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Endocytic mechanisms and drug discovery in neurodegenerative diseases

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1. ABSTRACT

Extensive research has been carried out to elucidate the mechanism of neurodegenerative diseases, with special emphasis on lysosomal storage disease (LSD) and Alzheimer's disease (AD). Studies have outlined complicated profiles in both types of disorders for the role of endocytosis in disease pathogenesis and progression. Recent discoveries relating endocytosis to the pathological origin and therapeutic strategy of the diseases have yet to be addressed. In this review, I attempt to demonstrate a comprehensive analysis on the endocytic mechanism of the disease. I propose that LSD could be classified as a late endosomal trafficking disorder. I also highlight that the most critical cellular event in AD – the producing, processing, and trafficking of A*beta*42 peptide –

Table 1. Endocytic compartments and the specific markers

Endocytic Pathway	Endocytic Organelle	Abbreviation	Morphology	Protein Marker	Labeling Probe
Early	Very Early Endosome	VEE	Very small vesicles adjacent to PM	Clathrin or caveolin1	Bodipy-LacCer or Bodipy-SM
	Early Endosome	EE	Small vesicles scattered in cytoplasm	EEA, Rab5	Fluorescent Tf/CtxB
	Sorting Endosome	SE	Small vesicles scattered in cytoplasm	Rab5 and 4	Fluorescent Tf/CtxB
	Endosomal Recycling Compartment	ERC	Perinuclear tubules	Rab11 and 4	Fluorescent Tf
Late	Late Endosome	LE	Perinuclear vesicles	M6PR, Rab7	Fluorescent dextran
	Late Endosomal Tubule	LET	Tubules when moving and vesicles when being stationary	Rab7 and 9, NPC1, LAMP2	Fluorescent dextran
	Lysosome	Lyso	Large vesicles	Rab7 and 9, LAMP2, NPC2, Cathepsin D	Fluorescent dextran, Lyso-Tracker, DiI-LDL, Filipin (for free cholesterol)

Abbreviations: PM, plasma membrane; EEA, early endosomal antigen; M6PR, mannose-6-phosphate receptor; NPC1, Niemann-Pick type C1 protein; LAMP2, lysosomal associated membrane protein 2; NPC2, Niemann-Pick type C2 protein; SM, sphingomyeline; Tf, transferrin; and CtxB, B subunit of cholera toxin

dynamically involves the entire endocytic system. I further analyze pipeline drug targets, summarize the development status of current new drugs, share thoughts on potential therapeutic strategies, and reveal that many such strategies are in close association with endocytosis. I emphasize that thoroughly understanding pathologically-relevant endocytic events is the key factor in speeding up discovery and development of novel drugs.

2. INTRODUCTION

Mammalian cells employ an intracellular digestive tract, the endosomal system, for uptaking extracellular nutrients and providing proper microenvironments for the interaction of signaling proteins within the system (1). Endocytosis is a process which is achieved by the functions of endosomal compartments in the endocytic pathway. Endosomes transport internalized materials via sequential delivery of the contents from the early to the late pathways. Endocytosed materials are processed by a variety of soluble and membranous enzymes within the endosomes, and subsequently transported out of the system (2). Ligands may be endocytosed and interact with their receptors within the endosomes to initiate cell signaling events (3). Lysosome, the final stage in the endocytic pathway, is a specific type of endosome. Abnormality of the endocytosis system can result in a variety of human diseases including neurodegeneration, atherosclerosis and diabetes**.** Additionally, many drugs targeting the central nervous system (CNS), such as G-protein coupled receptor (GPCR) modulators, are highly regulated by endocytosis (4). In this review, I will be focusing on lysosomal storage disease (LSD) and Alzheimer's disease (AD). I will give an overview of the normal endocytic system, address disease-related endocytic abnormalities, and analyze current and future therapeutic strategies for the disorders.

3. ENDOCYTIC PATHWAYS IN CONVENTIONAL AND NEURONAL CELLS

3.1. Endocytic pathways

3.1.1. Overview of endocytic pathways

The endocytic pathway in mammalian cells can be divided into early and late endocytic sub-pathways (Table 1). These two sub-pathways are sequentially involved in the transport of materials from endosomes of earlier stage to those of later stage (Figure 1). Besides its essential role of initiating internalization and delivering contents to the late endocytic compartments, the early endocytic pathway is also involved in recycling a large portion of internalized materials back to the plasma membrane or outside of cells (5). The late endocytic pathway accepts materials from the early pathway and is responsible for the enzymatic degradation of the accepted contents. In addition to this basic function, the late endocytic pathway is involved in the endocytic recycling function (6). Endocytic organelles of both early and late sub-pathways also communicate to multiple organelles such as plasma membrane, Golgi apparatus (Golgi), mitochondria and endoplasmic reticulum (ER) to transfer endocytosed materials out of the organelles (6, 7, 8, 9, 10). Over the past two decades, computer-based high speed digital imaging technology, and green fluorescent protein-based live cell labeling techniques, have jointly developed into a mature platform for live cell biology research (11). This platform allows us to image and analyze the dynamics of endocytosis in live cells with the capability of obtaining data of exceptional quality. Numerous studies of living cells have increased our knowledge about the role of endocytosis in the pathogenesis and progression of LSD and AD (12, 13).

3.1.2. Early endocytic pathway

The early endocytic pathway is composed of the plasma membrane (PM) and endosomes, which include very early endosome (VEE), early endosome (EE), sorting endosome (SE) and endocytic recycling compartment (ERC). These endosomes can be recognized and determined by using special protein markers and fluorescent probes (refer to Table 1) (14, 15, 16) . Early endocytic organelles are Rab proteins Rab5, Rab4 or/and Rab11-positive organelles. Most of the compartments can be tracked, in a time dependent manner, with fluorescent transferrin (Tf) in living cells (17, 18). The recycling of endocytosed receptors and transporters back to the plasma membrane mainly occurs in this pathway via the rapid and slow recycling machineries (19).

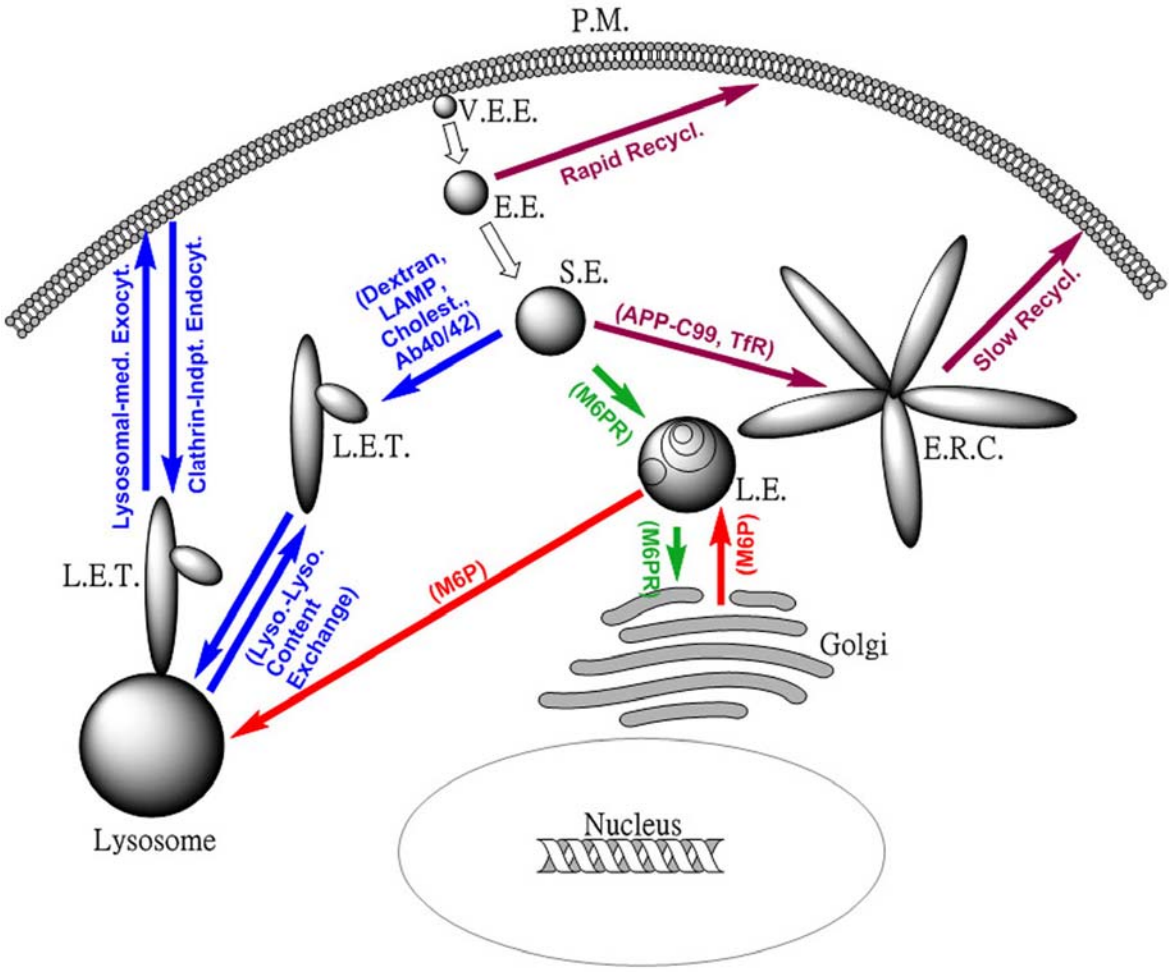

Figure 1. The endocytic pathway. This model shows that the endosomal pathway includes multiple sub-pathways. These pathways are depicted in the figure with arrows of different colors. The white arrows indicate very early steps in endocytic pathways. The purple arrows indicate early endocytic recycling pathway. The green and red arrows indicate the classical LE-mediated late endocytic pathway. The blue arrows indicate the newly defined late endosomal tubule-mediated late endocytic pathway. Abbreviations: P.M. – Plasma Membrane; V.E.E. – Very Early Endosome; E.E. – Early Endosome; S.E. – Sorting Endosome; E.R.C. – Endosomal Recycling Compartment; L.E. – Late endosome; L.E.T. – Late Endosomal Tubules; Lyso.-Lysosome; Cholest. – Cholesterol; Endocyt. – Endocytosis.

