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ABSTRACT

Neuronal regulation and functionality is dependent on polarized protein distribution. Appropriate protein targeting is particularly relevant for proteins that contribute to synapse formation. Neuroligin-1 (NLG-1) mediates heterophilic synaptic adhesion with the axonal receptor β-neurexin and their interactions trigger the assembly of functional presynaptic terminals. A prerequisite for such a role in synapse formation is that NLG-1 should be exclusively targeted to the somato-dendritic domain and excluded from axons. Mutational analysis within the cytoplasmic domain in NLG-1 demonstrates that three equivalent signals target NLG-1 to the dendritic plasma membrane. Additionally, using a fluorescently-tagged NLG-1, NLG-1’s intracellular trafficking route was shown to contain the potassium channel Kv2.1 and Rab11b, and found to be in transferrin negative compartments. This work is significant because it will lead to a greater understanding of cellular and molecular mechanisms of nervous system development.

Keywords:
Neuroligin-1
Dendritic Targeting
Fluorescence Microscopy
Cultured Primary Hippocampal Neurons
Sorting Motifs
Membrane Protein Trafficking
Neuronal Polarity
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<td>AIS Axon Initial Segment</td>
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<tr>
<td>AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor</td>
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<tr>
<td>AP Adaptor Protein</td>
<td></td>
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<td>APP Amyloid Precursor Protein</td>
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<tr>
<td>ARE Apical Recycling Endosome</td>
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<tr>
<td>Arp-1 Actin-Related Protein 1</td>
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<tr>
<td>ATP Adenine Triphosphate</td>
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<tr>
<td>β-NRX β-Neurexin</td>
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<tr>
<td>BSE Basolateral Sorting Endosome</td>
<td></td>
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<tr>
<td>ΔC Cytoplasmic Domain Terminal Truncation</td>
<td></td>
</tr>
<tr>
<td>CAM Cell Adhesion Molecule</td>
<td></td>
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<tr>
<td>CLD Cholinesterase-Like Domain</td>
<td></td>
</tr>
<tr>
<td>CD-MPR Cation-Dependent Mannose-Phosphate Receptor</td>
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<tr>
<td>CFP Cyan Fluorescent Protein</td>
<td></td>
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<tr>
<td>CI-MPR Cation-Independent Mannose-Phosphate Receptor</td>
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<tr>
<td>CRE Common Recycling Endosome</td>
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<tr>
<td>D:A Dendrite to Axon</td>
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<tr>
<td>DF Decay- Accelerating Factor</td>
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<tr>
<td>DD Aspartic Acid Couplet</td>
<td></td>
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<tr>
<td>DIV Days in vitro</td>
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<tr>
<td>EE Early Endosome</td>
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<tr>
<td>EEAT3 Excitatory Amino Acid Transporter</td>
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<tr>
<td>EGFR Epithelial Growth Factor Receptor</td>
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<tr>
<td>ER Endoplasmic Reticulum</td>
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<td>FRAP Fluorescence Recovery after Photobleaching</td>
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<tr>
<td>GFP Green Fluorescent Protein</td>
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<td>GGA Golgi-Localized, Gear-Containing, ARF Binding Protein</td>
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<tr>
<td>GPI Glycophosphatidylinositol</td>
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<tr>
<td>HA Hemagglutinin-Tag</td>
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<tr>
<td>HD Huntingtin Protein</td>
<td></td>
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<tr>
<td>Kv2.1 Potassium Channel 2.1</td>
<td></td>
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<tr>
<td>JIP JNK Signal Scaffolding Protein</td>
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<tr>
<td>LDLR Low Density Lipoprotein Receptor</td>
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<tr>
<td>LE Late Endosome</td>
<td></td>
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<tr>
<td>LRP9 Low-density Lipoprotein Receptor- Related Protein</td>
<td></td>
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<tr>
<td>LYS Lysosome</td>
<td></td>
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<tr>
<td>MDCK Madin-Darby Canine Kidney</td>
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<tr>
<td>MT Microtubule</td>
<td></td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MTOC</td>
<td>Microtubule Organizing Centre</td>
</tr>
<tr>
<td>Nav1.2</td>
<td>Sodium Channel 1.2</td>
</tr>
<tr>
<td>NgCAM</td>
<td>Neuro-glial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>NLG-1</td>
<td>Neuroligin-1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-Aspartate Receptor</td>
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<tr>
<td>PDK1</td>
<td>Protein Kinase D1</td>
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<tr>
<td>PDZ</td>
<td>Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post Synaptic Density Protein</td>
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<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
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<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>Tfr</td>
<td>Transferrin Receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi Network</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular Stomatitis Virus G</td>
</tr>
<tr>
<td>VVL</td>
<td>Hydrophobic Motif</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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<tr>
<td>YTLA</td>
<td>Tyrosine Motif</td>
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1.1 Overview

Cell polarity is a remarkable feature of neurons that underlies most aspects of their function, which is to receive and transmit information. Neurons typically can be divided into two distinct structurally, morphologically and biochemically distinct domains: the axon and the dendrites. To establish such polarity, proteins such as cytoskeletal proteins, ion channels, and cell adhesion molecules must be properly sorted and differentially targeted to their specific respective compartments. Appropriate polarized protein targeting is particularly relevant in the case of proteins that contribute to synapse formation. Contact of axons with dendrites initiates the development of synaptic structures which allow information to be transduced via the action potential. As axo-axonal contacts generally do not lead to synapse assembly, these functional properties of signal transduction are dependant on the asymmetric distribution of organelles, membrane proteins and cytoskeletal proteins (Craig and Banker, 1994; Horton and Ehlers, 2003). Some of these synaptogenic signals are provided by cell adhesion molecules (CAMs) that mediate either homo- or heterophilic interactions between the axonal and dendritic cell surfaces (Scheiffele, 2003; Washbourne et al., 2004). The mechanism by which these proteins are targeted to their specific cellular location is therefore an important aspect of the specialized cellular function of neurons.

This thesis identifies the cytoplasmic targeting motifs involved in the dendritic localization of neuroligin-1, and infers its intracellular trafficking mechanisms based on comparisons made between other dendritically targeted proteins. Neuroligin-1 (NLG-1) is a neuronal CAM that mediates heterophilic adhesion with the axonal cell adhesion molecule β-neurexin (βNRX). NLG-1 -
βNRX interactions can trigger the assembly of functional presynaptic terminals in axons. A prerequisite for such a role in synapse formation is that NLG-1 should be exclusively targeted to the somato-dendritic domain and excluded from axons. Previous work demonstrated that NLG-1 is dendritic and the targeting of NLG-1 relies on a cytoplasmic motif of 32 amino acids. In this study mutational analysis in the cytoplasmic domain in NLG-1 demonstrated that three equivalent signals target NLG-1 to the dendritic plasma membrane. Additionally, using a fluorescently-tagged NLG-1, intracellular trafficking routes were investigated. Models of protein trafficking in polarized cells are reviewed and compared to those of sorting and trafficking models in neurons, with a specific focus on the sorting and trafficking behavior of NLG-1.

1.2 Paradigms for Transmembrane Protein Trafficking: Epithelia vs. Neurons

Protein localization in neurons is important for neuronal function. However due to the technical experimental challenges in working with neuronal cultures much of what we understand about trafficking in polarized cells comes from polarized epithelia. Ectoderm is the primary tissue from which several types of epithelia and neurons are derived, and this developmental commonality led to the investigation of similarities in basic cellular processes, including sorting signals and polarized protein trafficking. The first to propose a common mechanistic sorting system was Dotti and Simons (1990), who suggested that polarized epithelia and neurons have similar protein sorting signals that target the proteins to specific locations in the cell. Polarized epithelia contain two distinct specialized domains, the apical domain and the basolateral domain. The apical domain faces the lumen of a cavity, and the basolateral surface adjoins underlying tissue. Two proteins that are independently sorted to each domain in Madin-Darby Canine Kidney (MDCK) cells, are the basolateral Vesicular stomatitis virus glycoprotein (VSVG) and the apical hemagglutintin (HA) (Rodriguez-Boulan and Sabatini, 1978). When expressed in primary neurons, Dotti and Simons (1990) showed that VSVG was polarized to the somatodendritic domain, and that the HA was
polarized to the axon. They concluded that polarized epithelia and neurons utilize the same sorting mechanisms to target their proteins to distinct cellular locations, and that a direct comparison can be made between the basolateral domain and the dendritic domain, and similarly between the apical and axonal domains. These observations established a paradigm to compare trafficking mechanisms between these two cell types. This enabled further study of polarity for neuronal systems using polarity experiments in epithelial models that were, from a technical standpoint, more tractable.

1.3 Trafficking in Polarized Epithelia

Having related polarized epithelia and neurons and establishing a level of similarity, the knowledge of trafficking in epithelia can be used as a guide for trafficking in neurons. Generally for both cell types, proteins bound for the plasma membrane or intracellular organelles typically are processed and trafficked in the cells secretory pathway. This pathway is comprised of the endoplasmic reticulum (ER), multi-compartmental Golgi apparatus, and the cytoskeleton which facilitates vesicle transport.

Membrane proteins are synthesized on the ER by ribosomes and then are translocated into the ER lumen where they undergo modification including proper folding, glycosylation and multimerization during their transit through the ER and Golgi (Rodriguez-Boulan and Musch., 2005). At the trans-Golgi (TGN) proteins are sorted and packaged into transport vesicles and shuttled to their destined cellular location. Adaptor proteins recognize and bind to sorting signals typically located in the cytoplasmic tails of the membrane proteins and facilitate protein concentration into membrane bound carriers that bud from the Golgi into vesicles and may help to recruit motor proteins. The motor proteins will transport the cargo along microtubules and actin to the plasma membrane, where the cargo will be released and inserted into the membrane.

There exist two distinct surface domains in epithelia: the basolateral and apical membranes, which are separated by a functional boundary between
adjacent cells which in part regulates selective diffusion across the paracellular space. This structure, the tight junction, prevents lateral diffusion of membrane proteins and lipids, and serves as an anchoring point between cytoskeletons of adjacent cells (Rodriguez-Boulan and Musch, 2005). Because there is a barrier between these cellular compartments membrane proteins must then be trafficked intracellularly to establish the two separate domains (Fig. 1). Basolaterally trafficked proteins (see fig 1: labeled routes 2, 5) can either be sorted by direct trafficking from the Golgi into post-Golgi vesicle intermediates or indirectly into common recycling endosomes (CRE) by interaction with specific adaptors. Apical sorting routes include the use of various modifications to the proteins within the Golgi to ensure their sorting from the TGN into CREs and apical recycling endosomes (AREs) (Routes 1 and 4). Proteins internalized from the surface into endocytic carriers follow separate apical and basolateral sorting routes. Some basolateral proteins are indirectly shuttled to the basolateral membrane by budding from the TGN directly into CREs (route 3a and 3b to route 5). Some apical proteins undergo transcytosis, whereby they are first trafficked to the basolateral membrane upon where they undergo endocytosis into AREs and are then sorted into the apical membrane (route 8). Proteins can also be recycled at the apical and basolateral membranes into AREs and (basolateral sorting endosomes) BSEs respectively and then back to their original membranes (route 4) (Rodriguez-Boulan and Musch, 2005).
Figure 1. Model for Protein Sorting in the Golgi Complex

Trafficking of membrane proteins follows three separate pathways in epithelia; I. Direct trafficking and sorting from the Golgi, II. Protein sorting occurs post TGN-exit whereby budding carriers from the Golgi contain a mixture of apical and basolateral proteins which are subsequently sorted into separate carriers, III. Trancytotic route via endosomal intermediates (Rodriguez-Boulan and Musch, 2005). Circled numbers in the figure represent sorting routes found in these pathways. (Rodriguez-Boulan and Musch, 2005)

1.4 Sorting Signals in Epithelia

Sorting signals are primary amino acid sequences or protein modifications that target a protein to specific cellular locations. Sorting signals in polarized epithelia are generally either amino acid motifs, N-linked or O-linked carbohydrates, glycoprophosphatidylinositol (GPI) anchors or other proteinaceous
modifications that are recognized either at vesicle formation from the Golgi or further downstream in the sorting process (Matter, 2000). Basolateral sorting signals are usually located in the cytoplasmic portion of the protein and two well-characterized motifs are NPXY and YXXΦ (Matter et al., 1992). Both are tyrosine-based amino acid sorting sequences where N is asparagine; P is proline; Y is a tyrosine; X is any amino acid; and Φ is a bulky, hydrophobic residue (Ohno and Nakatsu, 2003). YXRF is found to be required for TfR basolateral and dendritic sorting (Collawn et al., 1990; West et al., 1997) and both of the tyrosine-based sorting signals are involved in basolateral sorting and endocytosis in the LDL receptor. Amino acid changes in either motif will disrupt the basolateral targeting of LDLR in MDCK cells (Matter et al., 1992).

A second well-characterized basolateral sorting signal is the hydrophobic motif, e.g., LL, VL, that can be found in the macrophage IgG Fc Receptor (Hunziker and Fumey, 1994). Hydrophobic motifs are involved both in endosomal trafficking and endocytosis, and in many cases a flanking acidic residue confers additional sorting information. DXXLL constitutes a type of di-leucine-based signal found in several transmembrane proteins and receptors that cycle between the TGN and endosomes. The aspartic acid (D) residue is critical to sorting and is often found in the context of acidic patches, as in the case of human Sortilin, whose sorting sequence is GYHDDSDEDLLE (Bonaficino and Traub, 2003). Any mutation in the D residues inactivates endocytic signals and results in increased expression of the transmembrane proteins at the cell surface, as is the case for the cation-independent mannose phosphate receptor (CI-MPR) (Chen et al., 1997; Bonaficino and Traub, 2003). Another example where the DXXLL signals are involved in trafficking is in the case of CI-MPR and the cation-depandant mannose phosphate receptor (CD-MPR), whose sequences, SFHDDSDEDLHI, and EESEERDDHLLPM respectively, mediate incorporation of their proteins into clathrin-coated pits that bud off from the TGN to move into the endosomal system (Puertollano et al., 2001; Doray et al., 2002).

