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The Role Of Endolysosomes In Models Of Neurodegenerative Disorders And In Regulating Levels Of Intracellular Calcium

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THE ROLE OF ENDOLYSOSOMES IN MODELS OF NEURODEGENERATIVE DISORDERS AND IN REGULATING LEVELS OF INTRACELLULAR CALCIUM

by

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ABSTRACT

Neurodegenerative disorders are affecting increasingly numbers of humans, especially in our ever increasingly aged population. Here, I describe three projects whereby I attempt to better understand the degree to which endolysosomes contribute to pathological features associated with two neurodegenerative disorders and to the regulation of intracellular calcium levels in cultured neurons.

In previous studies conducted in a rabbit model of sporadic Alzheimer's Disease (AD), we found that elevated circulating ApoB cholesterol *per se* contributed to the pathogenesis of sporadic AD by affecting endolysosomes. To extend further our *in vivo* findings and to determine underlying mechanisms, we tested the hypothesis that; ApoB containing cholesterol directly altered the structure and function of endolysosomes and contributed to the development of AD-like pathology in primary cultured neurons. To test our hypothesis, we used a variety of methods including ratio-metric measurement of endolysosome pH, real-time RT-PCR, immunoblotting, immunostaining, and enzyme activity assays. Treating neurons with ApoB containing LDL cholesterol increased endolysosome accumulation of cholesterol, enlarged endolysosomes and elevated endolysosome pH. In addition, ApoB containing LDL cholesterol increased endolysosome accumulation of beta amyloid converting enzyme 1, BACE-1,

enhanced BACE-1 activity and increased production of Aβ levels. Our findings suggest strongly that the altered structure and function of endolysosomes play a key role in ApoB containing LDL cholesterol induced pathological features of AD.

HIV-1 transactivator protein Tat continues to be regarded as an important pathogenic factor for HIV associated neurocognitive disorders (HAND) because of its ability to directly excite neurons and increase Aβ. Based on the facts that endolysosomes not only play an important role in neuronal cell death and internalize HIV-1 Tat, but also contribute to amyloid genesis, we tested the hypothesis that; HIV-1 Tat directly altered the structure and function of endolysosomes and contributed to neurotoxicity and the development of AD-like pathology in primary cultured neurons. Following treatment of HIV-1 Tat into primary cultured rat hippocampal neurons, neuronal viability was determined using a triple staining method. Prior to noting statistically significant changes in HIV-1 Tat-induced neuronal cell death (48 hours), we observed as early as 24 hours after HIV-1 Tat treatment, HIV-1 Tat internalization in endolysosomes, increased endolysosome sizes, raised endolysosome pH, decreased specific activities of endolysosome enzymes, disrupted endolysosome membrane integrity and inhibited autophagosomes formation. In addition, we found that following endolysosome dysfunction, HIV-1 Tat increased Aβ generation, increased endolysosome accumulation of AβPP, Aβ and BACE-1 and enhanced BACE-1 activity. Our findings suggest that disturbed endolysosome structure and function contributes to HIV-1 Tat-induced neurotoxicity and the development of AD-like pathology.

Endolysosomes are ‘acidic’ calcium stores, however little is known about how calcium is released from these stores and how this release contributes to calcium signals both spatially and temporally. Using multiple assays such as calcium imaging, surface protein labeling, immunoprecipitation and RNA interference, we found in primary cultured hippocampal neurons that calcium released from acidic calcium stores triggered calcium influx. This novel phenomenon is similar to classical store-operated calcium entry (SOCE), which we termed ‘acidic store-operated calcium entry’ (aSOCE). Moreover, we found that the aSOCE was mediated by cell surface redistribution of N-type calcium channels and LAMP1, a regulator of lysosome exocytosis. Our results suggest that lysosome exocytotic insertion of N-type calcium channels might mediate this novel aSOCE in neurons. Such findings could provide a new insight into the spatiotemporal complexity of calcium signals and fundamental calcium-dependent cellular responses.