VEEs are associated with the plasma membrane as the initiation stage of endocytosis (20). Two kinds of distinct VEEs have been identified: clathrin-coated VEEs and caveolin-associated VEEs (21). Clathrin-coated VEEs are the major endosomes for receptor-mediated endocytosis. Caveolin-associated VEEs are the major endosomes mediating endocytosis of viruses and glycolipids such as lactosylceramide and globoside. These two VEEs then dissociate from the plasma membrane and form two distinct types of their downstream EE: Rab5-positive EE and Rab5-negative EE. Both types of EEs join in SE (positive for both Rab5 and Rab4) (22). SEs are a type of organelle in which the contents are subsequently sorted into downstream organelles at later stages of endocytosis. Sorted materials can be either directly recycled back to the plasma membrane, transferred to the downstream endocytic recycling compartment, or delivered to LEs.

3.1.3. Late endocytic pathway

The late endocytic pathway receives a portion of materials from the SE (23). One of the main functions of the late endocytic pathway is the enrichment of free cholesterol and glycolipids (24, 25). This pathway includes lysosome and two types of LEs. Shown in Figure 1, the classical LE is involved in endosome-to-Golgi trafficking; they are lysosomal associated membrane protein 2 (LAMP2)-negative, but mannose-6-phosphate receptor (M6PR)-positive, organelles which contain minimal fluid phase contents (1). The classical LEs are responsible for delivering Golgi-targeting proteins (e.g. M6PR) from SEs to Golgi, and M6P-labeled lysosomal enzymes from Golgi to lysosomes (26).

Another type of LE is the newly defined late endosomal tubule (LET) (9, 25). Although the LETs

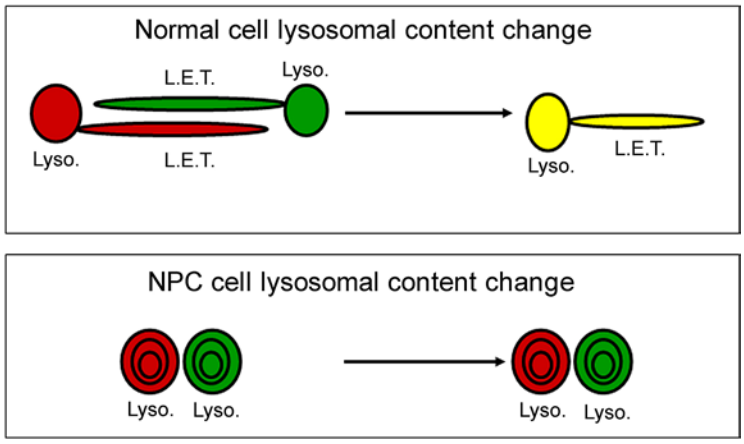

Figure 2. Difference in lysosomal content exchange mediated by late endosomal tubular trafficking between normal and NPC1 cells. This model shows a fundamental difference in the late endocytic pathway between normal and NPC1 cells. In normal cells, lysosomes exchange their content constantly and rapidly by the means of late endosomal tubular trafficking, contributing to glycolipid sorting function in the endocytic pathway. The late endosomal tubule-mediated lysosomal communication creates a dynamic network in the late endocytic pathway. However, in NPC1 cells, lysosomes do not exchange their contents, resulting in a discontinuation of the late endocytic networking. Abbreviations: L.E.T. – late endosomal tubule; Lyso. – lysosome.

contain late endocytic markers such as LAMP2, cholesterol, and glycolipids, they can be differentiated from lysosomes by loading cells with endocytosed low-density lipoprotein (LDL). LETs have the following characteristics: 1) They are enriched in membrane proteins such as Niemann-Pick type C1 (NPC1) protein, a cholesterol-binding protein involved in the transport of late endocytic cholesterol; 2) They are enriched in certain glycolipids such as GalNAcBeta1-4[NeuAcAlpha2-3]GalBeta1-4GlcBeta1-1'-ceramide (GM2) and lactosylceramide/galactosylceramide (Lac-Cer/Gal-Cer), but not NeuAc2-8NeuAc2-3GalBeta1-4GlcBeta1-1'-ceramide (GD3) and GalAlpha1-4GalBeta1-4GlcBeta1-ceramide (CTH); 3) They rapidly move along microtubules at speeds up to 5 microns/sec, driven by motor proteins kinesin and dynein; 4) The dynamic movement of LETs is negatively regulated by the cholesterol content in the endosomes; 5) Trafficking of LETs is necessary to maintain content exchange among lysosomes (Figure 2); 6) Efficient trafficking, sorting, and recycling of endocytosed glycolipids is dependent on the maintenance of LETs; and 7) LET trafficking is inhibited in NPC1 disease and other LSDs such as mucolipidosis type IV (ML4) and GM2 gangliosidosis by the accumulation of cholesterol and alteration of membrane fluidity (8, 9, 24, 25, 27 and unpublished data from Mei Sun, Lin Sun and Mei Zhang).

Endocytosed materials can be secreted out of cells after being delivered to the end stage organelle, lysosomes. It was noticed in the early 1980s that lysosomes in fibroblasts exocytosed materials out of cells (28). This phenomenon has been extensively studied recently and is now known as calcium-mediated lysosomal exocytosis, a spectacular function of lysosomes in mammalian cells (6). This exocytosis function is distinct from those of Golgi-mediated exocytosis and secretion. Lysosomal exocytosis can be a dominant function in immune cells that utilizes the machinery to execute their antigen-presenting function (29). In dendritic cells, lysosomal enzymes digest pathogen-associated proteins into smaller peptides that are subsequently carried to the lymph node by the cells. Mediated by lysosome-mediated exocytosis, dendritic cells couple the peptides to the major histocompatibility complex (MHC) class II molecules and display them on the cell surface. Defects in lysosomal exocytosis could result in immune deficiency as well as other diseases in humans. Lysosomal exocytosis appears to be a minor event in common cells, with less than 10% of lysosomes being involved in secretary function. However, it can be activated in specific situations such as plasma membrane damage, and is responsible for the repair of disrupted plasma membrane (30). Bulk influx of calcium from leaking plasma membrane elevates the concentration of intracellular calcium and triggers fusion of small lysosomes with the plasma membrane, a process regulated by the lysosomal isoform of synaptotagmin (Syt VII) (31). Loss of the ability to repair the plasma membrane by this mechanism in skeletal muscle cells can result in dysferlin-defecient muscular dystrophy in humans (32). Additionally, lysosomal mediated exocytosis and plasma membrane repairing function could well be using the trafficking machinery mediated by LETs (Figure 1, refs 9 and 33).

3.1.4. Cytoskeleton and endocytic pathways

Endosomal trafficking is a cytoskeleton-dependent event. Microfilaments and microtubules play differential roles in supporting endocytic dynamics (34). Formation of clathrin-coated pits and VEEs depend on microfilaments. Disruption of microfilaments with actin depolymerization inhibitor cytochalasin blocks receptor endocytosis from the cell surface (35). Microtubules, however, play a role in organizing EEs along the network. Disruption of microtubules with tubulin polymerization inhibitor colchicine results in dispersion of the EEs throughout the cytoplasm (36). Microtubules also serve as the intracellular 'highway' for the movement of late