Another family of sorting signals recently identified are acidic clusters of residues that are implicated in the retrieval from endosomes to the TGN. A
cluster of acidic amino acids is important in sorting the CI-MPR from the endosomal recycling compartment to the TGN, and also in sorting the v-SNARE, VAMP4, back to the TGN from recycling endosomes (Maxfield, 2007; Hong et al., 2007).

Apical sorting signals are less well defined than basolateral signals. Apical signals are localized in extra-cytoplasmic and membrane-associated domains of their proteins and are also comprised of lipid moieties, sugars or peptide motifs. The first apical sorting signal discovered was that of the apically-targeting GPI anchor found on the decay-accelerating factor (DAF) in MDCK cells (Rodriguez-Boulan, 1989). Other signals include N-glycans and O-glycans and GPI-anchored protein incorporation into lipid rafts that are sorted apically due to their affinity for microdomains of glycosphingolipids and cholesterol which are assembled in the Golgi (Scheiffele and Simons, 1995; Yeaman, 1997; Simons, 1988). Another set of apical targeting motifs are proteinaceous motifs present within the protein. The envelope glycoprotein of influenza virus HA, has its apical sorting signal present in the transmembrane domain and this allows association with lipid rafts (Scheiffele and Simons, 1997). The light-sensitive protein rhodopsin has an apical sorting signal located in its cytoplasmic domain, that mediates its binding to a light chain of dynein (Sung, 1998). The sorting signals encoded by amino acid sequences within membrane proteins are recognized by cellular machinery, typically in the form of adaptor proteins (APs), which bind to the signal sequences, concentrate the protein into a nascent vesicle, and facilitate their budding from donor membranes.

1.5 Adaptor Proteins in Epithelia

Protein transport between organelles of the secretory and endocytic pathways is mediated by vesicular membrane-bound carriers. Cargo proteins are packaged into these vesicles and pinch off from the membrane to be transported to the target organelle or membrane (Kuehn and Schekman, 1997). Adaptor proteins (AP) are cytosolic heterotetrameric complexes of adaptin molecules composed of two large subunits ~100kDa, (γβ1, αβ2, δβ3 and εβ4), one medium
subunit ~50 kDa (μ1-μ4), and one small subunit ~20kDa (σ1-σ4) (Nakatsu and Ohno, 2003). Four AP families have been identified to date; AP1-A/AP1-β, AP2, AP3-A/AP3-β and AP4 (Kirchhausen, 2000). Each AP complex has a characteristic distribution in the cell that underlies its function. AP1-β is epithelial cell-type specific and is involved in basolateral targeting of membrane proteins such as LDLR, recognizing the tyrosine-based sorting signal via its μ subunit (Meyer et al., 2000). AP1-A complexes are found at the TGN and endosomes and there interact via their μ1A subunits with cargo to concentrate them and direct them to endosomes. AP2 complexes work at the plasma membrane where they concentrate cell-surface receptors into clathrin-coated pits and internalize them (Folsch and Mellman, 2001). AP3 binds cargo at the TGN and endosomes, and plays a role in sorting cargo proteins that contain a tyrosine or a di-leucine motif to lysosomes (Peden et al., 2004). AP3-β is involved in synaptic vesicle formation and found specifically in neuronal cells (Blumstein et al., 2001). AP-4 is involved in two different sorting processes. In MDCK cells, the μ4 subunit interacts with different cargo proteins destined for the basolateral membrane such as the LDLR (low-density lipoprotein receptor) and with the CD-MPR. AP-4 is localized with the clathrin coat machinery in the Golgi complex and in the clathrin-coated membrane of early endosomes, and with the Cl-MPR (Barois and Bakke, 2005).

Apical APs have been elusive, perhaps in part due to the heterogeneity of sorting signals. Of the above APs, AP2, in conjunction with clathrin recycles proteins at the apical surface (Rodriguez-Boulan et al., 2005). Other apical regulators of vesicle movement are ARF6 and ARNO, members of the Ras GTPase family of proteins (Altschuler et al., 2003).

1.6 Clathrin Coats and Endocytosis

Vesicle coat proteins are part of the machinery required for cargo recruitment and vesicle formation. Clathrin, a well-characterized coat protein, works co-ordinately with APs to recognize a protein or lipid at the plasma
membrane or TGN and facilitate membrane invagination and endocytosis
(Kirchhaussen, 1999). Clathrin helps to stabilize the curvature of a growing pit
from which a vesicle will bud and increases the region of deformation to form a
closed vesicle. Clathrin is composed of three heavy-chain and three light-chain
molecules that come together to form a triskelion and these assemble into cages.
The cages act to facilitate the pinching off of vesicles in three stages; initiation of
coated-pit formation, propagation of the triskelion lattice, and closure or budding
of the membrane vesicle (Hirst and Robinson, 1998). Coated vesicle detachment
from the membrane requires a GTPase-containing protein, dynamin. Dynamin
forms concentric rings around the membrane from its origin and mechanically
squeezes the connection between the membrane and the forming vesicle. Once
the vesicle buds off, the clathrin lattices are disassembled and the adaptor
complexes are released from the membrane (Chen et al., 1991).

1.7 Microtubules, Actin and Molecular Motors

To achieve efficient delivery of cargo proteins to their target destinations,
there must exist an organized and coordinated sorting and transport network
within the cell. This delivery is efficiently executed by the use of cargo-
recognizing motor proteins and an extensive actin and microtubule (MT)
cytoskeletal network. MTs are components of the cytoskeleton that are
composed of long polymers of α and β-tubulin dimers, that polymerize end-to-
end in protofilaments, which then bundle to form hollow cylindrical filaments. MTs
have a fast growing plus end and a slow growing minus end and are thus highly
polarized and dynamic structures (Meads and Schroer, 1998). They are
nucleated and organized by a microtubule organizing centre (MTOC) and their
orientation is critical to maintaining cell polarity, morphology and vesicular
transport. MT orientation can vary greatly among different cell types. For
example, in non-polarized epithelial cells such as fibroblasts the arrangement is
in a radial array with the slow-growing minus ends emanating from the MTOC
and the plus ends extending to the cell cortex. In MDCK polarized epithelial cells,
MTs form vertical arrays with the negative ends facing the MTOC and arrays of
mixed polarity at the apical and basal poles (Bacallao et al., 1989). MT mixed polarity at the apical surface implies that plus-end-directed motors might also participate in apical delivery. It has also been found that upon MT disruption post-Golgi exocytosis is inhibited, and there is an increase in protein mis-sorting. These findings outline the importance of MTs in polarized protein sorting (Musch, 2004).

MT asymmetry allows for efficient polarized vesicle transport accomplished with the use of molecular motors of the kinesin and dynein families, and provides directional cues recognized by these motors. Kinesins and dyneins are somewhat structurally similar (Hirokawa and Takemura, 2005). They have two major parts that contribute to their cargo-binding transport functionality, a globular motor domain composed of two heavy chains that provide the energy for motion by hydrolyzing ATP and bind reversibly to the cytoskeleton, and a tail portion, which is composed of light chains that interact with the cargo. Dynein and kinesin tail domains are divergent from one another which provides for the diversity of cargo binding. Typically it is the light chain that binds the cargo or it interacts with the adaptor protein that is bound to cargo. It is thus important to understand how the light chain in both motors identifies the various and distinct cargo proteins. The cargo they transport ranges from organelles, protein complexes, complexes of nucleic acids and proteins, mRNAs and even MTs (Gelfand, 2002; Hirokawa and Takemura, 2005).

Kinesins and dyneins move along MTs. The majority of kinesin family members move along the plus-end of MTs in an anterograde fashion, and the dyneins move along the minus-ends of MTs retrogradely (Hirokawa and Takemura, 2005). For example, dynein interaction with the C-terminal end of rhodopsin through the dynein light chain Tctex is essential for the apical targeting of rhodopsin in MDCK cells (Tai and Sung, 2003). The kinesin-family member KIFC3, another minus-end-directed microtubule motor, has been associated with the apical delivery of influenza HA and annexin-13b (Lafont and Simons, 1994). Together, with the help of the specificity of APs for sorting signals, kinesins and
dyneins provide an efficient highly polarized transport system for cellular trafficking.

Organelles and other cargo are linked to kinesin and dynein motors via membrane/organelle-bound adaptor proteins such as kinectin or dynactin, respectively. Dynactin is a multiprotein complex that associates with dynein and mediates the attachment of some cargos to the motor and links these to MTs. The 1-MD dynein heavy chain dimer and the 300-kD p150\textsuperscript{Glued} dimer of the projecting arm of dynactin contact microtubules (Culver-Hanlon et al., 2006). The actin-related protein 1 (Arp1) subunit forms a short filament at the base of dynactin and can bind membrane-associated \beta\text{III} spectrin, which probably acts as the membrane receptor for the dynein–dynactin motor complex (Holleran et al., 2001; Muresan et al., 2001). HAP1, for example, which binds to huntingtin HD protein, has been shown to interact with the p150\textsuperscript{Glued} subunit of dynactin (Engelender, 1997). The C-terminal tail of kinesin interacts with the 160 kDa integral ER membrane kinectin protein (Toyoshima et al., 1992). Several proteins have been identified that bind to kinesins aside from kinectin which include the nerve growth factor receptor (NGFR), trkA, the JNK signal scaffolding proteins JIP1/2 and JIP3, \beta\text{-adaptin} clathrin adaptor protein, amyloid precursor protein APP and NMDA receptor-binding protein, mlin-10 (Kamal and Goldstein, 2001).

Actin filaments are found predominantly as a mesh-like network at the plasma membrane where they provide structural support and shape to the membrane, drive vesicle movement to and from the membrane, moderate protein activities while in the membrane, and restrict and retain proteins within the membrane. Actin exists either as a globular monomer (called G-actin) that contains an ATP binding site in the centre of the molecule or as a filament (designated F-actin), that is composed of polymers of G-actin. Two G-actin bind weakly, but addition of a third stabilizes the complex (Salas et al., 1993). This forms a nucleation site and adds additional molecules and polymerization is initiated. Actin polymerizes in a polarized fashion with monomer addition at the positive-ended actin filaments. The filament network formed interacts with a variety of different proteins that facilitate the variety of processes involving actin.
(Korn and Pantalloni, 1987). Actin not only acts at the plasma membrane. Actin filaments have been implicated in trafficking along the endocytic and secretory pathways, and particularly at the Golgi complex (Stamnes, 2002). Actin has been shown to have a role in vesicles leaving the Golgi in the anterograde pathway to the basolateral membrane in polarized cells (Müsch et al., 1997; Müsch et al., 2001; Duran et al., 2003). Additionally, the actin cytoskeleton is also important in materials leaving the Golgi in retrograde trafficking to the ER (Duran et al., 2003).

There are several mechanisms by which actin can facilitate movement. It has been proposed that the force generated by actin polymerization moves membranous vesicles through the cytoplasm. The focalized polymerization of actin via Cdc42, a Rho GTPase, at the Golgi is likened to a comet's tail that provides force to move the vesicle through the cytoplasm (Ridley, 2001). In addition to actin polymerization, actin motor proteins (myosins) also generate force, which can promote the formation of transport carriers and/or their movement away from Golgi cisternae. They assist in the formation of a vesicle carrier by providing force driving the constriction/scission of the neck of the forming vesicle (Krendel and Mooseker, 2005).

Myosins, like kinesins and dyneins, are unidirectional molecular motors involved in biological movements and transport events. Most myosin heavy chains consist of three distinct regions: an NH$_2$-terminal motor or head domain, responsible for actin binding and ATP hydrolysis; a neck region and a COOH-terminal tail, which is responsible for cargo binding and/or dimerization of heavy chains (Thompson and Langford, 2002). Myosins are involved in polarized transport in the cell. For example, the minus-end directed myosin VI is involved in the polarized basolateral delivery of membrane proteins in MDCK cells whose sorting is mediated by AP1-B (Sui-Yan Au et al., 2007).
TRAFFICKING IN NEURONS

1.8 Mechanisms of Neuronal Sorting

Similar to polarized epithelia many neurons also contain distinct functional membranous domains, the axon and the dendrites that must contain the proper complement of proteins to allow for information transmission. A pyramidal neuron of the central nervous system is comprised of a cell body, multiple short and thick dendrites, and a long, thin branching axon. They are highly polarized and compartmentalized cells, which underlies their specific function of the transmission of information from one cell to another (Mainen et al., 1996). Precise polarized protein targeting and localization is critical to every aspect of the neuron’s ability to function efficiently. Protein targeting in neurons is fundamentally the same as in polarized epithelia. It begins with the segregation of transmembrane proteins to distinct populations of carrier vesicles from the TGN. Their incorporation into transport vesicles is determined by the sorting signals they contain. They are then recognized by adaptor proteins and carried by motors along MTs to their specified destination.

