992). Therefore a monoclonal antibody that specifically recognizes GD1a (Schnaar et al., 2002) was used to probe its levels on westerns and dot blots. GM1 was probed using the binding subunit of cholera toxin. While CTxB was shown to also bind fucosylated GM1 present in rat glioma C6 cells (Masserini et al., 1992), the lack of detectable expression of Fuc-GM1 in N2a cells (Hitoshi et al., 1996) indicates that binding of CTxB to Fuc-GM1 is unlikely. In cells treated for lipid raft disruption by the cholesterol sequestering mechanism of filipin, gangliosides were analyzed by western blot. An increase in both GM1 and GD1a relative to actin from whole cells was observed at both 12 and 24h after disruption of lipid rafts. These results agree with those obtained from fluorescent microscopy experiments in which more intense fluorescence was observed for both lipids on filipin-treated cells than on controls. In MβCD-treated cells, the increase in GM1 and GD1a observed by dot blot analysis corresponded with the appearance of greater fluorescence of both GSLs on MβCD-treated cells relative to controls. These changes in ganglioside expression are in agreement with observations of changes in ganglioside content during differentiation. For example: studies done in hippocampal neurons indicated that major changes in complex gangliosides such as GM1, GD1a, GD1b, and GT1b occurred during axonogenesis with little change occurring during dendritogenesis and synaptogenesis (Hirschberg et al., 1996). In neuroblastoma cells, neuritogenic agents that stimulated
axonal growth (e.g. KCl and ionomycin) caused a significant increase in GM1 and GD1a while agents that induced dendritic outgrowth (retinoic acid and dibutyryl cyclic AMP) induced an increase in GM2 on the plasma membrane (Wu et al., 1998; Wu et al., 2001).

The use of two high-density sucrose gradients to isolate nuclei from control cells gave nuclei that had little to no detectable GM1 colocalizing with NPP. At 12 and 24h after lipid raft disruption with MβCD, the appearance of GM1 was seen surrounding nuclei, and was still found after a 24h recovery period in regular media. These results were also observed in filipin treated cells, however GM1 was not observed around the nucleaus at 12h but was apparent at 24h. These observations agree with those made by Wu et al. (2001). Using FITC-labeled CtxB, they found very little GM1 associated with nuclei from undifferentiated N2aW28 cells (a subclone of N2a cells), while nuclei from both N2aW28 and N2a cells induced to differentiate in an axonal-manner had a marked increase in nuclear GM1 (Wu et al., 2001; Kozireski-Chuback et al., 1999). Localization of GM1 to the nuclear membrane has been shown to be both at the outer membrane, and the inner membrane where it associates with a Na$^{+}$/Ca$^{2+}$ exchanger (Xie et al., 2002). As the NPP complex spans both the inner and outer leaflets of the nuclear membrane, colocalization of GM1 with NPP does not indicate whether the GM1 is on the outer or inner membrane.

It has been postulated by Ledeen and colleagues (Xie et al., 2002; Ledeen and Wu, 2006) that the association of GM1 with the Na$^{+}$/Ca$^{2+}$ exchanger in the nuclear membrane may serve to protect the nucleus during prolonged increases in cytosolic Ca$^{2+}$. 
While filipin is a reagent frequently used to disrupt lipid rafts as well as to detect cholesterol via its intrinsic fluorescence (Castanho et al., 1992), evidence also indicates that it stimulates Ca\textsuperscript{2+} flux (Kinsky et al., 1966; Adams et al., 1970; van Zutphen et al., 1971; Spielvogel and Norman, 1975). It is therefore possible that treatment to disrupt lipid rafts with filipin could result in an intracellular increase in Ca\textsuperscript{2+}, triggering GM1 to localize to the nuclear membrane to protect the cell from apoptosis induced by the increase in intracellular Ca\textsuperscript{2+}. Additionally, in studying the importance of cholesterol efflux in regulating ion transport, Francis et al. (1999) reported that MDCK cells treated with MβCD for 30 minutes resulted in changes in permeability. Hence it is possible that perturbation of the plasma membrane by MβCD could increase permeability of ions to which the N2a cells respond to by elevating nuclear GM1 as a protective function. Further studies are necessary to determine whether disruption of lipid rafts by filipin induces a change in intracellular Ca\textsuperscript{2+}.