CHAPTER I

ENDOLYSOSOME STRUCTURE AND FEATURES

1.1 Historical Review

One day in 1882 when a Russian scientist Elie Metchnikoff was looking at cells of a starfish larva under his microscopy at home, he was suddenly struck by an idea that cells might possess defensive mechanisms against foreign intruders. Such a sparkling idea was confirmed in his later studies on interactions between Daphnia (a small planktonic crustacean) and fungus. In those studies, he demonstrated that in some cases phagocytes engulfed and destroyed attacking fungus, whereas in other cases phagocytes failed to handle the fungus, thus resulting in disseminated infection and death of the hosts. Such findings formed the basis of his phagocyte theory and prompted his next 25 years of experimentation on relationships between cells and microbes in the course of infection (James, 1971). His findings in phagocytes actually touched several important characteristics of lysosomes and included findings that phagocytes have a digestive tract (vacuolar system) to deal with extracellular materials (pinocytosis and phagocytosis), phagocytes have stomachs that do not uptake extracellular materials (autophagic vacuoles) and phagocytes maintain a lower pH that provides an environment favorable for lysosome enzymes to clear intruders. Due to Metchnikoff's great contributions to the study of

lysosomes (which he named as cytases), he was considered to be the true discoverer of lysosomes by Belgian cytologist Christian de Duve who expanded greatly our knowledge about lysosomes in the mid-20th century (De Duve et al., 1955).

In 1949, De Duve and his colleagues first successfully isolated lysosomes from rat liver by ultracentrifugation. However, they spent another six years to eventually observe the morphology of lysosomes using newly developed electron microscopes. This new technique resulted in De Duve naming this special group of acid hydrolases-labeled cytoplasmic particles as “lysosomes” (standing for lytic bodies) and in making a variety of seminal findings related to lysosomes (De Duve, 1966; De Duve et al., 1955). Subsequent studies demonstrated the ubiquitous presence of lysosomes in almost all kinds of animal (Weissmann, 1964; Weissmann, 1965) and plant cells (De Duve, 1966; De Duve, 1970) except red blood cells (Allison, 1967). In 1969, lysosomes were discovered in neuronal cells of the central nervous system (Holtzman, 1969). Since then, scientists have continuously uncovered physiological and pathological roles of lysosomes in neurons.

1.2 Lysosome Morphology

Although there has been some modification in the definition of lysosomes over the intervening 50 years, its original description made by De Duve still holds today - lysosomes represent a group of heterogeneous acidic organelles that

contain a variety of hydrolytic enzymes surrounded by a single membrane. Lysosomes can secret their contents after fusion with the plasma membrane in some cell types (Andrews, 2005; Luzio et al., 2000; Saftig and Klumperman, 2009; Stinchcombe and Griffiths, 1999). Beyond a traditional concept as end-points of a garbage-disposal unit, lysosomes are now considered to be a central point in the regulation and quality control of cells.

Morphologically, the sizes and shapes of lysosomes vary in different cells and tissues but they all appear as large vacuoles containing electron dense material (De Duve, 1970; de Duve, 1975). The sizes of lysosomes range between 0.1-1.2 μm in diameter (Elmlinger et al., 2003) and the sizes of neuronal lysosomes are normally less than 1 μm. Like lysosomes in other cells, neuronal lysosomes carry a high content of lysosome membrane proteins and active lysosome hydrolases, but do not contain mannose-6-phosphate (M6P) receptors (Kornfeld and Mellman, 1989). In addition, these acidic organelles in neurons are often located in the perinuclear region (Renate, 2005).