The epithelia-neuron model has been a useful tool in attempting to identify trafficking and sorting mechanisms in neurons. Originally it was proposed that neurons and polarized epithelia shared common sorting signals (Dotti and Simons, 1990). In 1998, Jareb and Banker, employing rigorous measure of cell surface protein distributions, further investigated the parallels between the epithelia and neuronal equivalency sorting model. They expressed three basolateral marker proteins, mis-targeted sorting mutants of these proteins, three apical markers and an axonal protein in cultured hippocampal neurons. They found that the basolateral proteins, polyimmunoglobulin receptor (plgR) and low density lipoprotein receptor (LDLR) were all targeted to the dendrites. The apically-targeted proteins in MDCK cells that were expressed in neurons were p75/NGFR, HA, CD8α and the sorting mutant of plgR and LDLR, and were observed to have roughly equal distribution in both axons and dendrites (Jareb and Banker, 1998). The results confirmed the functional similarity between
basolateral sorting signals and dendritic sorting signals, although they also demonstrated that apical sorting information is not sufficient to target proteins to the axon. The basolateral/dendritic parallel model is a useful tool to study dendritic targeting, though the apical/axonal model is delusive and does not hold true in all instances.

The evidence of dendritic and basolateral parallels are seen within the sorting signals for the TfR and LDLR. Both are targeted to the dendrites by the same tyrosine motif that targets them to the basolateral membrane in epithelial cells (West et al., 1997; Matter and Mellman, 1992; Jareb and Banker, 1998). After expression of the receptors in neurons, mutation of these motifs results in disruption of somatodendritic targeting (Jareb and Baker, 1998), although it has not been shown whether they are sufficient to mediate somatodendritic targeting.

Motifs associated with dendritic targeting have also been found in ion channels and receptors that are endogenously expressed in neurons. The potassium channel, Kv4.2, a specifically somatodendritic transmembrane protein, has a 16 amino acid, dileucine-containing motif, that is both necessary and sufficient to mediate dendritic targeting of membrane proteins in neurons (Rivera et al., 2003). The epidermal growth factor receptor (EGFR) is basolaterally targeted in MDCK cells and when expressed in neurons is somatodendritic. The EGFR uses a sorting signal composed of a proline-based motif and two weaker dihydrophobic motifs (He et al., 2002). Mutations that disrupt its targeting in MDCK cells also disrupt its targeting in neurons (Silverman et al., 2005). Despite the importance of GluRs little is known about their sorting. Only large regions of 30 amino acids or more in the C termini of the metabotropic glutamate receptors mGluR2 (Stowell and Craig, 1999) and mGluR1a (Francesconi and Duvoisin, 2002) and of the AMPA receptor GluR1 have been identified (Ruberti and Dotti, 2000).

However, not all basolateral and dendritic signals overlap. Telencephalin (TLCN), a CAM found in the telencephalon brain segment, is strictly localized to the somatodendritic compartment (Oka et al., 1990; Murakami et al., 1991), and
is localized there by a 17 amino acid motif at the C terminus that contains a critical phenylalanine residue. This signal acts as a dendritic targeting signal in neurons but does not function as a basolateral targeting signal in epithelial cells (Saito et al., 2005). A notable interesting scenario is that of the glutamate transporter excitatory amino acid transporter 3, EAAT3, which is targeted apically in MDCK cells and dendritically in hippocampal neurons (Cheng et al., 2002).

Comparisons of dendritic/basolateral sorting eventually led to investigations into mechanisms of dendritic transport. Basolaterally targeted transmembrane proteins such as TfR are exclusively found at the dendritic surface when expressed in neurons. Axonal proteins such as NgCAM are trafficked to both axons and dendrites, although they are only concentrated in the axonal plasma membrane (Burack et al, 2000). Directed transport has been documented for various other dendritic proteins that include the EEAT3 excitatory amino acid transporter and the EGF receptor (Cheng et al. 2002; He et al., 2003). Therefore vesicles destined for dendrites are trafficked by direct delivery to the dendritic plasma membrane, whereas axonally targeted vesicles travel both into the dendrites and axons, but they are selectively retained only in the axonal membrane (Fig. 2). Thus, selectivity of transport in dendrites occurs at the level of direct transport from the Golgi, whereas axonal protein sorting occurs at the level of retention. It is the specific sorting signals in each of the cargo proteins that determine the path the protein will take.
Figure 2. Direct Trafficking and Selective Retention in Neurons.

(A) Dendritic proteins (red) are sorted and then transported in a subpopulation of transport vesicles destined for dendrites.
(B) Axonal proteins (blue) are not selectively sorted, and thus these transport vesicles are found in dendrites as well as the axon.
(Shah and Goldstein, 2003)

Additionally, axonal targeting signals have been described that are involved in direct axonal transport routes as well as transcytic routes (Sampo et al., 2003; Wisco et al., 2003). For example, NgCAM, an axonal transmembrane cell adhesion molecule in neurons (Burack et al., 2000; Winckler et al., 1999), is also apically targeted in MDCK cells (Anderson et al., 2005). When NgCAM is observed intracellularly, it is not polarized to axons but also has carriers located within the dendrites. Interestingly, NgCAM inserted at the cell surface is highly polarized to the axon and nearly absent from the soma and dendrites (Burack et al., 2000). A proposed mechanism of trafficking as described by Wisco et al. (2003) suggests that NgCAM accumulates preferentially on the axonal membrane. Signals necessary for this transcytotic pathway, both an ankyrin binding domain and a YXXΦ endocytic signal, were also found in the cytoplasmic tail of the protein (Schaefer et al., 2002). The extracellular domain contains an
axon targeting determinant containing fibronectin type III-like repeats (Sampo et al., 2003). During the initial time it occupies in dendrites, NgCAM overlaps transiently with the endosomal markers transferrin (Tf) and EEA1. It then sorts away from Tf into mobile carriers and via fast axonal transport is sorted into the axon and retained in the axonal plasma membrane (Yap et al., 2008). The same dendritic delivery and transcytotic behavior is seen with VAMP2, a synaptic vesicle v-SNARE that is also present on the axonal surface (Taubenblatt et al., 1999; Sampo et al., 2003), however its targeting sequence has not yet been described.

Though less well defined and frequent than basolateral sorting signals, parallels between apical and axonal sorting are present in some proteins and absent in others. Although typically dendritic, one type of glutamate receptor is targeted to the axon, specifically the presynaptic bouton. The axonal sorting of mGluR1b has a tripeptide (RRK) axon-targeting signal in its cytoplasmic tail which acts recessively to basolateral or dendritic targeting signals (Francesconi, 2002). The sodium channel Nav1.2, which is localized at the axon initial segment, is targeted by a 27 amino acid residue motif that contains clusters of acidic amino acids (Garrido et al., 2003). Amyloid precursor protein (APP) is used as a model to study the signals for axonal transport in neurons. APP is a ubiquitously expressed transmembrane glycoprotein with a large N-terminal extracellular domain, a single transmembrane domain and a short 47 residue cytoplasmic tail (Kang et al., 1987). It contains within its extracellular domain a βA4-dependant signal and a weaker N-glycosylation signal that directs it to the axon (Tienari et al., 1996).

APP sorting in hippocampal neurons compared to that in MDCK cells demonstrates that these cell types decode slightly different signals from APP (Sheets et al., 1998). Newly synthesized APP is sorted axonally in neurons and basolaterally in epithelial cells. APP holoprotein transport is regulated by ectodomain determinants in neurons, whereas in MDCK cells it is regulated by signals in the cytoplasmic tail (De Strooper et al., 1995; Haas et al., 1995). These examples demonstrate that the same sorting signals will not always target a
protein to equivalent regions of epithelial and neuronal cells. This suggests there must be a difference in the factors and machinery whose function is to recognize and sort the targeting signals within each cell type.

1.9 Neuronal Adaptor Proteins

In neuronal cells, sorting signals are used as a means to polarize membrane proteins to the axon or the dendrites, and adaptor proteins are most often required for the sorting of these proteins. Neuronal adaptor protein complexes have been discovered for endocytic pathways, however very little is known about adaptor proteins that are responsible for post-Golgi network trafficking of neuronal transmembrane proteins (Nakatsu and Ohno, 2003). A potential model for neuronal adaptor proteins can be inferred from adaptor proteins of polarized epithelial cells that bind to basolateral sorting signals and are involved in basolateral sorting. Although it has not yet been tested, AP4 is a likely candidate because it binds to the tyrosine sorting signal present in furin, LDLR, MPR46 and TfR and thereby mediates their basolateral sorting in MDCK cells (Simmen et al., 2002). It was also shown that AP1-β uses a similar tyrosine-based signal to localize LDLR and TfR in the porcine kidney cell line LLC-PK1 (Sugimoto et al., 2002). As evidenced by this basolateral and dendritic sorting parallel, it is conceivable that there is an adaptor protein in neurons that acts similarly to AP-4 in polarized epithelia. It should be noted however that not all proteins depend on AP-4 for basolateral sorting, thereby implicating other adaptors working in parallel.

One neuronal specific adaptor has been identified; the AP-3 complex. The AP-3 complex is unique among adaptors because three of its subunits are encoded by two alternative genes: β3A/β3B, μ3A/μ3B, and σ3A/σ3B (Dell'Angelica et al., 1997; Simpson et al., 1997). β3B and μ3B are exclusively expressed in neurons, suggesting that they assemble AP-3 complexes for the purpose of neuron-specific sorting (Pevsner et al., 1994; Newman et al., 1995). The neuronal and ubiquitously expressed subunits have nonredundant functions, and thus the AP3 complexes functions are divergent and distinct (Seong et al.,
Neuronal AP3 complexes are expressed in the cell body, axons and dendrites, whereas ubiquitously expressed AP3 complexes are present only in the cell bodies (Seong et al., 2005). Both AP-3 forms reside in the same endosome. Neuronal AP-3 sorts proteins into the axon or a synaptic vesicle pathway and generates synaptic vesicles, while ubiquitous AP-3 sorts proteins to the late endosomal or lysosomal pathway (Ohno, 2006; Robinson, 2004). Both the μ3A and μ3B subunits present in ubiquitous and neuronal AP-3 complexes recognize different tyrosine-based sorting motifs (Ohno et al., 1998).

Recently, it was shown that protein kinase D1 (PKD-1), a member of the DAG-binding Ser/Thr kinases, acts within the Golgi and mediates sorting of dendritically targeted membrane proteins leaving the TGN. PKD-1 knockdown disrupts the intracellular trafficking of TfR and LDLR, and they are sorted into a vesicle population that contains VAMP2 and are sorted into both the axon and the dendrites (Bisbal et al., 2008). Thus protein sorting occurs at the level of biogenesis of Golgi-derived dendritic vesicles.

1.10 Neuronal Microtubules and Motor Proteins

Prior to the establishment of polarity, neurite MTs are of uniform polarity with plus-ends distal to the cell body. Once polarity has been initiated, the axon becomes specified and only then do the other neurites become specified as dendrites. The dendritic MTs in the proximal region become of mixed orientation, and in the distal region they face only plus-end distal (Baas et al., 1989). The axon retains its plus-end distal MT orientation, and at its hillock forms a barrier that deters diffusion of proteins from dendrites to the axon (Winckler, 1999; Chieko et al, 2003). The establishment of a unique pattern of MT tracks allows for molecular motors to begin polarized transport to the cell periphery. Dendritic proteins such as TfR are only ever trafficked in the dendrites and do not enter the axon, which indicates that polarity must be established by selective MT-based transport (Burack et al., 2000). There must then be a mechanism that targets axonal and dendritic proteins to the pre-ordained locations.
MTs, or modifications on MTs present an opportunity for efficient polarized vesicle targeting accomplished with the use of molecular motors of the kinesin and dynein families. For example, KIF5, takes a directional cue from MTs, as its preference is for MTs at the axon initial segment in neurons (Nakata and Hirokawa, 2003). MTs in both dendrites and axons are found to be of the plus-end distal form, therefore KIF5 must be able to distinguish between the MT populations, and their interaction at the axon initial segment must contribute to polarized axonal transport.

Another example of directional motor-cargo interactions was shown with the KIF17 motor, also called a ‘smart’ motor because it can recognize dendritic MTs, traffics glutamate NMDA receptors directly to the dendritic membrane at postsynaptic sites, while it remains absent from the axon (Setou et al., 2000). The NMDA receptor is made up of 2 subunits, NR2β and NR1, of which the NR2β is bound by a mln-10 complex to Kif17, a neuronal-enriched plus-end directed motor, at NR2β’s COOH terminal PDZ domain (Moriyoshi et al., 1991; Meguro et al., 1992; Sheng et al. 1994; Setou et al. 2000). Kif17 thus specifically will traffic the NMDA receptor to dendrites. This depicts mechanistically one specific example of selective dendritic transport based on the motor and cargo interaction.

How are axonal proteins trafficked to establish polarity if they can enter both dendrites and the axon? Both the dendrites and axons have plus-ended MTs, and consequently both utilize plus-end directed motors (Horton and Ehlers, 2003). It has been shown that, unlike with Kif17 that traffics only to the dendrites and thus displays selective transport, axonally targeted proteins such as VAMP2 are not selective and rely on fusion and retention in the axonal membrane. Thus the cargo must define the role for the molecular motors that transport axonally-targeted proteins. Because the cargo provides the directionality of trafficking, the sorting signals it contains must be the drivers for the motors. It is important to understand the variety of ways that sorting signals will interact with adaptor proteins and consequently motors to identify a, or several, clear mechanism(s) that involve trafficking of axonal and dendritic proteins.
ESTABLISHING POLARITY IN NEURONS

1.11 Cultured Hippocampal Neurons

Hippocampal neurons are the primary accepted model system to study neuronal polarity and are used exclusively in this study. Hippocampal neurons in culture can recapitulate those in vivo in form and function, thus allowing for a detailed means of studying cell polarity and molecular trafficking in neurons (Goslin and Banker, 1989). This is supported by examples such as the dendritic microtubule associated protein, MAP2 and axonal GAP-43 growth-associated protein that will localize in cultured hippocampal neurons as they would in vivo. Additionally, if cultured at low density where the cells are isolated from one another, the cells will still polarize. This implies that there is an endogenous cellular genetic program that governs polarity (Craig and Banker, 1994).