The mechanism by which disruption of lipid rafts promotes neurite outgrowth that is axonal in nature, remains to be determined. However, gangliosides have been shown to modulate activity of lipid raft associated signaling proteins that play a role in cell proliferation or dendritogenesis [such as the protein kinases Kit and CLICK-III (Jahn et al., 2007; Takemoto-Kimura et al., 2007)]. It is possible that disruption of these ganglioside-protein interactions by filipin disruption of lipid rafts translates into the observed cellular responses.
Differentiation of neuroblastoma cells can be induced by numerous stimuli (retinoic acid, serum deprivation, neuraminidase treatment) that cause changes in ganglioside content (Wu et al., 1998; Wu et al., 2001; Valaperta et al., 2007). The results described in this work indicate that N2a cells can be induced to reversibly differentiate by disruption of lipid rafts using either filipin or MβCD. Both agents induced N2a cells to express processes that were axonal in nature. Induction of axon-like processes was accompanied by an increase in GM1 and GD1a, with a marked increase in GM1 on the membrane of nuclei isolated from lipid raft disrupted cells. While the mechanism for these changes is undefined, the fact that the changes were induced by disruption of lipid rafts indicates that lipid rafts function in regulating ganglioside expression as well as process formation by N2a cells.
Chapter 4

Conclusions and Future Directions

The mechanism by which BoNT/A binds and enters neurons is not well defined. Previous studies have indicated that gangliosides in the plasma membrane were necessary for the activity of BoNT/A. TeNT, which also binds to a ganglioside on the plasma membrane, requires intact lipid rafts to bind and internalize and act on VAMP-2. Since TeNT is from the same clostridial family of toxin-producing bacteria as BoNT/A, it is commonly used as a model for the behavior of clostridial neurotoxins. The similarities between TeNT and BoNT/A as seen in their sequence homology and their interaction with gangliosides, coupled with the evidence that gangliosides are enriched in lipid rafts led to the hypothesis that the ability of BoNT/A to inhibit neurotransmission might be dependent upon lipid rafts. However disruption of lipid rafts of N2a cells did not prevent the activity of BoNT/A; in fact, activity was actually enhanced by their disruption. This suggests that the mechanism for the interaction of BoNT/A with the cell plasma membrane and its subsequent internalization is different than that of TeNT. The difference may be indicative of the fact that while both toxins bind to the pre-synaptic membrane of peripheral neurons, TeNT undergoes axonal retrograde transport and after release at the terminal synapse is taken up by inhibitory neurons in the central nervous system.

The data also supports the conclusion that BoNT/A uptake into the cells is probably by clathrin-mediated endocytosis. Testing the effect of biochemical disruption
of clathrin-mediated endocytosis on internalization of BoNT/A proved to be difficult because: 1) long-term potassium depletion/hypotonic shock would likely be injurious to the cells and 2) the effect of disruption was reversed upon restoration of medium containing potassium. Consequently, N2a cells were incubated for only 1h with BoNT/A after treatment to disrupt clathrin-mediated endocytosis, which meant that BoNT/A had little time to bind to the plasma membrane. To more thoroughly investigate the involvement of clathrin-mediated endocytosis and BoNT/A internalization, N2a cells could be transiently transfected with a dominant-negative mutant form of dynamin (lys$^{44}$ to ala$^{44}$; K44A) as described by Ehehalt et al. (2003). Dynamin is a GTPase that regulates clathrin-mediated endocytosis by cinching-off clathrin-coated pits from the plasma membrane. The K44A dominant-negative mutant has been shown to inhibit internalization of transferrin (a marker for clathrin-mediated endocytosis), while having little effect on clathrin-independent pathways (Damke et al., 1994). This approach could yield a more conclusive indication of whether clathrin-mediated endocytosis is needed for BoNT/A internalization.

The fact that lipid raft disruption enhanced the activity of BoNT/A on SNAP-25 was an unexpected result and one that should be further analyzed. It is possible that disruption of rafts leads to greater accessibility of either: 1) the protein receptor SV2 or 2) the substrate SNAP-25.

1). Increased availability of SV2: Increased availability of SV2 would likely be advantageous to the toxin only immediately after lipid raft disruption. Immunostaining
for SV2 using an anti-SV2 mouse monoclonal antibody available from the Developmental Studies Hybridoma Bank, followed by a labeled secondary antibody analyzed by confocal microscopy could be used to determine whether more SV2 is present on the plasma membrane of lipid raft disrupted cells compared to controls. Because BoNT/A binds to a luminal portion of SV2, the binding epitope is only accessible when synaptic vesicles fuse with the plasma membrane to initiate exocytosis. To ascertain the effect of lipid raft disruption on exo- and endocytosis by N2a cells, two different fluorescence-based methods could be used. First, an FM43-1 assay can be employed. FM43-1 is a lipophilic probe that binds rapidly to the plasma membrane. Extracellularly, the probe fluoresces weakly but its fluorescence is markedly enhanced after internalization into a membrane environment. Therefore, it is used to study vesicle recycling. The second method capitalizes on the fact that SV2 is a transmembrane vesicle protein with the luminal portion as the epitope for the anti-SV2 mouse monoclonal antibody. Conjugation of this antibody with a fluorescent label would provide a probe for studying the movement of SV2 before and after disruption of lipid rafts.