1.3 Lysosome Enzymes

After the initial identification of acid phosphatase as a lysosome enzyme, more than 80 acidic hydrolases have been found in lysosomes (Yamashima and Oikawa, 2009). Lysosome enzymes are synthesized in the rough endoplasmic reticulum, modified and packed in the Golgi apparatus, transported into late endosomes with the help of M6P receptors, and are eventually fused into

lysosomes (Desnick et al., 1976; Mukherjee et al., 1997). These lysosome enzymes are responsible for degradation of almost all major constituents (i.e. carbohydrate, lipid, protein, nucleic acid and mucopolysaccharidoses) (Weissmann, 1964) from the intracellular and extracellular milieu (de Duve, 1975) under a favorable acidic pH (de Duve, 1975; Deduve, 1964). As such, we evaluated lysosome function in our studies by examining the expression levels and activities of three acidic hydrolases; cathepsin D (aspartic protease), cathepsin B (cysteine protease) and acid phosphatase (phosphatase).

1.4 Lysosome pH

All lysosome enzymes are contained in structures surrounded by a single lysosome membrane and this single membrane keeps the enzymes inside to compartmentalize digestive functions of enzymes and to prevent their destructive effects by releasing them into the cytosol. Furthermore, lysosome membranes carry multiple regulatory machineries that maintain an optimum acidic environment (pH ≈ 4.5) inside lysosomes. These regulatory machineries include proton influx mechanisms (vacuole type H^+-ATPase), proton efflux mechanisms (passive diffusion, proton-coupled antiporters/symporters) and ion conductance mechanisms (chloride channels, cation channels, Na^+/K^+ exchange) (Van Dyke, 1996). The principle mechanism is the vacuole H^+-ATPase (V-ATPase), a multimeric complex functioning as a proton pumping rotary nano-motor that regulates lysosome pH. V-ATPase has two components; the cytosol V_1 sector is

a large ball-shaped head subunit that hydrolyzes ATP for energy supply, and the transmembrane V_0 sector is a subunit that contains the proton channel responsible for proton translocation. Thus, ATP hydrolysis and the proton pump are coupled to rotary mechanisms (Marshansky and Futai, 2008). V-ATPase shares similarities with F-ATPase (ATP synthase) in subunit structure and rotational catalysis but they display distinctive features in distribution and mechanisms. F-ATPase is located exclusively on the mitochondrial inner membrane where it functions as an ATP synthase coupled with proton motive force (pmf) produced by the respiratory chain. By contrast, V-ATPase is a proton pump found in diverse endomembrane organelles and plasma membranes where V-ATPase functionally not only acidifies the lysosomes but also provides a potential energy source for driving a variety of organelle-specific coupled transporters. V-ATPase can be inhibited by the specific inhibitor bafilomycin A1 (a fungal antibiotic) at nanomolar concentrations. It is suggested that bafilomycin binds to V_0 complex in or around the proton channel, but the exact binding sites and inhibitory mechanisms of this inhibitor are not clear (Crider et al., 1994; Gluck, 1993; Zhang et al., 1994; Zhang et al., 1992).

1.5 Lysosome Calcium

Endolysosome compartments have been recognized as intracellular calcium stores that contain readily releasable pools of calcium and have distinctive machinery for sequestering and releasing calcium (Christensen et al.,

2002; Churchill et al., 2002; Docampo and Moreno, 1999; Patel and Docampo, 2010). Because of the low pH in their lumen, these endolysosome calcium stores are often referred to as “acidic calcium stores”. Acidic calcium stores have high concentrations of calcium ranging from 400 - 600 μM (Christensen et al., 2002). This high concentration of calcium is maintained by the pH gradient across these acidic organelles, where vacuolar H^+-ATPase pumps H^+ into the lumen and drives Ca^{2+} uptake by Ca^{2+}/H^+ exchanger (Moreno and Docampo, 2009; Patel and Docampo, 2010). Therefore, V-ATPase is responsible for sequestering calcium in acidic stores (Churchill et al., 2002; Haller et al., 1996; Shigaki et al., 2006; Srinivas et al., 2002). On the other hand, there are two types of calcium channels expressed in endolysosomes; two pore channels (TPC) (Galione et al., 2009) and possible TRPML channels (a subfamily member of TRP channel) (Puertollano and Kiselyov, 2009). Besides, endolysosomes have their own calcium buffering systems such as luminal proteins and polyanionic matrixes (Patel and Docampo, 2010), although the current information on endolysosome calcium buffering systems is relatively limited. Endolysosomes function as a group of distinctive calcium stores. For instance, endolysosome calcium release from TPC channels by nicotinic acid adenine dinucleotide phosphate (NAADP) is sensitive to either inhibition of organelle acidification (by bafilomyocin A1) or osmotic bursting of lysosome membrane (by GPN), but insensitive to ER calcium release (by thapsigargin) (Pandey et al., 2009). However, communications between acidic calcium stores and other calcium stores may exist (Calcraft et al., 2009; Ruas et al., 2010).