Hippocampal neurons consist of a cell body, a single, long, branching axon that is of uniform caliber, and several shorter, thicker dendrites that taper along their whole length and are amassed with synapses. In culture they develop in five defined stages (Fig.3). When placed in culture cells will adhere to the substratum within the first hour or two and then change appearance by the development of lamellipodia around the cell body (Dotti and Banker, 1988). Approximately 6 hours post-plating, the dissociated neurons extend from the lamellipodia 4-7 small neurites. Between 24-48hrs in culture, after a seeming 'dance' that the neurites undergo, stretching and retracting, one major process will become twice the length of the other processes, then this typically becomes the axon. The default state of the other shorter neurites is dendritic (Dotti and Banker, 1988). Polarity has now been established in the cell. It has been proposed that the mechanism that determines axon specification is a biochemical tug-of-war, whereby the axon is the winning neurite to receive less inhibition from its neighbors and more growth-promoting signals (Da Silva et al., 2005). The now-defined axon and dendrites continue to develop morphological, biochemical and structural characteristic properties. These properties allow the cell to assume
its physiological function by establishing biochemical signal-transduction cascades, which are dependent for activation of the synapse.

![Diagram of neuron stages](image)

**Figure 3. Growth stages of hippocampal neurons in culture.**

Hippocampal neurons in culture develop in predictable stages. Following plating neurons begin as a round cell body haloed by lamellipodia from which 4-5 neurites will develop. In just over a day one neurite will extend out and become an axon followed by dendritic outgrowth.

*(Dotti and Banker, 1988)*

### 1.12 Synapse Structure and Formation

Synapses are junctions that allow the transmission of information from a neuron to another cell. Synapses must be polarized structures to allow for an efficient functional connection. The formation of synapses in the vertebrate nervous system occurs over a protracted period of development, beginning in the embryo and extending into early postnatal life *(Mcburney et al., 1988)*. It also occurs in the adult, the mechanisms of which have been shown to be involved in learning and memory. In the vertebrate nervous system, most neurons communicate via chemical synapses, which transduce electrical signals in the form of action potentials into chemical signals, in the form of neurotransmitters, and then back to electrical impulses in the dendrite. Synaptic vesicles containing neurotransmitters are found docked at the presynaptic bouton at the tip of the axonal plasma membrane, and once an action potential arrives and depolarizes the cell, the vesicles fuse with the membrane and release their cargo into the synaptic cleft. The neurotransmitters diffuse across the cleft where they bind to
neurotransmitter-gated ion channels that are present in the postsynaptic dendritic membrane (Craig, 2005).

The ion channels present in the dendritic plasma membrane are concentrated into clusters throughout the membrane called the post-synaptic density (PSD). The PSD is characterized by not only these ion channels, but an electron-dense meshwork of proteins that contains neurotransmitter receptors, various second-messenger molecules, a variety of scaffolding proteins and cell adhesion molecules (CAMs) (Breseler et al., 2004). Many of these molecules contain PDZ-binding domains, which allow them to interact with various PDZ proteins that comprise the postsynaptic density, such as PSD-95 and glutamate receptors (Craig, 2005). The assembly of this complex of proteins occurs as a gradual accumulation of molecules, the first of which is PSD-95, followed by AMPA and NMDA glutamate receptors (Qin et al., 2001). The presynaptic bouton protein recruitment appears to differ, involving presynaptic components being trafficked in pleiomorphic vesicles containing preassembled components of the complex (Ahmari et al., 2000).

Synapse formation is a highly dynamic and regulated process essential for neurodevelopment and plasticity. Synapses between mature neurons can be assembled and disassembled rapidly and depends on the adhesion systems between the pre- and postsynaptic neurons. Specific signals ensure directionality to generate the fundamentally different structures of pre- and postsynaptic terminals. Differentiation of pre- and postsynaptic membrane specializations can occur once initial axon-target interactions develop and is aided by signaling molecules which engage in bidirectional signaling. Several classes of molecules are capable of directly inducing various aspects of synapse formation, and these include both secreted proteins as well as CAMs (Scheiffele et al., 2000).

1.13 Neuroligins

The roles of neuroligins (NLGs) have become studied in depth based on their role in synaptogenesis and neurological disorders (Levinson et al., 2005;
Scheiffele et al., 2000; Chih et al., 2004). NLGs belong to family of brain-specific cell adhesion molecules present at the synapses: NLG-1, NLG-2, NLG-3, and NLG-4. NLG-1 was originally identified in an affinity assay by associating with its binding partner, β-neurexin, another cell adhesion molecule located at the presynaptic bouton and which is bound to synaptic proteins such as CASK and Mints and synaptotagmin. NLG-1 is a type 1 transmembrane protein with a large extracellular domain, a single transmembrane region, and a cytoplasmic tail (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Biederer and Sudhof, 2000). Its cytoplasmic domain interacts via its PDZ binding region at the postsynaptic density with the structural molecule postsynaptic density molecule PSD-95. PSD-95 is a scaffolding molecule located at PSDs and contains 3 PDZ domains that bind to the N-methyl-D-aspartate receptor subunit 2 (NMDA2R), voltage-gated potassium ion channels, and NLG-1. The extracellular domain of NLG-1 consists of 2 EF-hand (a helix-loop-helix structural domain) motifs that bind Ca+2, a cholinesterase-like domain (CLD) that contains the β-neurexin binding site, and an O-glycosylation region (Fig. 4) (Lise and El Husseini, 2006). The extracellular domain contains a variable region and a catalytically inactive CLD region (which lacks an active site serine) that allows the receptor to participate in receptor/ligand-like interaction (Ichtchenko et al., 1995, Ichtchenko et al., 1996). This interaction is achieved with the extracellular domain of NLG-1 binding to the extracellular domain of β-NRX, and achieves to promote synaptic cell adhesion and assemble pre and post-synaptic specializations intracellularly (Dean and Dresbach, 2006; Lise and El Husseini, 2006).
Figure 4. Structure and Domains of NLG-1 and β-Neurexin

Neuroligins: consist of a cytoplasmic C-terminal tail that contains a PDZ-binding domain, a transmembrane domain, and an extracellular domain that contains a shared sequence homology with acetylcholinesterase and an O-glycosylation region. Orange: the acetylcholinesterase homology domain and is necessary for β-neurexin binding. Pink: oligomerization domain. β-neurexins: extracellular N-terminal sequence. Purple: LNS domain that binds to neuroligins, an O-glycosylation region, a transmembrane (TM) domain, and a cytoplasmic tail that contains a PDZ interaction site on the C-terminus.
(Adapted from Dean and Dresbach, 2006)

1.14 Neuroligins and Synapse Formation

There are two types of synapses that are formed: excitatory and inhibitory. The expression of NLG-1 and β-NRX in heterologous cells establishes the specificity of excitatory synapse formation. Scheiffele et al. (2000) demonstrated that NLG-1, when expressed in HEK293 cells and co-cultured with explants from pontine axons, induce presynaptic structure development in the contacting axon. Synapsin-1 in the pontine axons was demonstrated to accumulate and cluster at the terminal bouton presynaptically and recruit ion channels and neurotransmitter receptors postsynaptically (Scheiffele et al., 2000; Dean et al., 2003). Scheiffele also demonstrated that formation of NLG-1 multimers is necessary for its activity, as well as binding to β-NRX (Fig. 5), (Scheiffele et al., 2003). NLG-mediated adhesion promotes the functional recruitment of post-synaptic NMDA receptors via the interaction with PSD-95 in non-neuronal cells, suggesting that β-NRX-NLG-1 adhesion complexes provide a platform for postsynaptic neurotransmitter receptor recruitment and promote synapse assembly in a bidirectional manner (Washbourne et al., 2004).
Figure 5. Molecular Interactions at the Synapse

The cytoplasmic tails of neuroligins and β-neurexins contain a C-terminal PDZ-binding domain. β-neurexin binds the scaffolding proteins via the PDZ-binding domain and brings together components of neurotransmitter release machinery. Neuroligin contains a PDZ-binding motif that binds postsynaptic scaffolding proteins, including PSD-95, S-SCAM and others. PSD-95 and S-SCAM link neuroligin to glutamate receptor recruitment. (Adapted from Dean and Dresbach, 2006)

1.15 Neuroligins and Autism

Autism is a brain developmental brain disorder that impairs social interaction and communication, and causes restricted and repetitive behavior in children even prior to 3 years of age. This corresponds to the developmental time period where neuronal elaboration and remodeling in children occurs (Chih et al., 2004). It is highly heritable, although the genes responsible are still ambiguous. NLGs have been implicated in the cause of autistic disorders. There are five NLG gene forms in humans: NLG-1, -2, -3, -4, and -4Y, and 3 of those genes (NLG-1, -3, and -4) map to loci which are associated with a predisposition to autism (Chih et al., 2004). Mutations in the genes that encode NLG-3 and NLG-4 genes on the X chromosome and the gene that encodes for NLG-4Y is encoded on the chromosome. This helps to explain the fact that incidence in males is four-fold higher than in females (Lord et al., 2000; Volkmar and Pauls, 2003). In dissociated cultured hippocampal neurons the mutant neuroligins NLG-3 and
NLG-4 genes were retained in the ER and not properly delivered to the synapse, and thereby reducing the number of synapses formed (Chih et al., 2004). This example underscores the importance of proper protein sorting in neurons. In addition, a decrease in dendritic branching and arborization has been noted in autistic individuals, characteristic of neuroligin deficiency in culture (Chubykin et al., 2005; Dean and Dresbach, 2006). NLGs involvement in synapse formation and neural development exemplifies the requirement for proper sorting, targeting and retention at the synapse.

1.16 Neuroligin-1: Subcellular Localization and Mechanism of Polarization

In 2005, Rosales et al. investigated the cytoplasmic sequence that directs the dendritic targeting of NLG-1. They demonstrated that NLG-1 is polarized to the dendritic surface and that a 32 amino acid sequence in the cytoplasmic domain of NLG-1 was necessary and sufficient for targeting. Within this 32 amino acid sequence two motifs were identified that are responsible for the dendritic targeting of other neuronal proteins. A tyrosine motif is found, which targets the low density lipoprotein receptor (LDLR) and the tyrosine receptor (TfR) to dendrites (Jareb and Banker, 1998). The second motif includes a Val-Val and Val-Leu that conforms to the hydrophobic motif similar to that required for the sorting of the K+ channel Kv4.2 to dendrites (Rivera et al., 2003). Notably, by chimeric and deletion analysis they demonstrated that the extracellular domain was not involved in sorting of the WT protein. This study set out to identify the specific amino acid motifs that are required to target NLG-1 to the dendritic membrane. Initially it was investigated whether one, or both, of these motifs are involved in the dendritic polarization of NLG. Amino acid substitutions within NLG were created, one for each identified motif, and also a third mutant that had both motifs altered. Mutations in the two putative motifs disrupt the dendritic targeting of NLG-1, although not to the level of an unpolarized protein. There was no additive effect with the combined point mutations in the double mutant construct.

Within the 32 amino acid there lies another possible motif, an acidic couplet (DD) of which similar sequences have been implicated in other
membrane protein endocytosis and trafficking. Acidic patches consist of a wide variety of acidic amino acid combinations and their roles vary widely based on the specific protein they comprise. For example, many proteins are trafficked back to the Golgi after endocytosis, such as the v-SNARE protein VAMP4, via a well-characterized distal acidic cluster that is involved in the efficient delivery of VAMP4 from the recycling endosome to the TGN (Hong et al., 2007). Mutations and C-terminal truncation constructs were created to determine the role of the aspartic acid couplet in the NLG-1 32 amino acid sorting sequence. Using quantitative, wide-field fluorescence microscopy, this thesis demonstrates that a small acidic couplet within the 32 amino acid cytoplasmic domain of NLG-1 contributes to the polarization of NLG-1 to the dendrites, and that all three sorting motifs, VVL, YTLA, and DD, are required for the proper polarization of NLG-1 to the dendritic membrane.

1.17 Neuroligin Trafficking

Trafficking of membrane proteins can occur by either intracellular transport followed by deposition into the membrane or by insertion into the membrane at a non-target location and diffusing to its final location. This study also focused on identifying the targeting mechanism of NLG-1 to its location in the dendritic membrane. To investigate intracellular routes of NLG-1 transport, a NLG construct containing an HA tag for cell surface detection via immunocytochemistry, as well as a GFP sequence for intracellular detection via direct fluorescence was made. HA-GFP-NLG was co-expressed with several other proteins that have been characterized to be transported via a vesicular system, including the proximo-somatodendritically targeted Kv2.1 potassium channel (Rivera et al., 2005), the dendritically targeted transferrin receptor (Burack et al., 2000), and the axonally targeted protein NgCAM (Jareb and Banker, 1998). To characterize NLG-1’s behavior in an endocytic population, Tf was added as a free ligand which functions for marking endocytic uptake from the cell surface into early endosomes (Richardson and Ponka, 1997). Using Z-stack acquisition, confocal imaging and deconvolution, expressed fluorescent
proteins were colocalized in a variety of pair-wise and triplicate combinations to identify trafficking relationships. Based on the relationships established via correlation coefficients and colocalization between the various protein combinations a model for the behavior of NLG trafficking is proposed.