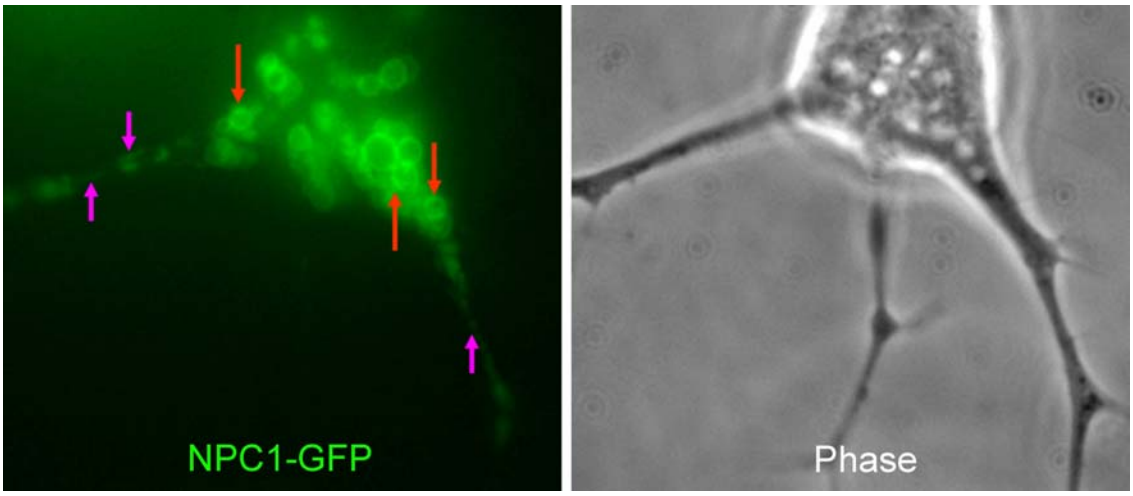

Figure 3. NPC1-GFP late endosomal tubular trafficking in neuron. This projection image merged from multiple time-lapse frames shows the movement tracks of late endocytic compartments in live neuron. Late endocytic compartments were visualized with NPC1-GFP (introduced with associate adenovirus (AAV)-mediated gene delivery) in cultured primary cortical neurons isolated from Sprague-Dawley rats. Red arrows point to the ring-shaped large lysosomal structures in the cell body of neuron. Purple arrows point to the LETs at the neurites interacting with the lysosomes. The ring-shaped large lysosomes are rarely found in the neurites (axon and dendrites), while the LETs are distributed and traffic throughout the neurites (data not shown here; interested readers can refer to reference 37). NPC1-GFP-AAV was provided by Dr. Robert Maue at Dartmouth Medical School (Hanover, Hew Hampshire USA). Cellular imaging was carried out in Drs. Joan Blanchette-Mackie and John Hanover's laboratories at National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in National Institutes of Health (NIH) (Bethesda, Maryland USA), with a CoolSnap HQ digital camera and 100x oil immersion objective mounted on a Zeiss microscope system. Detailed methods can be found in previous publications (9, 37).

endocytic compartments and delivery of early endosomal contents to the late endocytic pathway**.** Neuronal cells are particularly sensitive to microtubule-depolymerization drugs because long distance endocytic transport depends on functional axonal microtubules.

3.2. Endocytic pathways in neuronal cells

In non-polarized, adherent cells, endocytic organelles traffic bi-directionally along microtubules between the cell center and the cell periphery (9). In polarized cells, endosomes in neuronal cells traffic between the cell body and the processes, (37), or between the apical and basal plasma membranes in epithelial cells (38).

Neurons share the common stages of endocytosis of ordinary cells (1). For example, materials are endocytosed into neurons via early and late endocytic pathways, and sorting and recycling of endosomal contents are constantly occurring. However, as a polarized and extremely elongated cell type, neurons have spectacular endocytic features.

3.2.1. Long distance endosomal trafficking

Neurons need long distance 'highway' networks for the transfer of materials inside the cells. Proteins produced in ER and packed in Golgi are transported to the synapses along the axon (39). Similarly, endosomal contents are constantly transported along long distance microtubule-based 'highway' networks in neurons (40). Drugs that can damage microtubule networks often cause neurological symptoms in patients. For example, administration of microtubule-depolymerization drugs (e.g. Vincristine and colchicine) produces neurological symptoms due to disruption of microtubule networks. Microtubule stabilization drug Taxol also causes neurological toxicity due to a loss of microtubule dynamics and a malfunctioning microtubule network (41).

EEs and LEs/lysosomes are differentially distributed in neurons (42). EEs can be found in various parts of neurons such as axons, dendrites and presynaptic terminals. These compartments move from one place to another. For example, Tf can be transported from the cell body to the presynaptic regions via the early endosomal pathway (43). EEs can also provide signaling environments for initiating critical protein functions. Nerve growth factor (NGF) and its receptor TrkA may interact in endosomes to initiate neuronal signaling (44). Large lysosomes are predominantly located at the cell body (Figure 3). However, the late endocytic compartment can be transferred to distal axons where they get further acidified near the growth cones, as demonstrated with lysosomal membrane glycoprotein LEP100 (45). We also found that the highly dynamic NPC1-containing late endosomes can be found abundantly as small endosomal structures in the middle of axons and the terminal of neurons (Figure 3) (46).

3.2.2. Intercommunication between endosomes and synaptic vesicles

Intercommunication between endosomes and synaptic recycling compartments is a unique feature of neuronal endocytosis (47). Synaptic transmission is a

Table 2. Lysosomal storage disorders with glycosphingolipid accumulation

Diseases	Defects	Neurodegeneration	Disease specific therapies
Fabry Disease	*alpha*-galactosidase A deficiency	Maybe	ERT
Farber Disease	N-acylsphingosine amidohydrolase (ASAH) (acid ceramidase, AC) deficiency	Yes	N/A
Fucosidosis	*alpha*-L-fucosidase (FUCA) deficiency; *alpha*-L-fucosidase 1 (FUCA1) deficiency	Yes	N/A
Gaucher Disease	Glucocerebrosidase (acid*beta*-glucosidase) deficiency	Maybe	ERT
GM_1 Gangliosidoses, Morquio disease, type B; mucopolysaccharidosis type IVB (MPS IVB)	*beta*-galactosidase-1 (GLB1D) deficiency, or Elastin-binding protein deficiency	Yes	N/A
GM_2 gangliosidosis type I, Tay-Sachs Disease, Tay-Sachs AB Variant	*alpha* subunit of hexosaminidase A deficiency, or Hexosaminidase activator (GM2A) deficiency	Yes	N/A
GM_2 gangliosidosis type II, Sandhoff-Jatzkewitz Disease	*beta* subunit of hexosaminidase (HEXB) deficiency	Yes	N/A
Krabbe Disease	Galactocerebrosidase deficiency/ galactosylceramide*beta*-galactosidase deficiency	Yes	N/A
Lactosylceramidosis	Prosaposin and saposins A, B, C, and D deficiency	Yes	N/A
Metachromic Leukodystrophy, Sulfatide Lipodosis	Arylsulfatase A deficiency, or Pseudoarylsulfatase A deficiency	Yes	N/A
Multiple Sulfatase Deficiency, Austin Disease	Multiple sulfatase deficiency	Yes	N/A
Mucolipidosis Type IV	Mucolipin-1 defect	Yes	N/A
Niemann-Pick Disease, Type A and B	Acid sphingomyelinase deficiency	Yes	N/A
Niemann-Pick Disease, Type C1 and Type D	Niemann-Pick type C1 (NPC1) protein defect	Yes	N/A
Niemann-Pick Disease, Type C2	Niemann-Pick type C2 (NPC2) protein deficiency	Yes	N/A
Schindler Disease	N-acetyl-*alpha*-D-galactosaminidase deficiency	Yes	N/A

Abbreviations: ERT, enzyme replacement therapy; N/A, not available

critical and vulnerable function of neurons in the brain. Maintenance of the homeostasis of synaptic vesicle trafficking is regulated by endocytosis. Endosomes have been shown to serve as the storage compartments of recycling receptors at dendritic spines (48). Two important events in neurons, long-term depression (LTD) and long-term potentiation (LTP) have been linked to vesicle trafficking. For example, postsynaptic endocytosis plays an important role in long-term depression (LTD) at the synapses of hippocampal neurons (49). The availability of receptors might be maintained in intracellular organelles that are capable of rapidly delivering proteins to the synapses (50, 51, 52, 53). Loss of communication between endosomal compartments and synaptic vesicles would result in severe pathological events. The actual change of this mechanism needs to be further studied in LSD and AD to unravel the mechanism of disease.

3.2.3. Intercommunication between neurons and non-neuronal cells

Neuronal and non-neuronal cells show differential activities of the early and late endocytic pathways. The analysis in Figures 4, 5 and 6 suggests that the early endocytic pathway might be more active in neurons, while the late endocytic pathway may be more dominant in glial cells. I propose a model showing that the late endocytic pathway in glial cells plays a critical role in maintaining normal functions of neurons in the brain (refer to the model in Figure 7). Glial cells can serve as critical scavengers in clearing harmful metabolic materials around neurons, such as neurotoxic peptide A*beta*42, with their highly active late endocytic pathway (54, 55, 56).

Intercellular signaling between neurons and astrocytes might also regulate synaptic communication (57). In both neuronal and non-neuronal cells, late endocytic compartments can also be integrated in the plasma membrane during ionophore-stimulated exocytosis (58, 59). Proteolipid protein (PLP), a myelin membrane protein, can be transferred from the late endocytic compartment to the cell surface in oligodendrocytes triggered by cyclic adenosine monophosphate (cAMP)-dependent neural signals (60).

4. ABNORMALITIES OF THE ENDOCYTIC PATHWAY IN NEURODEGENERATIVE DISEASES

Alterations in endocytic trafficking are associated with the pathogenesis and progression of a variety of human diseases such as neurodegenerative disease, atherosclerosis, muscular dystrophy, and infectious diseases. In this review, I will analyze the abnormality in LSD and AD.