CHAPTER II

PHYSIOLOGICAL FUNCTIONS

Back to the De Duve's era, lysosomes were considered as catabolic organelles dealing with extracellular and intracellular materials, which correspondingly generated two major featured functions in lysosomes; heterophagy (endosome-lysosome) and autophagy (autophagy-lysosome) (De Duve, 1966). Later, exocytosis was introduced as another major function of lysosomes at least for some cell types (Allison and Davies, 1974). In the CNS, neuronal lysosomes participate in endocytosis and autophagy (Larsen and Sulzer, 2002; Parton and Dotti, 1993) and they conditionally undergo exocytosis as well (Arantes and Andrews, 2006).

2.1. Endocytosis (Heterophagy)

Heterophagy, initially proposed by De Duve, is now known as endocytosis, in which extracellular materials are internalized into membrane-confined vacuoles or microvesicles and destined to lysosomes for degradation. Endocytosis plays important roles in multiple cellular functions such as; internalization of nutrients, regulation of membrane receptor expression, and maintenance of cell polarity, as well as uptake of viruses, toxins and

microorganisms into cells (Mukherjee et al., 1997). Apart from these functions, neuronal endocytosis displays some tissue-specific characteristics such as recycling of plasma membrane after neurotransmitter release and playing an essential role in early development stages (Parton and Dotti, 1993). Based on the modes of internalization, endocytosis can be further divided into several subgroups; phagocytosis (eating particular substances), pinocytosis (drinking soluble substances), clathrin-dependent receptor-mediated endocytosis, and clathrin-independent endocytosis (Mukherjee et al., 1997). Here, we are mainly focusing on clathrin-dependent endocytosis.

The concept of receptor-mediated endocytosis was formulated in 1974 from the biochemical observation of cellular uptake of cholesterol. Included in the uptake process is cholesterol binding to low-density lipoprotein (LDL) receptors, internalization, recycling and/or degradation (Goldstein et al., 1976; Goldstein and Brown, 1974). In LDL receptor-mediated endocytosis, receptors were recycled to plasma membrane while ligands were degraded in lysosomes. Depending on the different fate of the ligands/receptors, three other subtypes of receptor-mediated endocytosis exist; receptors and ligands are both recycled, receptors and ligands are both degraded in lysosomes, and receptors and ligands are both transcytosed (cross the cells) (Brown and Goldstein, 1982; Brown and Greene, 1991; Goldstein et al., 1985; Wileman et al., 1985).

My research is focusing on receptor-mediated endocytosis of ApoB containing LDL-cholesterol and HIV-1 Tat peptide, both of which bind to LDLRs highly expressed on neurons. Once those ligands bind to those receptors, the