This thesis identifies the sorting motifs involved in NLG-1’s dendritic targeting and additionally introduces possible models of intracellular trafficking behavior of NLG-1 in hippocampal neurons. This work is important in understanding the molecular and cellular aspects of synapse formation and maintenance and thus neuronal development.
CHAPTER 2: METHODS

2.1 Hippocampal Cell Culture and Expression of Transgenes

Primary cultures of dissociated neurons from rat embryonic day 18 (E18) hippocampi were prepared essentially as described (Kaech and Banker, 2006). The dissociated neurons are plated onto poly-L-lysine pretreated glass coverslips and then the coverslips are placed with the neurons facing down. After 8 days in vitro, the neurons were co-transfected with a plasmid containing the protein of interest and for all experiments involving sorting signal identification, a plasmid containing enhanced green fluorescent protein (eGFP), pJPA-eGFP (Sampo et al., 2003). In preparation for transfection, the DNA (1 µg of each plasmid) was mixed with Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions as demonstrated by Sampo et al., 2003. Coverslips were then placed neuron-side down and neurons were allowed to express for a time conditional to each experiment from anywhere between 14-24hrs at 37°C under a controlled atmosphere containing 5% CO2.

2.2 Constructs

Each neuroligin construct was subcloned into the plasmid JPA (pJPA) for optimal neuronal expression (Sampo et al., 2003). Point mutations were created on both the tyrosine and hydrophobic motifs by utilizing mutagenizing primers and overlap PCR (Fisher et al., 1997). The external primers used for the PCR were the same for all the reactions. The external 5’ primer (5’NLG-ext) GTTCTAAGCTTGGGACCATG is complementary to the HindIII site near the 5’ end of neuroligin and the external 3’ primer (3’NLG-ext) GGTCCCTCTAGAGTGC is complementary to the XbaI site near to the 3’ end of neuroligin. Complementary internal overlap primers, 3’NLG-YTLA mutant AGGTGACCTCCTCATAGCAGCAGCAGCATCTGGG and 5’NLG-YTLA
CCCAGATGCTGCTGCTGCTATGAGGAGGTCACCTG provided mutations for the tyrosine motif. 3’NLG-VVL mutant CAGGCGGTCCGAGCAGCCGCCTCATG and 5’NLG-VVL mutant CATGAGGCGGCTGCTCGGACCGCCTG provided mutations for the hydrophobic motif. The overlap primers replaced the two motifs with alanines. Standard inverse PCR as described by Fisher and Pei (1997) was used to generate CD8-NLG constructs. To generate CD8-NLG-WT, 5’CD8-NLG Bspl primer was used on CD8-NLG construct (5’Phos/GCG GAT CCA GCT AAG CAA TAC TAC AAG) with 3’CD8-NLG primer (5’Phos/GAC GTA TCT CGC CGA AAG GCT GGG) to replace the AgeI site with a Bspl restriction site. CD8-NLG VVL was made with the CD8-NLG PCR product and primers to mutate NLG-VVL were 5’NLG-mut primer (ATA TGC TAA GCC TGC GGC TGC TCG G) and 3’NLG-Xbal-stop primer GCT CTA AGC CTG TGG TIC TIC GG. To generate NLG-YTLA insert, 5’NLG-WT primer was used TAG CTA AGC CTG TGG TIC TIC GG with 3’NLG-Xbal-stop primer as previously described onto NLG-YTLA template. NLG-VVL-YTLA insert was generated using 5’NLG-mut primer and 3’NLG-Xbal-stop primer. The CD8-NLG product was digested with Dpnl and ligated, transformed into E.coli and the isolated plasmid was then used as the parent vector for the point mutant sequence insert subcloning.

For the cloning involving CD8-NLG-3Mut: point mutations were created in the aspartic acid acidic couplet by utilizing mutagenized primers in fusing DNA fragments by inverse PCR. The 5’NLG-VVL-YTLA-DD primer /5Phos/TCA CCT GCC GCG ATT CCA CTA ATG was used with 3’NLG-VVL-YTLA-DD primer /5Phos/CTA CGA CGA CGA CGA TAC TCC TCC in a standard inverse PCR technique to incorporate alanine point mutations into the two aspartic acid residues. All CD8-NLG mutant constructs contain only the 32 amino acid sorting portion of the cytoplasmic domain.

NLG-WT C-terminal truncation and NLG-VVL-YTLA C-terminal truncation constructs were generated using inverse PCR and incorporating a stop codon and Xbal site after the YTLA residues, as performed by GenScript Corporation, USA. Fidelity of all mutations was confirmed by DNA sequencing.
HA-TEV-GFP-NLG was made from the full length HA-NLG-WT plasmid by first incorporating a TEV cleavage site after the HA sequence in the extracellular domain by standard overlap PCR using two 5' primers; 5’NLG-HindIII (GTTCTAAGCTTGGGACCATG) and HA-TEV-reverse (5’ATTTTCCGGTCAATTAGGTGCGAGCTGCCTGAAAGTACAGGTTCCTCGG AACCACATGTTCCTGCCTAGTCCGGAACGTC3’) with the 3’ sequence 1plusNLG (5’ATTTTCGGTCAATTAGGTGCGAGTCTGAGAC). Then 3’NLG-Xbal (AGACTCTAGACTATACCTGGTGGTTGAAT3’) and TEVAgel BsRGI-NLG (TAATTTCGGCAGGAAATCTGTATTTTCAGGGGATAGGACCGGTGACGGTCT G TACAGGGGAGTGAGACTACTCCACAGAGCTAAAG) and 2plusNLG (TAATTTCGGCAGGAAATCTGTATTTTCAGGGGATAGGACCGGTGACGGTCT G TACAGGGGAGTGAGACTACTCCACAGAGCTAAAG) and the products of the two reactions were run together with 5’NLG-HindIII and 3’NLG-Xbal primers to generate the overlap product. The products were then digested with appropriate restriction enzymes and ligated to make HA-TEV-NLG. The GFP was incorporated by subcloning the GFP sequence in behind the TEV sequence.

CMV-Rab11b-RFP plasmid was kindly provided by Dr. Amandio Vieira, Simon Fraser University, Canada. TfR-YFP-Mutant was a gift from Dr. Gary Banker, Oregon Health Sciences University, USA. Kv2.1-GFP was kindly provided by Dr. James Trimmer, University of California, USA. ER-CFP construct was purchased from Clontech, USA.

2.3 Immunostaining

To detect expressed proteins on the cell surface of live neurons, the primary antibody against the influenza HA-epitope-tag (1:500) was diluted in culture medium and placed on wax in a humidifying chamber at 37 °C. For CD8 constructs, antibody against CD8 was used (1:250). Coverslips containing transfected cells were exposed to the primary antibody-containing medium. After 8mins, the cells were rinsed in phosphate buffered saline (PBS) and fixed in a solution of 4% paraformaldehyde/4% sucrose in PBS. Cells were incubated in fish skin gelatin and BSA (0.5%, 10%, Sigma) in PBS for 1hr at 37°C to block nonspecific antibody-binding sites. Finally, coverslips were incubated with the
appropriate fluorescently conjugated secondary antibody for 1 hr at 37 °C and mounted on slides with Elvanol (Banker and Goslin, 1998). For intracellular staining, coverslips were fixed immediately, placed into a solution of 0.25% Triton X-100 for 5 mins and then rinsed in 1 XPBS and placed into fish skin gelatin and BSA for block at 1 hr at 37 °C. The appropriate primary antibody was diluted in fish skin gelatin and BSA and placed on wax in a humidifying chamber at 37 °C for 1-2 hrs. Subsequent steps are as described above for secondary antibody staining. A complete list of antibodies used is found in Table 1.

2.4 Microscopy and Quantitative Measurements of Immunofluorescence

Images of fluorescently labeled cells were visualized using standard wide-field fluorescent microscope (DMI 6000B, Leica), and images were taken with a digital fluorescence cooled CCD camera (Orca-ER Hamamatsu Photonics) controlled by MetaMorph Software (Universal Imaging, Downingtown, PA). Images were taken with a 20X 0.75 N.A. plan apochromat or 63X 1.4 plan apochromat objective (Leica). Exposure time was adjusted so that the maximum pixel value did not reach saturation. Only co-transfected cells whose labeled processes did not overlap with other labeled cells were selected for quantification.

All of the image processing was performed using MetaMorph (Molecular Devices, U.S.A.) software. Image analysis for Dendrite:Axon (D:A) ratio data was performed essentially as described by Sampo et. al (2003). After acquiring the image, two corrections were performed on the live-stained image before analyzing its fluorescence levels. First, a shading-correction was applied to compensate for the uneven illumination of the field (based on an image of a uniformly fluorescent specimen). Next, the average background calculated from several regions that did not contain labelled neurites was subtracted and this corrected image was used for analysis. To quantify the fluorescence in the cellular processes of the live-stained cells, several one-pixel wide lines (300-500 microns total length) were drawn medially along the axon and dendrites on the GFP image. The line traces were transferred onto the corrected images and the
average fluorescence intensity of those lines was tabulated. An image of that same neuron labeled for MAP2 (anti-MAP2, 1:1000, Chemicon) was used to assist in identifying dendrites. The average intensities from the axon and dendrites of each construct was calculated from 10-15 cells from at least three different cultures. Data was entered into an excel spreadsheet and used to generate (D:A) ratios. Statistical significance was assessed using a t-test for independent groups and a Tukey's test as a comparison of multiple means using JMP7 software. The natural log of the D:A ratios was taken to obtain a normal distribution and these values were used for statistical comparisons. To ensure that overexpression was not influencing protein distribution, all constructs were expressed for the minimum time where cell surface staining could be detected (12 hours).

Table 1. Summary of Primary and Secondary Antibodies used in Immunocytochemistry.

<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Source</th>
<th>Manufacturer</th>
<th>Secondary Ab</th>
<th>Source</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HA (3F10)</td>
<td>Rat 1:500</td>
<td>Roche, Mannheim, Germany</td>
<td>α-Rat-CY3</td>
<td>Goat 1:500</td>
<td>Jackson ImmunoResearch, West Grove, PA</td>
</tr>
<tr>
<td>α-MAP2</td>
<td>Mouse 1:1000</td>
<td>Chemicon</td>
<td>α-Mouse AlexaFluoro 350</td>
<td>Goat 1:500</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>α-CD8</td>
<td>Mouse 1:250</td>
<td>DAKO Corp</td>
<td>α-Mouse CY3</td>
<td>Goat 1:500</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
</tbody>
</table>

2.5 Colocalization/Correlation Analysis

Neurons were fixed and mounted unless transferrin uptake was required, whereby cells were then incubated live with 20μg/ml Alexa-Fluoro-647-transferrin (Molecular Probes) dissolved in water for 8mins to label the early endocytic pathway. Fixed neurons expressing a variety of fluorescently tagged constructs in various combinations were analyzed by deconvolution. A series of images,
through-focal (20 planes, approx. 0.2um apart) were acquired using a 63X, 1.32 N.A. Plan Apo objective, then processed by adaptive blind deconvolution (3 iterations) using Autoquant Deconvolution Software (Mediacybernetics). Single planes from image stacks were overlaid and subjected to correlation analysis using the Correlation Plot function in Metamorph. Thresholds for fluorescence intensity were set for each image and for each overlay to eliminate background.