4.1. Abnormalities in lysosomal storage disease (LSD)

LSD is a group of genetic diseases caused by defects of lysosomal enzymes, cofactors, transporters, or proteins that are involved in biogenesis of functional lysosomal enzymes. They are characterized by the presence of enlarged late endocytic compartments in cells (61). Mutations of more than 40 genes have been identified to be responsible for these disorders (62). The enlarged late endocytic compartments include LEs and lysosomes (63), suggesting that LSD should be more accurately called a *late endocytic storage disease*.

Many LSD patients suffer from lipid storage in brain cells and neurodegeneration (64). Various membranous lipids, often glycolipids, accumulate in the enlarged late endocytic organelles. LSDs with accumulation of glycospingolipids, a major type of the disease, are listed in Table 2. The molecular mechanisms for selective involvement of the central nervous system (CNS) in most LSDs are poorly understood. However, jamming of membrane lipids (especially glycolipids) in the compartment is commonly evident in neuronal cells. The malfunction of the late endocytic system could be the initiating event in the deterioration of CNS abnormalities. I

am proposing several possible cytological mechanisms of the pathology.

4.1.1. Defective late endosomal tubular trafficking

In NPC1 disease cells, the lysosomal system is a 'quiet' system with significantly reduced communications among the endosomes (9). Excessive cholesterol accumulates in neurons in NPC1 disease (65). When the function of transfected NPC1 protein clears excessive cholesterol in late endosomal / lysosomal compartments, the highly dynamic late endosomal trafficking system can be efficiently restored. The reduction of the activity of late endosomal trafficking was found to be a consequence of cholesterol accumulation in the organelles of NPC1 cells. The jamming of rigid lipid molecules such as free cholesterol is most likely the cause of immobilizing late endosomal dynamics by reducing the fluidity of late endosomal membranes. This study led to the visualization of a novel intracellular organelle, the NPC1-containing LET, and the characterization of newly defined intracellular organelle dynamics, late endosomal tubular trafficking (9).

The blockage of late endocytic trafficking appears to also be present in other LSDs, including Niemann-Pick type C2 disease (NPC2) and ML4 disease (66 and unpublished data from Mei Sun). Taking NPC1 as a model disease we propose that neurodegeneration in LSD is caused, at least partially, by the trafficking block of the late endocytic pathway in neuronal cells (9). These storage disorders may be classified as a *late endosomal trafficking disorder*. Intriguingly, the defective cholesterol trafficking in NPC1 cells can be rescued by overexprssing Rab7 or Rab9 via Rab protein-activated vesicular trafficking in the late endocytic pathway, suggesting a potential therapeutic strategy for NPC1, and preferably other LSDs (see later chapters).

4.1.2. Aberrant endocytic glycolipid sorting

In addition to the problem of late endosomal tubular trafficking, lipid sorting mechanism is also impaired in NPC1 cells (25). Two major Golgi and plasma membrane-residing glycolipids (GD.3. and CTH) are accumulated in the lysosomes of NPC1 cells instead of being transported to the Golgi or plasma membrane. Certain types of lysosomal resident glycolipids (GM2 and Lac-Cer/Gal-Cer) could not be found in the late endosomal compartment of NPC1 cells. Cholesterol also regulates the sorting of other lipids in other glycolipid storage disease cells (67). Several types of glycosphingolipids, including BODIPY-lactosylceramide, BODIPY-globoside and BODIPY-GM1 gangliosides that are sorted into Golgi in normal cells, accumulate in lysosomes, and in turn down-regulate trafficking of other lipids from late endocytic compartment to Golgi**.** The sorting machinery in the late endocytic pathway is inhibited by the accumulation of excessive cholesterol in the membrane of those glycolipid storage disease cells. Further study of the lipid sorting mechanism in neuronal cells is critical for the understanding of the diseases. Additionally, restoration of the functions of late endocytic trafficking in neurons could eventually benefit LSD patients.

4.1.3. Reduced lysosome-mediated retrograde transport and exocytosis

In NPC1 cells, lysosomal content exchange mediated by late endosomal trafficking is significantly impaired (9). NPC1 cells also have a functional defect – retrograde exocytosis of lysosomal materials to the culture medium (34). Inhibition of late endosomal trafficking is accompanied by the redistribution of late endocytic compartments to the perinuclear compartments and the reduction of vesicles at the periphery of cells (9, 28). In addition, late endosomal trafficking defects may be a general cytological phenotype in various LSDs. As discussed previously, lysosome-mediated exocytosis is essential for plasma membrane repair in normal cells (68). During the repair process, small late endosomal/lysosomal compartments are delivered to the impaired regions of the cell membrane. We have noticed that late endosomal retrograde trafficking is significantly reduced or inhibited in NPC1 cells and the highly dynamic late endosomal compartments are absent at the cell periphery (9). I suspect that there is a defective mechanism in repairing the plasma membrane. This possible defect needs to be further clarified because it could be a general problem in disease cells from LSD patients and potentially a pathophysiological defect in the neurons of LSD brains.

4.1.4. Summary

The mechanisms by which LSDs selectively affect neuronal cells are not well understood. Late endocytic pathways are primarily affected in neurons (refer to Figure11). The defect of the late endosomal trafficking in NPC1 cells may represent a general cellular pathology in LSDs. This pathological defect is reversible by either depleting the organellar contents or activating functional machineries including Rab protein-mediated vesicular trafficking. These intriguing findings suggest that we need to think creatively while developing novel therapeutic strategies for the disease (see chapter 5.3.1).

4.2. Abnormalities in Alzheimer's disease (AD)

AD is a neurodegenerative disease suffered by people over the age of 65. AD is the most common cause of dementia accompanied by progressive memory loss and declined daily activities (69). Early pathological findings include loss of neurites and synapses in the hippocampus region of the brain (70). Late pathological changes are characterized by the presence of both senile plaques and neurofibrillary tangles (71). Deposition of aggregated A*beta*42 peptide in the brain has been considered one of the key causal factors of AD (70). Mutations in presenilin-1 (PS1), presenilin-2 (PS2) or amyloid precursor protein (APP) can cause early onset forms of the disease, but apolipoprotein E (ApoE) gene polymorphisms that result in apolipoprotein E4 (ApoE4) genotype are the major genetic factors for late onset AD (70). Synaptic transmission damage is believed to be the earliest change in AD, especially at the stage of pure memory impairment (72). In AD brains, Ab*eta* predominantly accumulates at the synapses, and the surviving synapses are always surrounded by gliosis (71).

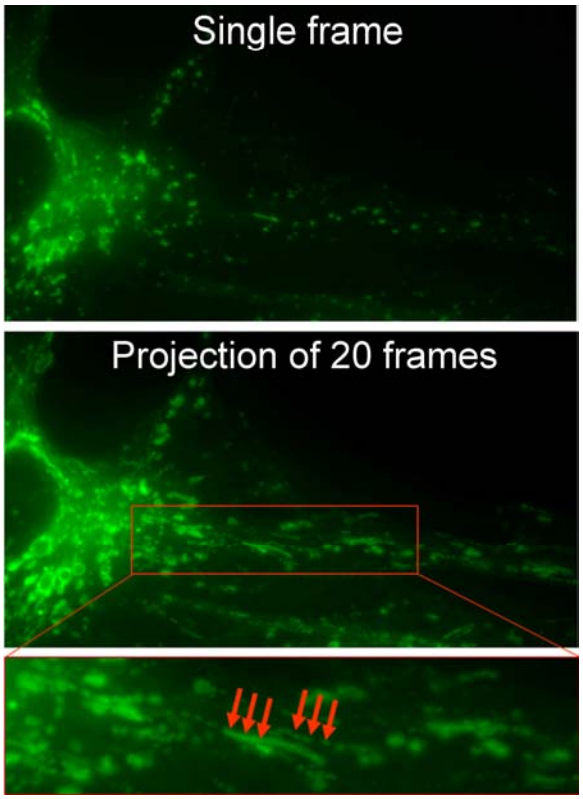

Figure 4. NPC1-GFP late endosomal tubular trafficking in glial cell. This projection image with multiple time-lapse frames shows the movement tracks of late endocytic compartments in live glial cell. The compartment was visualized with NPC1-GFP (introduced with AAV) in cultured primary glial cells isolated from Sprague-Dawley rats, demonstrated by a single frame and projection image of multiple time-lapse frames. Time-lapse digital imaging was taken with a frame rate of 1 frame/second. Red arrows indicate the tracks of single tubulo-vesicular endosomes in the projection image. Similar structures can be found elsewhere in the projection image. NPC1-GFP-AAV was provided by Dr. Robert Maue. Cellular imaging was carried out in NIDDK, with a CoolSnap HQ digital camera and 100x oil immersion objective mounted on a Zeiss microscope system. Detailed methods can be found in previous publications (9, 37).