complexes undergo invagination from the plasma membrane to clathrin-coated vesicles (Wileman et al., 1985). Shortly after, vesicles lose their coat while complexes are transported to a group of large smooth-faced vesicles and tubular structures referred to as endosomes. Endosomes were first named by Helenius and colleagues (Helenius and Marsh, 1982) to describe an intermediate state of pre-lysosome vacuoles during endocytosis. In addition to internalized components, endosomes receive biosynthetic components. Subsequent studies divided endosomes into two distinct subcompartments; early and late endosomes (Schmid et al., 1988), because of the facts that theses two subpopulations demonstrate a great distinction although they are kinetically related. Early endosomes maintain a mildly acid interior (pH ≈ 6) and distribute throughout nerve terminals of neurons (Parton and Dotti, 1993; Parton et al., 1992). Early endosomes are further divided into two groups; sorting and recycling endosomes (Gruenberg and Maxfield, 1995). Once sorting endosomes terminate ligand-receptor signaling by separating ligands from their receptors (Mellman, 1996; Mukherjee et al., 1997), recycling endosomes return receptors to the plasma membrane and ligands were transported to late endosomes. Late endosomes maintain an acidic environment (pH ≈ 5.5) and locate around the cell body of neurons (Parton et al., 1992). Late endosomes not only receive internalized material from early endosomes in the endocytic pathway, but also receive materials from other pathways e.g. trans-Golgi network (TGN) in the biosynthetic pathway and phagosomes in the phagocytic pathway (Stoorvogel et al., 1991). In turn, late endosomes are responsible for delivering components such as ligands

and newly synthetic lysosome glycoproteins and enzymes to lysosomes. Late endosomes transport lysosome enzymes to lysosomes with the help of Cation-Independent Mannose 6 Phosphate (CI-M6P) receptors, but these receptors don't stay in lysosomes. Those ligands, once separated from receptors in sorting endosomes, are transferred to late endosomes and eventually reach their destination, lysosomes. Lysosomes are the last stop of the endocytic pathway, where internalized materials are degraded into simple compounds and transported out of lysosomes. Lysosomes maintain a more acidic state (pH ≈ 4.5) and predominantly distribute around the neuronal cell body (Parton et al., 1992). Therefore, endocytic events in neurons are confined to nerve terminals and synaptic vesicles in axons whereas they occur along the whole dendrites (Parton et al., 1992).

In the present studies, I will evaluate endocytosis by using two endolysosome protein markers; EEA1 and LAMP1. EEA1 (early endosome antigen 1) is an early endosome marker (Simonsen et al., 1998). EEA1 has a strong binding ability for multiple proteins, which potentiates its role in intracellular membrane trafficking including endocytosis. For example, EEA1 is required for endocytic membrane fusion because it can bind to PI3K (lipid kinase) and Rab5 (GTPase), both of them are involved in regulation of membrane trafficking (Simonsen et al., 1998). In addition, the observation of EEA1 interacting with syntaxin-6 might suggest its participation in the trans-Golgi network to early endosome trafficking (Simonsen et al., 1999). As a well-known Rab5 effector, although EEA1 demonstrates diverse locations in brain (Bartlett et

al., 2001), it has been implicated in neuronal synaptic vesicle function, axon transport & growth and synaptic transmission (Selak et al., 2004; Selak et al., 2006); LAMP1 (lysosome associated membrane protein 1, or CD107a) is a late endosome and lysosome marker. It is expressed differentially in various cells (Huynh et al., 2007; Moreno, 2003; Sarafian et al., 2006) and are required for fusion of lysosomes with phagosomes (Binker et al., 2007; Huynh et al., 2007)

2.2. Autophagy

In addition to heterophagy, De Duve and his colleagues also proposed another essential feature of lysosomes, autophagy (Deter and De Duve, 1967). It is a conservative turnover process of intracellular components including degradation of long-lived stable proteins, and recycling of entire organelles especially mitochondria (Larsen and Sulzer, 2002; Marino and Lopez-Otin, 2004). In contrast to heterophagy that responds to sampling the extracellular environment, autophagy manipulates the intracellular milieu and helps to maintain homeostasis (Marino and Lopez-Otin, 2004).