Analysis using the correlation function in Metamorph software provides an effective measure of overlap in a pair of deconvolved images. This method not only measures the presence of pixel labelling in both channels, but also the level of intensity in the signal from the two channels and is sensitive to small differences in spatial distribution of fluorescence. The analysis generates a coefficient (r). This is a measure of the correlation of fluorescence intensity in two channels, and whose scale runs from 1, being a perfect correlation, to 0, which indicates no correlation (Costes et al., 2004). Correlation plots depict graphically the pixel intensity to overlap ratio (for details see Fig. 19, Appendix 1). To confirm reproducibility and accuracy of this analysis, colocalization analysis was performed in conjunction with several of the protein pair-wise combinations. This identifies individual puncta by using judgement from the analyst in thresholding and defining the dimensions and intensity parameters for each deconvolved structure used in the analysis. The values generated were consistent and congruent among all co-analyzed images. Fluorescence from cell bodies was not included in the analysis and regions were drawn around the dendrites for colocalization. Cell surface fluorescence does not contribute significantly to intracellular staining. This was shown by deconvolution of cell-surface stained images that were thresholded and compared to intracellular fluorescence. Upon thresholding to focus on signal over background, no significant amount of fluorescence contributed to the analysis. Statistical significance for the differences in correlation between groups was calculated using Tukey's test for multiple comparisons for independent groups by JMP7 software.
CHAPTER 3: RESULTS

3.1 Identification of the Dendritic Sorting Signals of NLG-1: An Intermediate Polarity

A stretch of 32 amino acids in the cytoplasmic domain of NLG-1 was previously shown by Rosales et al. (2005) to target this protein to the dendritic plasma membrane. Within this sequence exist amino acid sequences that potentially conform to known dendritic signals; a tyrosine and hydrophobic motif. Alanine site-directed mutagenesis in combination with quantitative cell surface immunofluorescence was employed to test the role of these two different potential sorting motifs. Two separate plasmids were constructed, one containing alanine substitutions for amino acids 782-785 and the other having substitutions in amino acids 772-774, mutating the putative tyrosine and hydrophobic motifs respectively. The VVL motif was selected based on the likelihood that the sorting motif could be either VV or VL and for simplicity is referred to as the hydrophobic motif, and the YTLA region based on its tyrosine residue. The resulting constructs are termed NLG-VVL and NLG-YTLA, respectively (Fig. 6). All constructs contain an N-terminal extracellular hemagglutinin-tag (HA) tag that allows for cell surface detection. The plasmids were transfected into hippocampal neurons, along with soluble GFP to outline cell morphology, and expressed for 18hrs (Fig. 7A). The cells were then immuno-stained live using an anti-HA antibody directed against the extracellular HA tag. Comparison of the distribution of protein in the dendrites vs. axons was analyzed as described by Sampo et al. (2003), whereby the ratio of average cell surface fluorescence intensity in dendrites was compared to that of the axon (D:A ratio). The D:A ratio for HA-NLG-WT was 9.9:1 ± 0.45, n = 34 (Fig. 7A). For comparison, the D:A ratio of a lymphocyte transmembrane protein that is uniformly distributed with no known sorting signal, CD8, is 1.8 ± 0.4 (Rosales et al., 2005). NLG-VVL and NLG-YTLA
were found to be present in both the axon and the dendrites with values that indicate an intermediate polarity (4.2 ± 0.75, n = 13 and 4.4 ± 0.86, n = 18, respectively). Because sorting was not completely abolished the mutants were combined to determine if together they would have an enhanced effect, a double mutant (NLG-VVL-YTLA) was created. This mutant contained alanine substitutions in both the tyrosine and hydrophobic motifs (Fig. 7: E, F). NLG-VVL-YTLA demonstrated an intermediate polarity with a D:A ratio of 4.7 ± 0.77, n = 17 (Fig. 9, 10). Statistical analysis revealed that though all three mutants were significantly different from NLG-WT, they were also different from the CD8 (Fig. 10), and not different in value from one another. These D:A ratios imply that the tyrosine and hydrophobic motifs both play roles in the dendritic polarity of NLG, however targeting is not completely abolished when these motifs are disrupted. The intermediate polarity observed in all mutant constructs, including the NLG-VVL-YTLA with both motifs absent, indicated that there must be other cytoplasmic residues that contribute to NLG polarization. The intermediate polarity could be explained by the possibility that expressed NLG mutants multimerize with endogenous NLG thus influencing cell surface distribution. However this scenario is unlikely as previous findings have shown that the extracellular domain does not contribute to sorting (Rosales et al., 2005).
Figure 6. 32 Amino Acid Cytoplasmic Sequence of Neuroligin-1 and Mutant Constructs

32 amino acid cytoplasmic sequence of neuroligin wild-type and mutant constructs generated to identify dendritic sorting signals. Numbers indicate the amino acid number starting from the N-terminal (1) and ending at the C-terminal (844). The 32 amino acid region extends from 772-804. The hydrophobic motif (VVL) includes amino acids 772-774, the YTLA tyrosine motif includes amino acids 782-785, and the acid couplet DD includes amino acids 791-792. The C-terminal-truncation constructs are both truncated after amino acid 785.
Figure 7. Cell Surface Distribution of Wild-type and Mutant Neuroligin in 8 Days In Vitro Cultured Hippocampal Neurons.

Cotransfected cells expressed soluble GFP (A, E) to identify cell morphology and HA-tagged NLG construct (B, F) for 18 hours prior to fixation. Wild-type NLG is restricted to the dendrites (B), the NLG double mutant (NLG-VVL-YTLA), lacking two sorting motifs, is found to have an intermediate polarity (F). A phase image of the wild-type expressing cell is provided in C. anti-MAP2 Antibody staining was performed to accurately distinguish the dendrites from the axon (D). Arrows direct attention to the axon; dendrites are indicated by cell arrowheads, and a black arrow marks the cell body.
3.2 Additional Signals in the 32 Amino Acid Domain are Essential for the Polarization of NLG-1

To test the possibility of another sorting signal present in the C-terminal portion of the 32 amino acid region, two truncation mutants were constructed. In the first a stop codon was inserted at amino acid position 785 behind the YTLA sequence creating NLG-WT-ΔC. A stop codon was inserted at the same position using the template for the NLG-VVL-YTLA and generating the mutant NLG-VVL-YTLA-ΔC. The expectation was that the NLG-WT-ΔC should have an intermediate polarity, whereas the NLG-WT-ΔC should be completely unpolarized if there is a sorting signal present in the truncated region. NLG-WT-ΔC had a ratio of D:A = 4.73 ± 1.73, n = 31, and the NLG-VVL-YTLA-ΔC had a polarity of D:A = 2.57 ± 1.15 n = 26 (Fig. 9A, 10). The NLG-WT-ΔC had an intermediate polarity equivalent to that of the NLG-VVL, NLG-YTLA and NLG-VVL-YTLA constructs. The NLG-VVL-YTLA-ΔC was found to be significantly less polarized than all of the previous constructs, and was similar to the unpolarized chimera, NLG-CD8 (D:A = 2.5 ± 1.0, n = 15) (Fig. 9, 10, 11) indicating there are amino acids present between positions 785 and 804 that contain additional sorting information.

3.3 An Acidic Couplet is Necessary to Direct the Polarization of NLG-1 to the Dendritic Membrane

To further define the specific amino acids within the 785-804 region responsible for conferring polarity to NLG the sequence was analyzed for potential sorting motifs. Because acid residues are known to sort other membrane proteins (Maxfield, 2007; Hong et al., 2007), two adjacent aspartic acid residues within the region were investigated. To test if the acidic couplet is the third sorting motif and to observe the mutant sequences in isolation, the extracellular domain of NLG was replaced with that of CD8. Five constructs were created; CD8-NLG-WT, CD8-NLG-VVL, CD8-NLG-YTLA, CD8-NLG-VVL-YTLA, and a triple mutant (CD8-NLG-VVL-YTLA-DD). The triple mutant was created by an alanine point mutation replacing the two acidic residues from the CD8-NLG-
VVL-YTLA construct, generating a new triple mutant called CD8–NLG-VVL-
YTLA-DD (Fig. 8 B). Their D:A ratios were determined as previously described. 
The CD8-NLG-VVL-YTLA-DD was found to have a polarity similar to that of the 
unpolarized NLG-CD8 control (D:A = 2.68 ± 0.71, n = 15).

Thus the NLG-VVL-YTLA-ΔC and CD8-NLG-VVL-YTLA-DD both had 
polarities similar to those of the unpolarized CD8 protein (D:A ratios of; 2.57 ± 
1.15, n = 26, 2.68 ± 0.71, n = 41 respectively) (Fig. 9, 10, 11). The intermediate 
polarity of the NLG-WT-ΔC demonstrates that the acidic couplet alone is not 
sufficient to direct NLG-1 to dendrites, however the acidic couplet contains 
sorting information that is necessary, in addition to the VVL and YTLA motifs, in 
directing NLG-1 to the dendritic membrane. Taken together these results indicate 
that several motifs are required for the sorting of NLG-1 to the dendritic 
membrane in hippocampal neurons.
Figure 8. Mutant Construct Cell Surface Staining and Polarization

Soluble GFP outlines cell morphology for three different HA-tagged mutant NLG constructs (A, C, E). HA-cell surface staining for each construct can be seen in B, D, F). CD8-NLG-VVL-YTLA-DD and NLG-VVL-YTLA-ΔC, which are lacking all three sorting motifs, both exhibit a cell surface polarity to that of a uniformly polarized membrane protein as evidenced by axonal fluorescence. NLG-WT-ΔC, lacking only the acidic couplet motif, displays an intermediate polarity (F).
<table>
<thead>
<tr>
<th>Constructs</th>
<th>Cell surface Staining</th>
<th>Dendrite:Axon Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLG WT</td>
<td>D:A = 10.4 +/- 0.70</td>
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</tr>
<tr>
<td>CD8 NLG</td>
<td>D:A = 2.50 +/- 1.00</td>
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<tr>
<td>NLG YTLA</td>
<td>D:A = 4.42 +/- 1.06</td>
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</tr>
<tr>
<td>NLG VVL</td>
<td>D:A = 4.62 +/- 0.90</td>
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<tr>
<td>NLG VVLYTLA</td>
<td>D:A = 5.23 +/- 0.93</td>
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<tr>
<td>NLG WT ΔC</td>
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<td>CD8 NLG VVLYTLAADD</td>
<td>D:A = 2.68 +/- 0.71</td>
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Figure 9. Schematic Representation and Cell Surface Quantification (Dendrite: Axon Ratio) of Neuroligin-Wild-Type and Mutant NLG-1 Constructs, Truncations and CD8 chimeras.

NLG controls (WT, CD8) and tyrosine and hydrophobic point mutants are depicted in A. NLG C-terminal truncations and those constructs with the extracellular domain of CD8 are seen in B. DD marks the aspartic acid couplet in the cytoplasmic domain.
Figure 10. NLG Construct Whiskerplot of Polarization Trials

A whiskerplot displays the data for all constructs. D:A Ratio values appear on the left. C1 and C2 truncation constructs were previously used to isolate the 32 amino acid sorting sequence as described by Rosales et al. (2005). Bars represent individual group D:A means. CD8-NLG-VVL-YTLA-DD and NLG-VVL-TYLA-ΔC are all unpolarized similarly to the D:A ratio of CD8 and the C1 truncation lacking the 32 amino acid domain.
Construct

<table>
<thead>
<tr>
<th>Construct</th>
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<tbody>
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<td>CD8-NLG</td>
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</tr>
<tr>
<td>CD8-NLG32aa</td>
<td>A</td>
</tr>
<tr>
<td>NLG-C2</td>
<td>A</td>
</tr>
<tr>
<td>NLG-WT</td>
<td>A</td>
</tr>
<tr>
<td>NLG-WT-ΔC</td>
<td>B</td>
</tr>
<tr>
<td>NLG-VVL-YTLA</td>
<td>B</td>
</tr>
<tr>
<td>NLG-YTLA</td>
<td>B</td>
</tr>
<tr>
<td>NLG-VVL</td>
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<tr>
<td>NLG-C1</td>
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<td>CD8-NLG-VVL-YTLA-DD</td>
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<td>CD8</td>
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</tr>
</tbody>
</table>

Figure 11. Statistical Comparisons of Neuroligin-1 Polarization Trials

Statistical comparisons in polarity of all experimental constructs as determined using Tukey's test for comparisons for multiple means. Levels not connected by the same letter are significantly different. To obtain a normal distribution, D:A ratios had their natural log taken and used for statistical analysis. Constructs lacking all three sorting motifs are equivalently unpolarized as that of a uniform membrane protein (CD8). Constructs lacking only one or two of the three motifs exhibit an intermediate polarity (VVL, YTLA, VVL-YTLA, WT-ΔC).

3.4 Identification of NLG-1 Vesicle Population in Dendrites

Because vesicular trafficking is the primary means for intracellular membrane protein transport, this study attempted to identify the vesicle population responsible for the intracellular trafficking of NLG-1. To test for the presence of NLG-1 in a dendritic vesicle population, a combination of well-characterized fluorescently tagged proteins were expressed in cultured hippocampal neurons in pair-wise and triplicate combinations. The dendritic proteins TfR and Kv2.1 were used to identify vesicles in the dendrites (Burack et al., 2000; O'Connell et al., 2005), and NgCAM was used to mark carrier vesicles in the axon (Burack et al., 2000) (Table 2). To evaluate the sorting of these proteins, two and three-colour imaging was performed on 8-day in vitro cultured rat hippocampal neurons that had been allowed to express the constructs for 18hrs.
Table 2. Fluorescent Markers and Corresponding Cellular Locations

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fluorescent Tag</th>
<th>Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLG-1</td>
<td>GFP</td>
<td>Dendritic</td>
</tr>
<tr>
<td>Kv2.1</td>
<td>Cherry</td>
<td>Dendritic</td>
</tr>
<tr>
<td>TfR</td>
<td>GFP or Cherry</td>
<td>Dendritic</td>
</tr>
<tr>
<td>NgCAM</td>
<td>Cherry</td>
<td>Intracellular: Uniform Extracellular: Axonal</td>
</tr>
<tr>
<td>Tf</td>
<td>AF-647</td>
<td>Dendritic: Early Endosomes</td>
</tr>
<tr>
<td>Rab11b</td>
<td>RFP</td>
<td>Recycling Endosomes</td>
</tr>
<tr>
<td>ER</td>
<td>CFP</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>TfR-Mut</td>
<td>YFP</td>
<td>Uniform vesicles</td>
</tr>
</tbody>
</table>

The compartments labelled with GFP- or Cherry-tagged dendritic markers were restricted to the dendrites (shown for TfR in Fig. 12). In contrast, the axonal marker NgCAM-Cherry labelled intracellular tubulovesicular structures throughout the dendrites as well as the axon. These results are consistent with previous studies based on expression of these markers individually, which showed that carriers containing TfR are excluded from the axon while carriers containing NgCAM are transported into both axons and dendrites (Burack et al., 2000; Silverman et al., 2001). To evaluate the extent of overlap between various pairs of markers and NLG-1, Z-stack acquisition in wide-field fluorescent microscopy and deconvolution analysis was employed to enhance signal-to-noise ratio by enhancing out-of-focus and cell-surface fluorescence. Faintly labelled structures were thus resolved and analyzed in two and three-colour overlays (Fig. 12). To ensure there is a zero pixel shift between filters during Z-stack acquisition 5μM beads were imaged as controls under separate wavelengths and their stacks were deconvolved and overlain (for more detail see Fig. 18, Appendix 1). Correlation coefficients were used as a measure of overlap and are expressed in r values.
As a positive control to validate this approach to overlap analysis, TfR-GFP and TfR-Cherry were co-expressed and their measure of overlap determined (Fig. 12). The markers exhibited a near perfect correlation coefficient ($r = .96 \pm .06$).