4.2.1. Increased Abeta generation in the early endocytic pathway

AD-causing mutations in APP (about 50% of all mutations) are always located near the *gamma*-secretase cleavage site of the protein, causing over-production of A*beta*42 peptide (73). A*beta*42 is broadly recognized as the number one causal factor of human AD. A*beta*42 is generated by the combinatory cleavage of APP by *beta*- and *gamma*-secretases in cell membrane (74). The endocytic pathway is the primary cellular site of *beta*-secretase, *gamma*-secretase, and A*beta* peptide generation (Figure 8) (75). *Beta*-secretase has been clearly localized in the Tf-containing early endocytic recycling compartment (76). However, it was difficult to pinpoint the cellular location of *gamma*-secretase due to the complexity of the enzyme in composition, as well as its sparse presence at functional sites (77). Using high resolution and high speed digital imaging technology, we carefully examined the dynamic trafficking of APP and APP-C99 (the *beta*-carboxyl terminus fragment) in live cells stably expressing GFP-fusion proteins. We discovered, for the first time, that the direct substrate of *gamma*-secretase APP-C99 accumulated in Tf-labeled early endosomal recycling compartment in cells treated with various *gamma*-secretase inhibitors (DAPT or L685,458) (18), which is also the site for *beta*-secretase producing APP-C99 from full length APP. As discussed before, this compartment is the route for the trafficking of endocytic materials between the plasma membrane and the EEs (1). The majority of APP-C99 molecules normally traffic between the plasma membrane and the EEs and are efficiently cleared from the endosomes. Upon inhibition of *gamma*-secretase (i.e. disruption of the clearance machinery for the substrate proteins), the APP-CTF degradation is blocked along the route. This finding pinpointed the site of the functional *gamma*-secretase complex in the early endocytic pathway**.**

Subcellular fractionation localized *gamma*-secretase and APP process to a specific sub-cellular structure, the lipid rafts, in cell membrane. Free cholesterol, instead of total cellular cholesterol, in the cell surface membrane regulates A*beta* production (78). Further studies confirmed the cellular location of A*beta* generation and *gamma*-secretase activity (79). Together with these findings and our previous observation (18), I propose that fresh A*beta* peptides are produced in VEEs (small endosomes associated with the plasma membrane) (refer to the endosomal structures in Figures 8 and 9). The VEEs may also be the site for *gamma*-secretase to execute its cleavage function on the substrates including APP-C99.

Early endosomal abnormality was found in sporadic AD brains. Enlargement of the compartment is an early pathological change in neurons (80). A*beta* accumulation was also found in the abnormally enlarged endosomes (81). We also found that in PS1/PS2 double knockout (dKO) cells, the Tf and Rab11-GFP-labeled endocytic recycling compartments were disorganized - instead of forming the typical morphology of the compartment - suggesting that loss of PS1/PS2 function could alter the endosomal organization in endocytic system (Figure 10). The impact of this type of abnormality on the pathology needs to be further studied in the future.

4.2.2. Generation of Toxic Abeta in the late endocytic pathway

While the early endocytic pathway provides compartments for the generation of the majority of A*beta*, the late endocytic pathway plays unique roles in processing A*beta* peptide. After being generated in VEEs, A*beta* is subsequently endocytosed in early, sorting, and late/lysosomal pathways. A clear difference between A*beta*40 and 42 is their differential associations with the cell membrane, suggesting that A*beta*42, but not A*beta*40, travels with the endosomal membrane from VEEs to the organelles in late stages, where they are further processed (70). Lysosomes collect the processed A*beta*42 which was

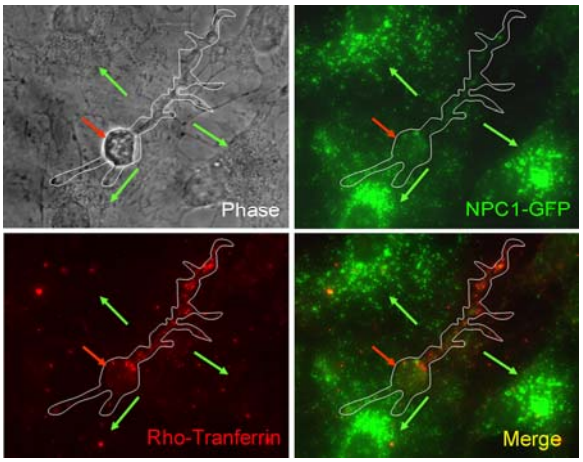

Figure 5. Comparison of early and late endocytic compartments in co-cultured neurons and glial cells. This panel provides a side-by-side comparison of the early and late endocytic pathways between live neuron and glial cell. The early endocytic compartment was labeled with endocytosed rhodamine-transferrin, while the late endocytic compartment was labeled with NPC1-GFP (introduced with AAV), in a co-cultured system with primary rat neurons and glial cells isolated from Sprague-Dawley rats. The comparison demonstrates a clear difference for the structures and activities of both pathways in cells (The early pathway is relatively high in neuron (red arrows), and the late pathway is relatively high in glial cells (green arrows)). Images were captured with a digital camera and 100x oil immersion objective. NPC1-GFP-AAV was provided by Dr. Robert Maue. Cellular imaging was carried out in NIDDK by the author with a CoolSnap HQ digital camera and 100x oil immersion objective mounted on a Zeiss microscope system. Detailed methods can be found in previous publications (9, 37).

found not to be easily digested in the compartment (82, 83, 84). Therefore the processed A*beta*42 molecules accumulate in the late endocytic compartment and are further processed in the lysosomes. In neurons, abnormally increased A*beta*42 were also found to accumulate in the multi-vesicular bodies (MVBs) in mouse AD brain (85). An *in vitro* study demonstrated that inhibition of lysosomal associated proteases could result in accumulation of toxic A*beta*42 (86). Both soluble and fibril A*beta* could be secreted out of cells from lysosomes (83). This is likely to be mediated via calcium-dependent lysosomal exocytosis at the synapses. Elucidating the role of the late endosomal and lysosomal pathways in degradation, processing, and secretion of A*beta*42, may ultimately help the design of novel drugs to enhance the elimination of A*beta*42 within lysosomes.

Endosomal processing of A*beta* can be also largely modulated by special proteins closely associated with the pathogenesis and progression of AD. One of the critical proteins is ApoE (87). All ApoE isoforms can strongly bind to A*beta* with nanomolar potency. However, only ApoE4, no other isoforms, can induce the increase of A*beta* production in cells. The isoform-specific effect of ApoE on A*beta* production may result from the alterations in APP recycling function in the endocytic pathway. More surprisingly, ApoE is an active molecule inside the endosomal system. The interaction of A*beta* and ApoE was shown to reduce stability and damage the function of lysosomes. Both ApoE4 and A*beta*42 should be efficiently degraded in normal cells to maintain the healthy lysosomes (88).

Lysosomes also play a regulatory role in keeping early endosomal proteins at proper levels to maintain the homeostasis of the early endocytic recycling pathway. The lysosomal degradation pathway down regulates the constitutive expression level of APP, APP-CTF, *beta*-site APP-cleaving enzyme 1 (BACE1) (89), *gamma*-secretase and A*beta*. For example, components of the early endosomal enzyme, such as *gamma*-secretase, could also be found in the late endocytic compartments under certain conditions (90). Full length APP could be artificially accumulated in the vesicles in cells treated with lysosomal protease inhibitor cholorquine, suggesting APP is normally down-regulated by lysosomal proteases in the late endocytic compartment (91). Part of APP-CTFs is also shuttled to the LEs and lysosomes for degradation (92). In an animal study, inhibition of late endosomal/lysosomal proteases resulted in accumulation of APP-CTFs (as well as full length APP) at the surface and in the early pathway, where some of those extra fragments were cleaved to generate more A*beta* (93). On the other hand, enhancement of APP internalization with an anti-APP antibody could significantly reduce A*beta* production (94). After delivery to lysosomes, excessive monomers and polymers of A*beta*42 can also damage lysosomal function, and result in decreased degradation of APP and APP-CTFs. The APP fragments could be subsequently redirected to the early endosomal pathway for the generation of A*beta*42 (95). Therefore, the late endocytic pathway serves as down-regulation machinery with its degradation function to eliminate excessive AD proteins that are constantly transferred through the compartments. Research effort should be prioritized for precisely understanding the relationship between the process and trafficking of A*beta* along these interconnected biological pathways.

4.2.3. Summary

The relationship between AD and endocytosis is summarized in the following: 1) APP is localized on the plasma membrane (PM) and the cleavage of APP by BACE occurs in EEs and the recycling compartment; 2) Functional *gamma*-secretase is located in VEEs where it meets recycled APP-CTF generated by BACE in the early endosomal pathway; 3) Most A*beta*42 is degraded in the late endocytic pathway, while undigested A*beta*42 is processed in multiple endocytic pathways during endocytic trafficking; 4) The ultimate process of A*beta* happens within the lysosomes; 5) The most toxic form of A*beta,* i.e. the lysosomal A*beta*42, is secreted out of cells via lysosomal-mediated exocytosis, which may be associated with the PM, with its membrane-association moiety; and 6) Cells use the late endocytic pathway to down-regulate APP as well as related enzyme activity. We could draw the conclusion that AD is closely associated with the entire endocytic system, and different stages of the system play

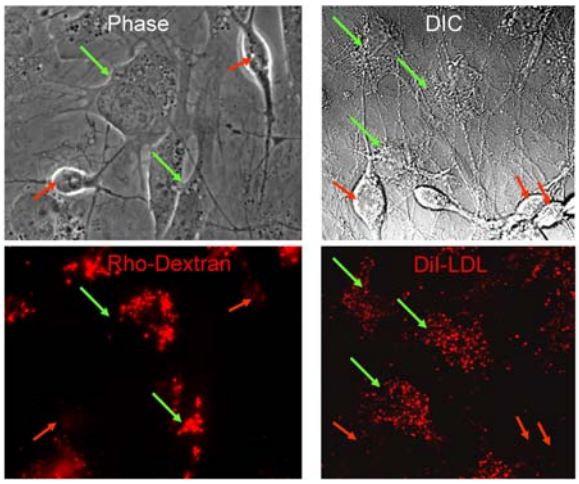

Figure 6. Comparison of internalization of late endocytic markers in co-cultured rat primary neurons and glial cells. This panel with two pairs of images shows the late endocytic compartments labeled with endocytosed rhodamine-dextran or DiI-LDL (red structures in the lower panels) in a co-cultured system with primary neurons and glial cells isolated from Sprague-Dawley rats. The comparison demonstrates a clear difference in the structures and activities of the late endocytic pathway between neurons (red arrows) and glial cells (green arrows). The uptake of both markers is much higher in glial cells than in neurons. Cellular imaging was carried out in NIDDK. The phase contrast and Rho-dextran images were captured with a CoolSnap HQ digital camera and 100x oil immersion objective, while the DIC and DiI-LDL images were obtained with a Zeiss LSM410 confocal system and 100x oil immersion objective. Detailed methods can be found in previous publications (9, 25, 37).

differential roles for the hostile peptide, A*beta*42 (Fig11). Thoroughly understanding the endocytic events should be the key for novel drug discovery.