Autophagy is currently categorized into at least three subgroups according to their distinctive targets: 1) Macroautophagy-bulk degradation of virtually all kinds of cytoplasmic components ranging from macromolecules to large organelles; 2) chaperone-mediated autophagy (CMA)-degradation of cytosolic protein with specific motif (KFERQ); and 3) microautophagy-degradation of small organelles and molecules (Klionsky and Ohsumi, 1999). The first two types of

mammalian autophagy have been implicated in CNS injury and diseases (Yamashima and Oikawa, 2009). Here, I will focus on macroautophagy.

Based on various roles of macroautophagy, it can also be divided into two forms; induced and basal autophagy (Mizushima, 2005). The former displays cells' responses to starvation and other multiple stimuli, thereby maintaining homeostasis and survival (Komatsu et al., 2005; Kuma et al., 2004; Shintani and Klionsky, 2004). The latter is responsible for turnover of cytoplasmic components in cells (Hara et al., 2006; Komatsu et al., 2006; Komatsu et al., 2007; Komatsu et al., 2005; Kuma et al., 2004). Despite both forms of autophagy being present in neurons (Yamashima and Oikawa, 2009), induced autophagy is reportedly less important in neurons as compared to other tissues (Zhao et al., 2008) while basal autophagy is more preferable in neurons because neurons need more economic mechanisms to maintain intracellular quality control as post-mitotic cells (Marino et al., 2011).

Macroautophagy consists of several sequential steps including sequestration (induction of autophagy and autophagosome formation), transportation to lysosomes (autolysosome formation), and degradation and utilization of products (Mizushima, 2007). In the process of sequestration, the exact mechanisms of induction of autophagy (autophagosomal membrane originates) remain unknown. In contrast, the mechanisms of autophagosome formation have been described (Marino and Lopez-Otin, 2004). During autophagosome formation, cytoplasmic constituents or organelles are wrapped with a double membrane to form autophagosomes. This process can be further

divided into three routes based on their relevant autophagy-related gene encoded proteins (ATG); two ubiquitin-like (UBL) conjugation systems (ATG12-ATG5 and ATG8), and one ATG9 related membrane complex. In the first UBL system, ATG12 forms conjugates with ATG5 with the assistance of ATG7 and ATG10. Then the ATG12-ATG5 conjugate interacts further with ATG16. The ATG12-ATG5-ATG16 conjugates are required for the elongation of the isolation membrane. However, they are not responsible for generation of the precursor structure, because they eventually separate from autophagosomal membranes before autophagosome completion, and they are absent in mature autophagosomes. In the second UBL system, ATG8 is transferred to ATG3 after being processed by ATG4 and ATG7. Then it completes its conjugation with PE (phosphatidylethanolamine) The process of ATG8-PE conjugation may also depend on the ATG12-ATG5 complex from the first UBL pathway. ATG8 could be recycled with the help of ATG4 once autophagosomes completion. Among those three mammalian orthologues of yeast ATG8, only MAP-LC3 (microtubule-associated protein light chain 3) is found in autophagosomes and its precursor (small membrane structure). The evidence that LC3 colocalizes with ATG12-ATG5 may suggest a possible connection between two UBL systems. LC3, a marker of autophagosomes, has three forms: (1) ProLC3 represents the full-length molecule; (2) LC3-I is the proteolytic form; and (3) LC3II is the membrane bound form (Marino and Lopez-Otin, 2004). In addition, LC3 also serves as a receptor of multifunctional protein, P62 (Bjorkoy et al., 2005). P62's ability to bind LC3 and ubiquitin might provide an opportunity to couple autophagy with

ubiquitin proteasome systems (UPS) while regulating protein aggregation and degradation (Komatsu et al., 2007). P62 is metabolized in lysosomes through autophagy and accumulated in autophagy-null cells (Komatsu et al., 2007; Nakai et al., 2007; Wang et al., 2006). In the present study, we mainly focused on three protein markers of autophagy formation: LC3, Atg5 and P62.