**Figure 12. Transferrin Receptor (TfR) Overlay**

Doubly transfected fixed hippocampal neurons expressing two different fluorescent versions of TfR as a positive control for colocalization. TfR-Cherry (A) and TfR-GFP (B) are overlain to create the image in C. Images were deconvolved and analyzed to generate correlation coefficients using Metamorph software as a means of determining overlap between marker pairs. Cell bodies as well as axon branches were excluded from the analysis. A portion of the dendrite is shown at high magnification (lower panels) to demonstrate that both of these markers label the same compartment; $r = 0.96 \pm 0.06$.

There is evidence for a common dendritic sorting pathway for both axonally targeted and dendritically targeted membrane proteins (Wisco et al., 2003; Sampo et al., 2000). Another control experiment was performed to demonstrate that similar structures can be identified and to further validate approaches used in this thesis. Axonally targeted NgCAM-Cherry was expressed with TfR-GFP and transferrin (Tf) labeled with Alexa Fluoro-647 as a control for axonal, dendritic and somatodendritic recycling pathway markers, respectively. NgCAM-Cherry was present in intracellularly in both the axons and dendrites, whereas TfR and Tf was localized only to the dendrites. NgCAM and TfR had a
correlation coefficient in the dendritic domain of 0.57 ± 0.02, where NgCAM and Tf had one of 0.44 ± 0.03 (Fig. 13). TfR and Tf colocalized with an \( r = 0.75 ± 0.02 \). This data is consistent with the results described by Wisco et al. (2003), which demonstrated a significant overlap of NgCAM and Tf within dendrites. This suggests there is a common dendritic sorting compartment in which both proteins are located.

Figure 13. Intracellular Distribution of NgCAM, TfR and Tf.

Intracellular distribution of NgCAM, TfR and Tf. Panels A, B and C were imaged in fixed hippocampal neurons and demonstrate the polarized distribution of TfR-GFP (A) and the axonally-targeted NgCAM-cherry (B). The cell was then imaged using Z-stack acquisition and images were deconvolved. A portion of the dendrite outlined in the white box was magnified and analyzed for colocalization using Metamorph software. NgCAM-Cherry and TfR-GFP (\( r = 0.56 ± 0.03 \)) (D), Tf-AF-647 and NgCAM-Cherry (\( r = 0.44 ± 0.03 \)) (E) and TfR-GFP and Tf-AF-647 (\( r = 0.74 ± 0.03 \)) (F) overlays of high magnification images can be seen in the lower panels.
There are also multiple other compartments located in dendrites, including ER and Golgi, which are well-characterized elements involved in secretory trafficking (Horton and Ehlers, 2004). To identify intracellular compartments that may contain NLG, the NLG-GFP construct was expressed and acted similarly to the untagged version in that it was polarized to dendrites and reached the plasma membrane (data not shown). Notably, NLG-GFP was both reticular and vesicular in appearance. Considering the presence of ER in dendrites and that protein folding can be completed in the ER, it was asked if NLG-GFP was present in the ER. ER-CFP and NLG-GFP were co-expressed and showed an $r = 0.60 \pm 0.02$ (Fig. 14). Despite this degree of overlap and reticular appearance, single labelled vesicular structures were detectable as indicated by white circles.

**Figure 14. NLG-GFP and ER Colocalization**

NLG-GFP (A) and ER-CFP (B) colocalization suggests NLG is present in the ER however NLG-GFP signal is evident in ER-minus compartments. They have a correlation coefficient of $0.60 \pm 0.02$ (C).
<table>
<thead>
<tr>
<th>Construct Combination</th>
<th>n</th>
<th>Least Sq Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfR-Cherry, TfR-GFP</td>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td>CD8-NLG-VVL-YTLA-DD, TfR-YFP-Mut</td>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>TfR-Cherry, Tf-AF-647</td>
<td>B</td>
<td>21</td>
</tr>
<tr>
<td>Rab11-RFP, NLG-GFP</td>
<td>B</td>
<td>7</td>
</tr>
<tr>
<td>Kv2.1-Cherry, NLG-GFP</td>
<td>B</td>
<td>32</td>
</tr>
<tr>
<td>ER-CFP, NLG-GFP</td>
<td>C</td>
<td>26</td>
</tr>
<tr>
<td>TfR-Cherry, NgCAM-GFP</td>
<td>C</td>
<td>23</td>
</tr>
<tr>
<td>NLG-GFP, NgCAM-Cherry</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>NgCAM-GFP, Tf-AF-647</td>
<td>D</td>
<td>12</td>
</tr>
<tr>
<td>NLG-GFP, TfR-Cherry</td>
<td>D</td>
<td>12</td>
</tr>
<tr>
<td>Tf-AF-647, Rab11-RFP</td>
<td>D</td>
<td>10</td>
</tr>
<tr>
<td>TfR-GFP, Kv2.1-Cherry</td>
<td>D</td>
<td>8</td>
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<tr>
<td>Tf-AF-647, Kv2.1-Cherry</td>
<td>D</td>
<td>12</td>
</tr>
<tr>
<td>NLG-GFP, Tf-AF-647</td>
<td>E</td>
<td>24</td>
</tr>
</tbody>
</table>

Figure 15. Statistical Results for Correlation Analysis

Statistical comparisons in colocalization of all expressed constructs as determined using Tukey’s test for comparisons for multiple means. Levels not connected by the same letter are significantly different. TfR-TfR has the highest level of correlation, acting as a positive control, where NLG and Tf have the lowest correlation.

To ask if NLG-1 is present in compartments that contain other known dendritic carriers, experiments to characterize the intracellular location of N LG-1 were performed by expressing the N LG-GFP and a Kv2.1 Cherry-tagged construct. Kv2.1 is a dendritic membrane protein that is trafficked in vesicles to the dendritic plasma membrane (Antonucci et al., 2001). A high degree of overlap was found between the two markers with $r = 0.73 \pm 0.02$. To identify post-Golgi carriers that may contain N LG-GFP, Tf-AF-647 was added to the media prior to fixation to label early endosomes. Three-colour imaging was used to compare the three proteins to one another. The overlap between N LG-1 and Tf was significantly lower, with an $r = 0.29 \pm 0.02$, and for Kv2.1 and Tf; $r = 0.39 \pm$
0.03 (Fig. 16). Because both NLG and Kv2.1 were found in a low Tf-containing vesicle population and found to have a high degree of overlap with each other, it suggested that they are both endocytosed in a Tf-minus endocytic pathway. To further characterize NLG-GFP behaviour, TfR-Cherry was expressed with each the NLG-GFP and a Kv2.1-GFP construct. When Kv2.1 and NLG-1 were expressed with TfR, an intermediate value was obtained between both pair-wise combinations; Kv2.1 and TfR; r = 0.40 ± 0.04, NLG-1 and TfR; r = 0.43 ± 0.03 (Fig. 15, 16).

Figure 16. High Magnification Images of Dendritic Membrane Proteins in Doubly Transfected Fixed Hippocampal Neurons.

Each panel illustrates each marker individually and in a two-colour overlay. The images were processed by deconvolution and analyzed by first thresholding each image and then calculating a correlation coefficient using Metamorph software. The white circles identify specific tubulovesicular structures. Panel A: NLG-GFP (top) and Kv2.1-Cherry (centre) images are overlain (bottom) with a correlation coefficient of 0.73 ± .02. Panel B: NLG-GFP and Transferrin Alexa-Fluoro-647 had a correlation coefficient of 0.29 ± .02. Panel C: NLG-GFP and TfR-Cherry demonstrated a correlation coefficient of 0.43 ± .03.

In an attempt to further define intracellular compartments that contain NLG-1 with the evidence that it is not present in a Tf-containing endocytic
population, Rab11b was co-expressed with NLG-GFP. Rab11b is a GTPase found in a non-Tf containing endosomal population in polarized cells and is a marker for the recycling endosomal pathway to the cell surface and traffic to the TGN (Lapierre et al., 2003). Rab11b-RFP and NLG-GFP were co-expressed and Tf-AF-647 was added to cell media prior to fixation. Consistent with previous results which demonstrate a low colocalization between Rab11b and Tf (Lapierre et al., 2003), Tf and Rab11b had a relatively low correlation coefficient \( r = 0.40 \pm 0.05 \), while NLG and Rab11b demonstrated high overlap with an \( r = 0.73 \pm 0.04 \) (Fig. 15). This provides evidence of Rab11b’s involvement in NLG’s endocytic trafficking and suggests NLG is endocytosed into the recycling endocytic pathway.

Because the NLG mutant is present uniformly on the cell surface, it was asked whether the misdirected NLG mutant is present in a uniform intracellular carrier population. A TfR-YFP sorting mutant used in the following experiment lacks dendritic sorting information and traffics intracellularly into both axons and dendrites. To test if the sorting mutant CD8-NLG-VVL-YTLA-DD is present in a uniform vesicle carrier, CD8-NLG-VVL-YTLA-DD was expressed with the TfR-YFP sorting mutant. The NLG-1 mutant was stained intracellularly with anti-CD8 antibody and the images were overlaid and analyzed. Distinct vesicles sharing both proteins could be detected both in the axon and the dendrites, and the correlation coefficient of overlap was 0.77± 0.05 (Fig 17). This demonstrates that mutating the sorting sequence of NLG-1 leads to its mis-sorting into a carrier population that enters both the axon and the dendrites.
Figure 17. NLG-1 Sorting Mutant and TfR Sorting Mutant Overlap in both Axons and Dendrites

A) An axonal segment of a neuron transfected with CD8-NLG-VVL-YTLA-DD and stained intracellularly with anti-CD8 is compared with B) a sorting mutant of TfR that lacks polarization. The correlation coefficient for the overlay in C) is 0.77± 0.05. The same constructs are compared in a dendritic region in D-F. White circles outline the colocalized puncta. The green box in G outlines the region of the axon that is magnified in A-C; the white box outlines the magnified region of the dendrite in D-F.

3.5 Additional Experiments for Identification of Post-Golgi Carriers

Attempts were made to visualize post-Golgi carriers that may bring NLG-1 and Kv2.1 to the dendritic and somato-dendritic membranes but to no avail. It has been a general difficulty in the trafficking field to isolate or capture evidence
of post-Golgi carrier organelles in polarized cells, particularly in neurons. Only recently have some TGN carriers been described, such as the trafficking of TfR using a photoactivateable construct, to show that TfR is trafficked into a Tf+EEA1+ endosomal compartment, also leading to the suggestion that endosomes can act as sorting stations in the endocytic pathway, not only for internalized proteins but also for newly synthesized ones (Luo et al., 2006). However, this finding was done in fibroblast cells, and thus far attempts to image such trafficking organelles in neurons have been difficult and unsuccessful. Several efforts were made to visualize NLG-1 post-Golgi trafficking in this thesis. These attempts included using a Golgi-temperature block that restricts resident proteins from exiting the TGN until a temperature above 20 °C is reinstated. Cells were fixed and imaged at various different time points after temperature release to identify carriers trafficked into the dendritic membrane, however no individual carriers were observed. A diffuse haze was the only visible shift seen between the time-trials of post-temperature block. Attempts at live imaging NLG-1 were ineffective due to a similar diffuse staining in the dendritic membrane but no evidence of vesicle carriers. FRAP (Fluorescence Recovery after Photobleaching) was used both in the cell body and within dendrites to try to identify moving vesicles back into an area of photobleaching. The only visible movement was a diffuse fluorescent signal moving back into the bleached area of the cell. As a new approach, a photoactivatable NLG-1 construct was created and expressed. The construct was only very weakly photoactivatable, and any visible signal was too weak to detect distinct movement during live imaging.

3.6 Conclusion

These results demonstrate that three amino acid signals in the cytoplasmic tail of NLG-1 govern NLG-1 dendritic localization and these motifs direct sorting in a coordinated manner. Additionally, the method of NLG-1 trafficking is inferred from a combination of information obtained from a pair-wise and triplicate co-expression of fluorescent membrane proteins of known intracellular locations. Results suggest that NLG-1 is trafficked to the PM from
the Golgi via the same carrier population that transports the Kv2.1 channel and TfR. NLG-1 appears to undergo endocytosis at the PM into a Rab11b positive and Tf negative compartment. Finally, a mutant dendritic protein that lacks dendritic sorting information is found in a vesicle population in the axon that also contains NLG-1.
CHAPTER 4: DISCUSSION

4.1 Summary

The heterophilic adhesion between NLG-1 and β-NRX, and thus synapse formation, depends on the polarized targeting of NLG-1 to the somatodendritic domain. Rosales et al. (2005) previously defined a 32 amino acid cytoplasmic sequence that targets NLG to the dendritic membrane. This thesis further investigated the specific motifs involved in NLG-1 dendritic sorting, and also asked which vesicle population, if any, NLG-1 is found in. This report elucidated the exact sorting motifs within this sorting sequence that direct NLG’s polarized targeting. Three sorting signals were identified; the tyrosine based motif (YTLA), the hydrophobic motif (VVL), and an acidic couplet (DD). These three motifs act as a tripartite sorting signal sequence and act commensurately to direct the dendritic sorting of NLG-1. Second, this study determined that NLG-1 is trafficked in a vesicle population that is also a carrier of the dendritic channel protein Kv2.1. Kv2.1 also exists in a Rab11b positive and Tf negative compartment, suggesting endocytosis at the PM via a Tf-independent pathway and into a recycling endosomal population.