5. DISCOVERY AND DEVELOPMENT OF NOVEL DRUGS FOR NEURODEGENERATIVE DISEASES

5.1. Current therapies for neurodegeneration

LSD can be caused by the defects in three types of proteins: late endocytic soluble proteins, late endocytic membrane proteins, and lysosomal enzyme supporting proteins. Lysosomal soluble proteins are secreted proteins that can be re-uptaken into late endocytic compartments via cation-dependent mannose 6-phosphate (CD-M6P) receptor or insulin-like growth factor-II (IGF-II) receptor-mediated endocytosis using the phosphorylated mannose residues on the lysosomal proteins (26, 96). This machinery has been adapted to therapeutic strategy for the replacement of lysosomal enzymes (97). The first lysosomal storage disease treated by replacement therapy was Gaucher disease. Gaucher disease can now be treated with recombinant human *beta*-glucocerebrosidase (Imiglucerase, or Cerezyme) as the second generation of tissue-derived *beta*-glucocerebrosidase (alglucerase, or Ceredase) (98). Following the success in Gaucher disease, Fabry disease can also be treated with *alpha*-galactosidase A replacement therapy (99). *Alpha*-galactosidase A is available from Shire (agalsidase *alpha* or Replagal) (100) and Genzyme (agalsidase *beta* or Fabrazyme) (101). The latter form was approved by the U.S. Food and Drug Administration (FDA) on 2001, while both drugs are currently used in Europe (102).

Inhibiting the production of certain critical glycolipids is another strategy for managing LSD symptoms (103). Glucocerebroside is the storage compound in Gaucher disease. The imino sugar derivative, N-butyldeoxynojirimycin (NB-DNJ, i.e., OGT-918, Oxford Glycosciences), can inhibit the glucosyltransferase (which catalyses the transfer of glucose to ceramide during glycospingolipid biosynthesis) and therefore prevents the formation of glucocerebroside (104). Although N-butyldeoxynojirimycin was shown to be efficacious in reducing glycolipids in certain diseases, improvement of neurological deficits has not been reported.

As a rare case, LSD cystinosis can be treated with a specific therapy using the oral drug cysteamine (Cystagon from Mylan Pharmaceuticals) (105, 106). Cysteamine cleaves accumulated cystine which then bypasses the harmful metabolic pathway thereby reducing renal failure risk and reliving eye damage.

In general the availability of targeted therapy for LSD is extremely limited. Only a few LSDs can be treated by correcting the enzymatic defects. Most of currently available therapies are supportive treatment for reducing symptoms of LSD.

Most of the existing drugs for AD do not target the defective molecular mechanisms of the disease. Current therapies for AD are therefore also limited to symptomatic management. Several drugs are currently on the market for the treatment of AD symptoms. These drugs are either acetylcholinesterase inhibitors including donepezil (Aricept; Eisai/Pfizer) (107), rivastigmine (Exelon; Novartis) (108), galantamine (Razadyne; Johnson & Johnson) (109) and tacrine (Cognex; First Horizon Pharmaceuticals) (110), or *N*-methyld-aspartate (NMDA)-receptor modulators such as memantine (Namenda; Forest/Lundbeck) (111). Acetylcholinesterase inhibitor drugs inhibit acetylcholinesterase (AChE) activity to increase acetylcholine level in the synaptic cleft and modestly improve cognition symptoms temporarily (112). Other symptom improving drugs include antidepressants, antipsychotics and anxiolytics, which are commonly used to help control the behavioral symptoms of AD patients (113).

5.2. Drug discovery for novel therapeutics on neurodegeneration

Significant research progress has been achieved by academia that is shedding light in the pharmaceutical industry on the development of new therapeutic strategies for LSD and AD. Co-development of novel therapeutics between academia and industry are now playing more and more promising roles in the translation of basic research

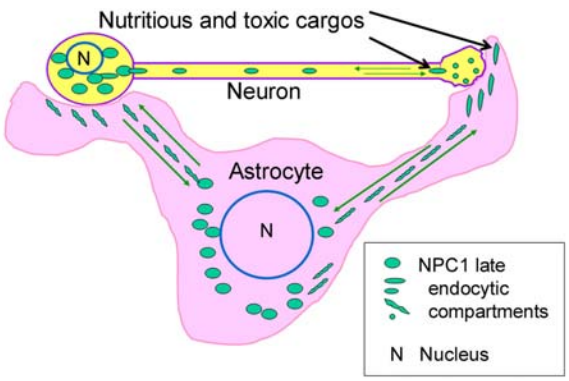

Figure 7. Role of late endosomal tubular trafficking in the interaction between astrocytes and neurons. This model shows the possible mechanisms in the late endocytic pathway by which astrocytes support neurons to clean the environment surrounding neurons, or/and secrete toxic endocytic cargos in the environment to damage neurons.

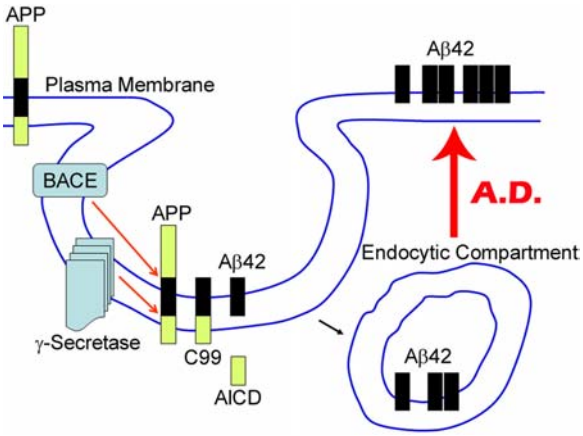

Figure 8. The fate of APP involves the entire endocytic pathway. This model depicts the overall trafficking routes for APP and its fragments in cells with normal condition or AD. The plasma membrane APP is shuttled into VEEs for *beta*-secretase cleavage, followed by *gamma*-secretase cleavage to produce A*beta*42 (and A*beta*40). A*beta*42 is further processed in late endosomes and lysosomes. Non-degradable lysosomal A*beta*42 is recycled back to the cell surface, inserted in or closely associated with the plasma membrane. In AD cells, this procedure is enhanced by the over-production of A*beta*42 in the EEs, or the protection of ApoE4 over A*beta*42 against lysosomal degradation, resulting in accumulation of excessive A*beta*42 (toxic forms) at the neuron membrane.

findings into developable drug pipelines for neurodegenerative diseases. Targeted therapies are the major focus for the development of drugs for AD, while cell-based therapies are the major focus for LSD.

5.2.1. Lysosomal storage disease

Although enzyme replacement therapy could successfully reduce the deterioration of a few LSDs, it has a general limitation of being undeliverable to the brain tissues resulting from the blood brain barrier (BBB). Therefore, there is hope that developing cell-based therapies will be able to overcome this tough problem.

5.2.1.1. Bone marrow and hematopoietic stem cell transplantations

Recently, the development of LSD therapy has been focused on cell-based strategies. The major advantage of cell-based therapy benefits from the cross-cell effect of functional lysosomal enzymes (114). The unique feature of inter-cellular trafficking of soluble lysosomal enzymes provides the possibility of developing bone marrow transplantation therapy (115). Transplanted bone marrow cells with normal or enhanced functions are transferrable to the brain via transmigration of cells across the patient's BBB (116). Several LSDs have been shown to be responsive to bone marrow transplants (117).

As a strategic expansion of bone marrow transplantation, stem cell technology (SCT) is an emerging new therapeutic method for LSD. SCT is highly effective in regenerating bone marrows to replace defective bone marrow in the human body. SCT may be also able to reduce the major problem of bone-marrow-transplantation (BMT)-related patient death. Drugs that can induce the elevation of hematopoietic stem cells in the periphery blood facilitate the harvest of bone marrow derived stem cells. Several protein markers have been shown to be stem cell specific. For example, chemokine receptor CXCR4 and its ligand stromal cell derived factor-1 (SDF1), is a pair of key regulators for the mobilization and homing of hematopoietic stem cells (118). Specific small molecule inhibitors, including CXCR4 inhibitor AMD3100 (AnorMed and Genzyme), have been developed to increase hematopoietic stem cells in the periphery blood of the donor (119). Although CXCR4 inhibitors are in current development for the transplantation in lymphoma patients after radiotherapy, this class of drugs could be further developed and applied to LSDs to speed up hematopoietic stem cell transplantation (120).