In addition to fluorescent assays to study exo- and endocytosis, the patch-clamp technique could be used to measure the transmembrane potential of the cells both before and after disruption of lipid rafts. In stimulated cells, exocytosis requires an increase in ion transport which causes a change in electrical current. The patch-clamp technique was previously used on N2a cells (Nobile and Lagostena, 2000) to study large conductance of anion channels. In this work, whole-cell patch-clamping was attempted with the N2a
cells. Because no change in current was seen when control cells were stimulated with high potassium no further studies were done.

2). *Increased availability of SNAP-25*: It is believed that BoNT/A has access to SNAP-25 during a very brief “physiological window” in which SNAP-25 is not in a complex with other SNARE proteins and is therefore available for cleavage by the neurotoxin. If lipid raft disruption decreases the association of SNAP-25 with the other SNARE proteins, BoNT/A would have more substrate to act on. An immunohistochemical method developed by Xiao et al. (2004) can distinguish free from complexed SNAP-25. Data obtained using this technique might answer the question of whether more SNAP-25 is accessible to the action of BoNT/A in lipid raft disrupted cells. Figure 4-1 illustrates the physiological window for SNAP-25 cleavage.

![Figure 4-1](image)

*Figure 4-1: Brief physiological window for proteolysis of SNAP-25.*

An open configuration of the SNARE proteins is adopted for a brief period during which the proteins are susceptible to proteolytic attack (Humeau et al., 2000).
Disruption of lipid rafts induced changes in the morphology of N2a cells as well as ganglioside content. A literature search indicated that this is the first study to demonstrate a correlation between lipid rafts, axonogenesis and nuclear GM1. While the mechanism of induced neuritogenesis remains unknown, future work could include studying the calcium concentration of N2a cells before and after disruption of lipid rafts. Lipid raft disruption for 12 or 24h resulted in the appearance of GM1 surrounding the nucleus. If treatment with either filipin or MβCD affects cell permeability to ions, an increase in cytosolic \( \text{Ca}^{2+} \) could induce the localization of GM1 to the nuclear membrane.

As previously discussed, GM1 has been shown to associate with a sodium-calcium exchanger at the inner nuclear membrane where it putatively functions to protect the cell from calcium flux (Ledeen and Wu, 2006). It has also been noted that agents that induce neuritogenesis can be accompanied by a concurrent increase in total cell GM1 and GD1a. Therefore, it would be of interest to determine whether the cytosolic \( \text{Ca}^{2+} \) concentration changes after: 1) initial exposure to either filipin or MβCD or 2) after expression of neurites induced by lipid raft disruption. This could be done using yellow cameleons which are genetically encoded fluorescent indicators for \( \text{Ca}^{2+} \). Calcium flux can be monitored in cameleon-transfected cells by confocal microscopy. Fig. 4-2 shows the mechanism of action of the cameleon indicator.
Figure 4-2: Schematic of the mechanism of action of the cameleon calcium indicator.

The different fluorescent units of the cameleon are connected by calcium-sensitive calmodulin. Upon binding calcium, the calmodulin moiety undergoes a conformational change that brings the fluorescent protein-domains into close proximity. The resulting FRET signal allows ratiometric measurements of free intracellular calcium.
Ledeen and Wu (2006) reported that GM1 is found in association with the sodium-calcium exchanger on the inner nuclear membrane, the results presented in this thesis only indicate that it could be on either the outer or inner nuclear membrane, or both. To distinguish between these possibilities the outer membrane could be striped from isolated nuclei using sodium citrate, a procedure shown to leave the inner membrane intact (Gilchrist and Pierce, 1993). The inner nuclear membrane could then be exposed to labeled CTxB to determine whether GM1 is present.

A greater understanding of why BoNT/A activity is enhanced after disruption of lipid rafts could be important for improving its therapeutic efficacy, as well as for preventing its action (in the case of bioterrorism). As there currently is no effective antidote to BoNT/A intoxication, further studies into its mechanism of transport into the cell and to its site of action are needed. In addition to learning more about BoNT/A, understanding the mechanism of how cholesterol depletion/sequestration affects cell morphology and ganglioside concentration could prove beneficial to the study of diseases in which lipid rafts may have a role. These would include not only those caused by pathogens that utilize lipid rafts as portals for entry but diseases such as Alzheimers which have been shown to be accompanied by alterations in the composition of lipid rafts.
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