4.2 Three Dendritic Targeting Motifs in the Cytoplasmic Domain of Neuroligin-1

Two of the three motifs isolated, YTLA and VVL, involved in sorting NLG-1 to the dendrites are involved in the dendritic sorting of other membrane proteins, such as the transferrin receptor (West et al., 1997), low-density lipoprotein receptor (Jareb and Banker, 1998), and the potassium channel, Kv4.2 (Rivera et al., 2003). The tyrosines present in the sorting sequences of Tfr and LDLR are similar to the tyrosine residue in NLG-1. The second motif described in dendritic targeting, such as is found in the Kv4.2 potassium channel (Rivera et al., 2003),
is the hydrophobic motif (Val-Val and Val-Leu). These motifs when removed from the NLG cytoplasmic domain do decrease NLG-1 polarity to an intermediate level, although it was expected that polarized sorting would be entirely eliminated. When the two mutations together are present in one NLG construct, they have a similar D:A ratio as each of the individual mutants, indicating they are co-linear and that other signals contribute to sorting.

The acidic couplet (DD) located at position 792 within the cytoplasmic domain is also a critical motif in the dendritic targeting of NLG-1. Acidic patches containing two adjacent aspartic acids play a large role in sorting and endocytosis and thus may function in a similar context in NLG-1. For example, in the basolateral sorting of LDLR, which contains two tyrosine-based sorting signals, each sequence is flanked by a cluster of negatively charged amino acids of the sequence EDE for the proximal determinant and EDD for the distal determinant. Both are necessary for basolateral targeting (Matter and Mellman, 1992). Furthermore, a di-leucine motif and an acidic cluster mediate the recycling pathway of the vesicle associated membrane protein (VAMP-4), whereby the N-terminal di-leucine motif is necessary for internalization from the cell surface, and the acidic cluster is involved in efficient delivery from the endosome back to the TGN (Hoai et al., 2007). Two acidic residues adjacent to a di-leucine motif has been shown to be involved in the sorting of the VMAT2 in the regulated biosynthetic pathway. The acidic sequence of two glutamic acids (EE) is required for sorting VMAT2 into large dense-core vesicles (LDCVs) containing monoamine neurotransmitters into the pathway for regulated exocytosis at the plasma membrane. The wild-type VMAT2 is not found at the cell surface due to immediate exocytosis, and is associated with LDCVs, whereas mutations in the two glutamic acids (EE) of VMAT2 increases cell surface expression without substantially affecting endocytosis. This is a result of a mis-sorting event into the constitutive pathway from the regulated pathway (Li et al., 2005). These examples provide suggestions of the potential role of the acidic couplet in NLG-1’s cytoplasmic domain. The DD cluster may well play a role in the endocytosis
of NLG-1 from the membrane into a recycling or endosomal pathway yet this signal helps NLG to maintain a polarized distribution.

4.3 Adaptor Proteins in NLG-1 Trafficking and Sorting to the Dendritic Membrane

A crucial step in the targeting of polarized proteins includes cargo selection and vesicle formation. Adaptor proteins are pivotal molecules that interact with the protein’s targeting signals in the amino acid sequence and thus mediate protein sorting into appropriate vesicle carriers. Two of the three motifs isolated in NLG-1 targeting suggest protein interactions with the family of adaptor proteins (APs) which are known to bind both tyrosine and di-leucine-based motifs (Bonifacino and Robinson, 2001). The most likely candidates would be the well-described adaptor complexes AP-1 and AP-2, which binds to tyrosine or di-leucine-based endocytosis signals in the cytoplasmic tails of a variety of plasma membrane proteins. These APs mediate their incorporation into clathrin-coated vesicles budding from the TGN or endocytic vesicles from the PM, respectively (Kirchhausen, 1999; Mellman and Warren, 2000; Bonifacino and Robinson, 2001). The role of acidic residues in recognition by these adaptors remains unclear. The monomeric Golgi-localized, gear-containing, ARF binding proteins (GGAs) require upstream acidic residues for recognition of a di-leucine motif in cargo proteins. For example, the cation-independent mannose-6-phosphate receptor (CI-MPR) and cation-dependent MPR TGN to late endosomal sorting is mediated by GGA recognition of an acidic cluster di-leucine motif within their cytoplasmic tails (Puertollano et al., 2001; Zhu et al., 2001; Doray et al., 2002). However the GGAs depend primarily on an acidic residue three positions upstream of the di-leucine and thus may not recognize the di-leucine-like sequence in NLG-1 with acidic residues 17 and 18 positions downstream. With three equivalent overlapping sorting signals, it could be likely that more than one form of adaptor is required for efficient targeting of NLG-1. The low-density lipoprotein receptor (LDLR)-related protein, LRP9, contains a C-terminal acidic
cluster di-leucine signal that is found in the GGA-binding consensus sequence (DXXLL) and also in the AP-1/AP-2-binding [DE]XXXL[LI] motifs, and it interacts with both these adaptors through these motifs (Doray et al., 2008). An alternative model for the acidic couplet in NLG sorting may be that delivery to the PM includes the aspartic acid couplet and VVL motif interaction with GGAs, and endocytic behavior may be regulated by AP-2 via the tyrosine and VV or VL motif.

It should be noted that signals that govern NLG-1 synaptic concentration are separate sequences than those required for its dendritic sorting. Two proposed synaptic targeting signals are either at the PDZ binding domain or within the juxtamembrane domain (Prange et al., 2004; Dresbach et al., 2004). Due to the importance of sorting for neuronal cell function, it is not surprising that multiple signals govern NLG localization.

4.4 Dendritic intracellular trafficking routes of NLG-1

By co-expressing fluorescently-tagged constructs of neuronal membrane proteins and using fluorescently-tagged markers, intracellular trafficking pathways were investigated. The colocalization experiments revealed that NLG-1 can be found in overlapping compartments with the Kv2.1 channel, is not endocytosed with TfR, does not colocalize with Tf-positive EEs although is found in a Rab11b positive compartment. Additionally, a NLG sorting mutant is found in the same carrier population as that of a TfR sorting mutant.

The high level of colocalization of NLG-1 with Kv2.1, intermediate level with TfR and low level with Tf suggests NLG is trafficked from the TGN with both proteins, however is endocytosed only with Kv2.1 at the membrane. Dendritic targeting is dependent upon dendritic transport therefore the composition of a dendritic vesicle is important. Underscoring the importance of understanding dendritic transport is seen with the NR2β subunit that is trafficked by the transport complex m-lin10 which binds to Kif17, facilitating the transport of NR2β. Knockout of Kif17 leads to behavioural defects in mice (Setou et al., 2000).
Both NLG and Kv2.1 have little overlap with Tf, suggesting they may be endocytosed into clathrin-independent endosomal compartments. This is the case with other membrane proteins, including the EGF Receptor that uses a caveolin-dependent endocytic route (Sigismund et al. 2005). Caveolin in this case is an unlikely candidate due to previous findings that the Kv2.1 channel is trafficked to lipid raft domains where it clusters in the membrane that are caveolin-independent (Martens et al., 2000). The mGluR7 constitutively endocytoses via a non-clathrin pathway in which it traffics via an Arf6-positive endosomal route (Lavezzari et al., 2006). The high correlation seen with NLG-1 and Rab11b suggests that NLG-1 is endocytosed into a recycling endosomal pathway. It is also congruous with findings that Rab11b is distinct from Tf-containing endocytic populations in polarized cells (LaPierre et al., 2003). Consistent with the literature, the expressed Rab11b and Tf also had a low correlation coefficient.

Rab11b is involved in the trafficking of other transmembrane proteins, including the thromboxane A2 G-protein coupled receptor, TPβ, which is constitutively endocytosed by an agonist-induced mechanism to maintain an intracellular pool of receptors that recycle to the cell surface to preserve agonist sensitivity (Hamelin et al., 2005). This could be a similar situation with NLG-1, whereby synaptic remodeling would be a mechanism of increasing or decreasing synaptic connectivity. Remodeling at the synapse has been shown to depend on recycling endosomal populations, whose bidirectional transport regulates dendritic spine formation and loss. This can provide for both removal and addition of membrane, particularly during long term potentiation (LTP) or long term depression (LTD). This is the case with AMPA receptor recycling during LTP, whereby AMPA receptors increase at the plasma membrane due to enhanced transport from recycling endosomes (Park et al., 2004; Park et al., 2006).

NLG and TfR overlap resulted in a correlation coefficient of an intermediate value. Interestingly, Kv2.1 had a similar amount of overlap with TfR. As both Kv2.1 and NLG had a low correlation with Tf, the data suggests that NLG
and Kv2.1 may be trafficked out of the TGN in the same vesicle population as TfR, however once they are deposited into the membrane they may be endocytosed and recycled independently into Tf-minus, and thus TfR-minus, vesicle populations.

Frequency of turnover may explain the need for separate endocytic carriers. Given a case of synaptic rearrangement that is occurring without LTP or LTD, NLG and Kv2.1 would require much less turnover at the membrane than TfR, which recycles back to the plasma membrane within minutes to deliver bound Tf into the cell and recycle back for further Tf interaction at the surface.

4.5 **Mutant NLG-1 is Trafficked into the Axon with a TfR Sorting Mutant**

The mis-sorting of CD8-NLG-VVL-YTLA-DD into the axon along with the TfR sorting mutant provides evidence that signal dependent mechanisms drive sorting events for some membrane proteins in neurons. It demonstrates that NLG-1 trafficking requires targeting signals that act by governing the sorting of proteins into specific populations of transport carriers, most likely by recognition by APs. Dendritic carriers that contain TfR leave directly from the Golgi. Recently it has been shown that with Protein Kinase D (PKD1) inactivation, a Ser/Thr kinase located in the Golgi, TfR is mispackaged into carriers that contain VAMP2 and are found in both axons and dendrites (Bisbal et al., 2008). Thus, mutant NLG-1 may be unrecognized for dendritic carrier packaging by adaptor proteins initially at the Golgi, and immediately trafficked into a common carrier, such as VAMP2 containing carrier, that traffics to both the axon and the dendrites.

TfR sorting in neurons is mediated by short cytoplasmic sequences that include the tyrosine motif, YTRF, similar to that found in NLG-1 sorting sequence. The signals in TfR act in a colinear manner whereby alteration of polarized dendritic sorting also increased cell surface fluorescence. Thus TfR sorting and endocytosis are mediated by multiple overlapping signals. This demonstrates the importance of dendritic targeting, whereby TfR sorting can depend on several
signals and repeated recognition of these signals in the recycling endosome to ensure proper polarization (West et al., 1997). This may be the case for NLG-1, which contains multiple sorting signals, although it is unknown where these signals are acting. They are sufficient to place NLG-1 in the dendrites and maintain a polarized distribution. Having overlapping signals is not surprising considering the importance of polarized trafficking and its contribution to synapse formation.

### 4.6 Conclusions and Future Perspectives

The exclusive dendritic targeting of NLG-1 provides a polarizing activity for axonal-dendritic recognition during synapse formation. This role is functional only because of its heterophilic adhesion with its axonal binding partner β-NRX, not a homophilic system such as SynCAM. This polarized targeting is accomplished by the specific sorting signals and adaptors that recognize them. CAMs and scaffolding proteins are critical in maintaining and modulating the morphology, number and type of synapses formed. For example, excitatory and inhibitory synapse ratios can be altered based on the presence of PSD-95, which tethers NLG-1 to excitatory synapses (Prange et al., 2004). If the molecules that control synaptic specificity are mistargeted it may result in aberrant synapse formation and alter the ratio of excitatory to inhibitory synapses, leading to a change in neuronal excitation and inhibition, and thus animal behavior.

The importance of understanding the cellular machinery that contributes to sorting and its trafficking behavior is evident in cases of its mistargeting such as that of NLG-3, whereby its mis-targeting and inability to be trafficked to the cell surface are defects found in Autistic patients (Chubykin et al., 2005). Future experiments should now be geared towards understanding the key players required for dendritic targeting, for example identifying the cellular machinery, including adaptor proteins and molecular motors, that contribute to NLG-1 sorting and further characterizing the vesicle populations that transport NLG-1.
Figure 18. Bead Overlay for Z-stack Calibration
5μM Beads were imaged at 63X objective using Z-stack acquisition, deconvolved and overlaid to ensure that a zero pixel spectral shift existed between filters. A) Bead imaged in DAPI, B) Bead imaged in TXRed, C) Overlay between A and B demonstrating a zero pixel shift.

Figure 19. Correlation Plots Demonstrating High and Low Correlation
Correlation plots were generated using the correlation analysis function by Metamorph software. These are made by plotting the intensity of each colour at each pixel in the image and generating a correlation coefficient from the software. Dimmer pixels in the images are located toward the origin of the scatterplot, while brighter pixels are located farther out. Pure pixels of a colour tend to cluster toward the axes of the plot, and colocalized pixels cluster towards the middle of the plot. Thus, the more linear relationship demonstrates a strong correlation. (A) TfR-TfR overlay with $r = 0.94$, and (B) NLG, Tf with $r = 0.25$.
REFERENCE LIST