5.2.1.2. Virus vector-mediated gene therapy

Gene therapy is another type of therapy for LSD in preclinical development. Gene therapy has achieved several major improvements including the utilization of highly-efficient lentiviral vectors and virus-transduced therapeutic cells (121). However, gene therapy has been moving slowly for LSD, due to major limitations including virus-induced tumorigenesis (122). Clinical application of gene therapy on LSDs has not been reported.

5.2.2. Alzheimer's disease

Major progress has been made on targeted therapy for AD. These targets include A*beta*, *alpha*-secretase, *beta*-secretase and *gamma*-secretase. Inhibitors targeting newly identified AD symptom-related receptors or ion channels, such as 5-hydroxytryptamine (5-HT) or calcium channels were also generated. Numerous other symptom-releasing-orientated new therapies for AD are under investigation including: estrogens, anti-inflammatory agents, statins, monoamine oxidase inhibitors and antioxidants. Many new therapies have reached various clinical trial stages

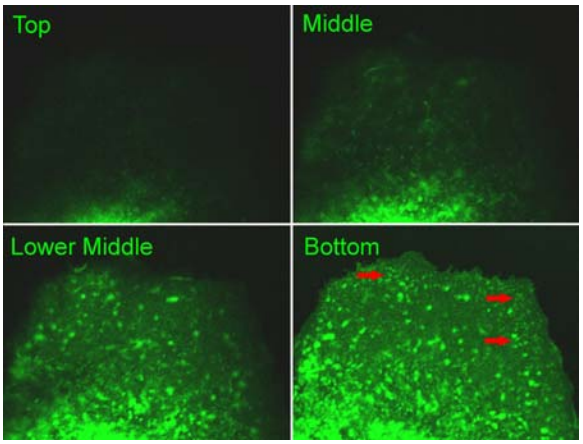

Figure 9. Loss of *gamma*-secretase activity causes APP-CTF accumulation at the plasma membrane and VEEs**.** This sequential image panel shows the association of APP-C99-enriched VEEs with the plasma membrane. CHO cells were stably transfected with GFP-tagged APP-C99 fragment (C99-GFP) and treated with *gamma*-secretase inhibitor DAPT (Calbiochem) for 24 hours. Sequential confocal scanning (Z-sectioning) was performed through a live cell. C99-GFP was visualized at the plasma membrane and numerous small punctate structures, shown in the bottom image (red arrows). In control cells without DAPT treatment, C99-GFP was not detectable under the microscope due to efficient clearance by the endogenous *gamma*-secretase (data not shown. Interested readers can refer to reference 18). Cell culture was carried out in Dr. Kovacs Laboratory at Massachusetts General Hospital (MGH) (Charlestown, Massachusetts USA). Images were obtained in the Confocal Imaging Center at MGH-East, with a Zeiss LSM510 Pascal confocal system and a 100x oil immersion objective. Detailed methods can be found in previous publication (18).

5.2.2.1. Disease protein-targeted therapy

5.2.2.1.1. Abeta inhibitor or antibody

Inhibiting A*beta*42 activity would be the most direct way to halt AD. This could be achieved by helping cells to efficiently clear the A*beta*42 peptide during the process and trafficking in the endocytic pathway. However, the exact mechanism for the clearance following treatment remains to be unraveled. Elan and Wyeth are co-developing humanized monoclonal antibody Bapineuzumab (AAB-001) against A*beta*, and have started a Phase III trial to treat mild to moderate AD in the U.S. The mechanism of action by which anti-A*beta* antibody significantly prevented AD in transgenic mice was shown through reduction of aggregation of A*beta* in the brain (123). Neurochem is developing small sulfonated molecule, Alzhemed, to inhibit the interactions between A*beta* with glycosaminoglycans for mild to moderate AD (124). However, data from the Phase III trial missed the primary endpoints in the US. Prana Biotechnology is developing compounds PBT-1 (Coloquinol) and PBT-2 to prevent the formation of toxic A*beta* oligomers by removing metals, such as copper and zinc, from the A*beta* peptide (125). A Phase II trial is on-going for PBT-1. Small molecule AZD-103, co-developed by Transition Therapeutics and Elan, was designed to inhibit A*beta* fibrillization and disassemble preformed amyloid fibrils (126). It is now in a clinical Phase I trial. TTP488 from TransTech Pharma/Pfizer, in Phase IIa, blocks the interactions between RAGE (Receptor for Advanced Glycation Endproducts) and A*beta* to reduce brain amyloid load (127). The interaction between RAGE and A*beta* could perturb neuronal functions and promote glial-mediated inflammatory responses and neuronal death.

5.2.2.1.2. Alpha-secretase activator

Activation of *alpha*-secretase activaty may shift APP processing towards the 'benign' pathway (cleavage of full length APP by *alpha*-secretase) and reduce A*beta* generation along the 'harmful' pathway mediated by *beta*-secretase cleavage of full length APP. TorreyPines Therapeutics is developing agonists for the GPCR protein M1 muscarinic receptor to activate *alpha*-secretase activator in cells. The lead compound NGX267 is currently in Phase I.

5.2.2.1.3. Beta-secretase (BACE1) inhibitor

Inhibiting BACE1 can reduce the production of A*beta*42 by decreasing *gamma*-secretase substrate formation without significantly affecting the Notch pathway (128). This may also shift the APP processing towards the 'benign' pathway – the *alpha*-secretase mediated cleavage of full length APP (129). Inhibiting BACE1 function may also improve patient brain function via normalizing voltage-gated sodium channel levels in neurons (130). CoMentis is generating small molecules as *beta*-secretase inhibitors (CTS-21166 is in Phase I and ATG-Z1 is in preclinical). Sunesis Pharmaceuticals and Merck have also been co-developing small molecule inhibitors against BACE1 (http://www.sunesis.com/pipeline/programs.php). Several other companies, such as Elan, are also developing this class of new drugs.

5.2.2.1.4 Gamma-secretase inhibitors

Inhibition of *gamma*-secretase activity increases the retention of APP-C99 on the cell surface and the recycling compartment, as revealed in the live cells. The excessive C99 fragment could then be shunted to the lysosomal degradation pathway. This could be an efficient way to prevent the production of the critical derivative of APP, A*beta*42, *in vivo*. Several large pharmaceutical companies are developing inhibitors targeting *gamma*-secretase protein complex, including: Eli Lilly with LY450139 in Phase II (131), Merck with MK0752 in Phase II (Rosen et al, Alzheimer's & Dementia 2:S79, 2006) and Eisai with E2012 in Phase I (http://www.pipelinereview.com/joomla/content/view/3142/113/).

5.2.2.2. Other mechanism of disease-based therapies

Several proteins have been recently identified as being related to the development of AD symptoms. 5-hydroxytryptamine (5-HT or serotonin) receptor-targeting drugs have been developed by several biopharma companies, including: agonist Xaliproden (Phase III) from Sanofi–Aventis (132), antagonist Lecozotan (Phase II/III)

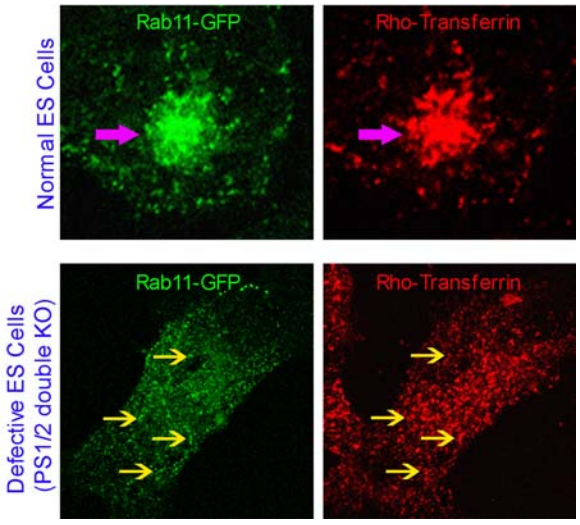

Figure 10. Abnormality of early endocytic recycling compartment (ERC) in an embryonic stem cell line generated from PS1/PS2 double knockout (dKO) mouse. This panel demonstrates the defective endocytic recycling compartment (ERC) with two distinct markers, Rab11 and transferrin, in cells lacking *gamma*-secretase function. The mouse embryonic stem cells were transfected with Rab11-GFP and loaded with rhodamine-transferrin for 7 min, and imaged immediately in live conditions. Note that typical ERC structures (purple arrows in normal cells on upper panel) were absent in mutant dKO ES cells (lower panel), shown by both ERC markers. Instead, EEs were present as scattered punctates throughout the cells (yellow arrows). Images were obtained with a Zeiss LSM510 Pascal confocal system and a 100x oil immersion objective. Cell culture and imaging was carried out in MGH-East. Images were captured with a Zeiss LSM510 Pascal confocal system and a 100x oil immersion objective. Detailed methods can be found in previous publication (18).

from Wyeth (133), and agonist PRX-03410 (Phase II) from Epix Pharmaceuticals/GlaxoSmithKline (http://www.epixmed.com/products/prx-03140.asp). A multi-target inhibitor Dimebon (Phase II) is being developed by Medivation, which could be inhibiting both NMDA (*N*-methyl-daspartate)-receptor and cholinesterase, and modulating mitochondrial permeability transitory pores in neurons (134). Calcium-channel (L-type) blocker MEM100.3. (Phase II), by Memory Pharmaceuticals, is generated from to reduce calcium overload in neurons (135). Antioxidant and anti-amyloid compound Oxigon (Phase Ia) is produced by Intellect Neurosciences to reduce neuronal ROS damaging (136). The microtubule binder and stabilizer AL-108 (Phase II) is developed in Allon Therapeutics for the purpose of reducing A*beta* aggregation (http://www.allontherapeutics.com/pd_AL-108.htm). All these compounds may modify the disease events indirectly associated with AD molecular defects.

5.3. Potential strategies for neurodegeneration therapy

Extensive studies provide direct and indirect indications for new therapeutic strategies for LSD and AD. Below are several possibilities of translating new discoveries into drug development. Since neurodegeneration is a rapid growing field, new concepts will be constantly emerging and current thoughts may soon be outdated. We should continue to keep track of new potential targets for neurodegenerative diseases.

5.3.1. Lysosomal Storage disease

5.3.1.1. Initiating Rab protein-mediated endocytic vesicular trafficking

It was shown by several independent groups that overexpressing late endosomal Rab proteins reduced cholesterol content in lysosomes of NPC1 fibroblasts and corrected NPC1 phenotypes in cells (137) (Mei Zhang, data not shown). Recently, the protein delivery technology using herpes simplex virus VP22 protein was successfully applied to cultured cells to correct the cellular defects of lipid trafficking in NPC1 and NPC2 cells (138). This result strongly suggests that Rab-mediated vesicular trafficking could be activated for efficiently transporting cholesterol from LEs to Golgi (139). It would be interesting to determine whether Rab protein-mediated vesicular trafficking will facilitate lipid mobilization out of lysosomes in a broad range of LSD cells. I have proposed in previous chapters that a general mechanism of vesicular trafficking defect may lie behind LSDs. Targeting improvement of vesicular trafficking in LSD cells would be a totally new therapeutic idea, especially for membranous lysosomal transporter defects, such as NPC1 and ML4 diseases.

5.3.1.2. Regenerating residual functions of mutant lysosomal proteins

Most newly synthesized proteins require the help of chaperone proteins to be delivered to the final (functioning) organelles (140). Inhibiting the function of chaperone proteins (such as HSP90) can dramatically reduce the stability and functions of their target (client) proteins (141). Chemicals may mimic chaperone proteins in maintaining protein function (142). This is currently a drug development strategy for other genetic diseases such as cystic fibrosis with CFTR gene defect (143). 1-deoxygalactonojirimycin, a competitive inhibitor of *alpha*-galactosidase A, was shown to be able to function as a chemical chaperone of the enzyme at low concentration (144). With this treatment, enzymes with Fabry disease mutations previously retained in the ER can be re-delivered to the lysosomes in which the residue enzymatic activity executes the function. Some small molecules have been demonstrated to be able to activate heat shock responses and protect neuronal damages (145). It would be interesting to further explore the possibility of improving protein folding by these chemicals. Similar ideas have also been described in some spectacular cases for the strategy towards AD therapy (88).

5.3.2. Alzheimer's disease

5.3.2.1. Interfering late endocytic toxic Abeta generation or secretion

The nature of the complicated post-translational fate of APP involves multiple proteolytic processes. Post-proteolytic process of A*beta*42 is extremely difficult to

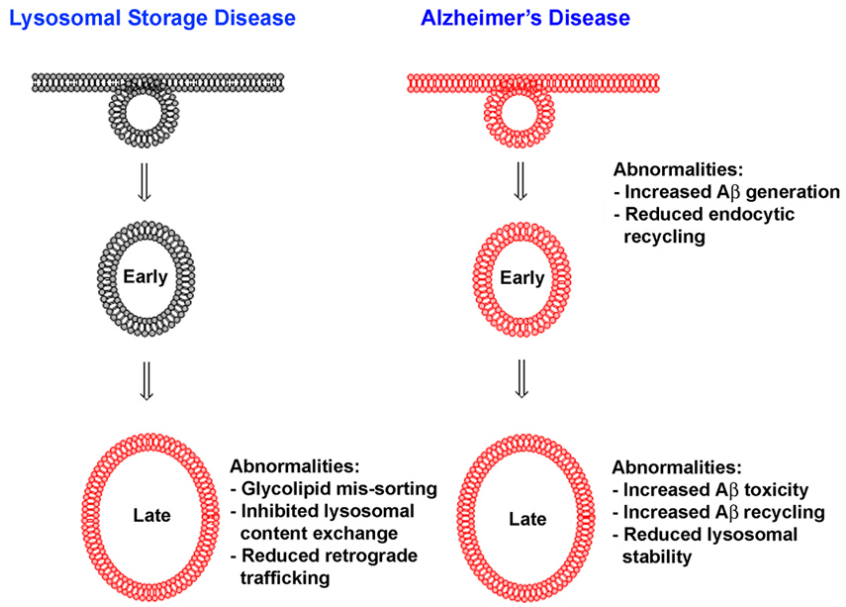

Figure 11. Differential involvement of the early and late endocytic pathways in LSD and AD. This model summarizes the abnormalities of the early and late endocytic pathways in LSD and AD. Vesicles labeled with the word *Early* represent early endocytic compartments while *Late* for late endocytic compartments. In summary, LSD mainly affects the late endocytic pathway and AD affects the entire endocytosis system.

clarify. In addition, A*beta* may behave differentially in different cell types. Therefore, interfering toxic A*beta* generation faces great challenges. To simplify the procedure for developing drugs that can reduce toxic A*beta* generation, cell-based phenotypic screen strategy would be very useful for small molecules that can inhibit the toxic effect of A*beta* on neuronal cells. I can propose several possible mechanisms for the potential drugs to take action: 1) Inhibiting secretion of freshly generated A*beta* from the VEEs; 2) Inhibiting formation of the toxic form of A*beta* in the late endocytic compartment; 3) Enhancing degradation of fresh or processed A*beta* in the late endocytic compartment; 4) Other mechanisms such as inhibiting *beta*-secretase, *gamma*-secretase, or ApoE-A*beta* interaction, as described in the previous chapters in this article.

5.3.2.2. Interfering ApoE-Abeta interaction

The neuronal toxicity produced by the interactions among cholesterol, ApoE4 and A*beta*42, is considered to be a key factor for understanding the pathogenesis and progression of AD. The possible mechanism of action for the enhancement of ApoE4 on A*beta* toxicity has been explored extensively (88). The endocytic pathway is involved in the mechanism of action of the disease. Direct interaction between ApoE4 and A*beta*42 contributes uniquely to the development of pathology for sporadic AD (146). A*beta*12-28P, a synthetic peptide homologous to the ApoE binding site on A*beta*, was reported to be able to significantly reduce A*beta* level, as well as related pathology in the brains of AD transgenic mice (147). The contribution of cholesterol should also be added to the picture of ApoE4 and A*beta*42 interaction for promoting AD (148). Therefore, it would be extremely beneficial to develop unique cell-based assays that mimic the interaction among cholesterol, A*beta*, and ApoE4 molecules, in the endosomal and lysosomal membrane. Cell-based assays can be great tools to screen chemical libraries for discovering small molecule drugs or antibodies that are able to perturb the critical interactions among cholesterol-ApoE-A*beta* in *in vivo* conditions.

6. PERSPECTIVES

As it has been strongly suggested, researchers must emphasize studies of the human A*beta* process in physiological conditions. Understanding the entire endocytosis process in cells, especially in brain cells, could be the most reliable way to access the truth behind neurodegeneration in AD and LSD. Practically, live cell-based assays are the key tools for the development of drugs targeting the roots of neurodegenerative problems heavily involving endocytosis.

Researchers must realize that neurons have strictly specified functions and very limited self-surviving potential. Glial cells play important roles in maintaining the scavenger functions for the microenvironment of neurons (149). Targeting glial cell functions can be an affective strategy for preventing the progress of neurodegeneration, especially at the very early stage of neurodegenerative diseases.

Considering the CNS to be the major pathological site for both types of diseases, small molecule compounds that can effectively accumulate in brain tissues are most favorable for drug development. The advantage of small molecule drugs is that they are relatively easily

distributed to the brain across the BBB, compared to larger molecules (150). For LSD, cell therapy, especially hematopoietic stem cell-based therapy, will play a major role for LSD since these cells are able to migrate across the BBB to compensate for the enzymatic defect.

The fact that neurodegenerative disorders are long term diseases strongly suggests that combinatory therapy might be the most desirable treatment against multiple aspects of disease mechanism (151). Single therapies could only be suitable for very few specific situations, such as enzyme-replacement therapy for non-CNS involving Gaucher's disease and Fabry disease, which are rare conditions in both LSD and AD.

7. ACKNOWLEDGMENTS

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