2.3. Exocytosis

Normally, exocytosis is a durable process, during which a cell releases its vesicular contents (signaling molecules, toxin, waste product) into the extracellular milieu and inserts vesicle membrane proteins/lipids into the cell plasma membrane (Morgan, 1995). There exist two types of exocytosis: 1) Constitutive exocytosis that is present in all cells and functions to incorporate membrane proteins as well as regulate cell sizes; and 2) regulated exocytosis that is restricted in certain cells (classic examples includes exocrine cells, endocrine cells and neurons) and requires an appropriate signal (e.g. a specific sorting signal on the vesicles, a clathrin coat, as well as an increase in intracellular calcium). In neurons, regulated exocytosis mainly deals with neurotransmission (Morgan, 1995) which is performed by secretory granules (small synaptic vesicles and large dense-core vesicles) (De Camilli and Jahn, 1990; Partoens, 2002). The process of exocytosis can be divided into five steps including trafficking, tethering, docking, priming and fusion. Exocytosis occurs by

consented mechanisms and most likely uses the same basic protein components (Burgoyne and Morgan, 2003).

Obviously, secretory granules have a reputation for exocytosis, but they are not the only players in the whole exocytosis game. Actually, there exist other forms of exocytosis in cells, for example, lysosome exocytosis. Even though De Duve had already described the ability of lysosomes to discharge their contents into the extracellular space from intact cells under pathological stimuli (De Duve, 1970; de Duve, 1975), he failed to claim exocytosis as a feature of lysosomes. It was Allison and his coworkers who proposed this secretion behavior in lysosomes as exocytosis (Allison and Davies, 1974). Subsequent studies confined the role of lysosomes as a type of secretory organelle in limited types of cells including neurons. These lysosomes were referred to as secretory lysosomes (Blott and Griffiths, 2002), as opposed to conventional lysosomes. The discovery of secretory lysosomes greatly modified the original concept of lysosomes. Instead of serving as digestive end points of endocytosis and autophagy, at least in certain cell types, lysosomes are now appreciated to be central quality control points integrating endocytosis, autophagy and exocytosis.

Secretory lysosomes probably evolved from conventional lysosomes because of a enhanced secretion need (Blott and Griffiths, 2002), but secretory lysosomes are more complex. Secretory lysosomes demonstrate diversity in their structure, contents and organization; for instance, dense cores structures are present in platelet, multilaminar structures are present in MHC class II compartments, and unique structures are present in melanosomes. Secretory lysosomes contain and

release a cell-specific set of secreted components resisting degradation by lysosome enzymes (e.g. apoptotic granzymes in CTLs, melanin in melanocytes). Secretory lysosomes have unique sorting mechanisms for some proteins (e.g. Fas ligand and gp75) (Blott and Griffiths, 2002). Secretory lysosomes have higher levels of secretion than conventional lysosomes, because of less strictness in organized cytoskeleton, vesicular transport, and docking (Andrews, 2000).

The study of secretory lysosomes reveals an additional and largely overlooked property of lysosomes (regulated exocytosis and secretion). Secretory lysosomes are only observed in limited cell types including hematopoietic cells, melanocytes, renal tubular cells, acrosomes and neurons (Andrews, 2000; Blott and Griffiths, 2002). Recent studies have shown that conventional lysosomes can fuse with the plasma membrane for membrane repair in response to increased intracellular levels of calcium (Andrews, 2000; Reddy et al., 2001). One possible explanation about secretory behavior of conventional lysosomes is that conventional lysosomes can transform into regulated secretory organelles. Several published reports seem to support this concept (Arantes and Andrews, 2006; Martinez et al., 2000). Conventional lysosomes share common regulatory machinery with secretory lysosomes, e.g. the exocytotic process of these two lysosomes could be regulated by intracellular calcium level and SNARE complexes. Collectively, these observations suggest that the secretory behavior of lysosomes occurs beyond the previous limitation in cell types and deserves further discussion in a broad dimension.

LAMP1, a lysosome marker mentioned above, has been used as an indicator of lysosome exocytosis for years. Using this indicator, lysosome exocytosis was first found in human epithelia; calcium influx triggered lysosome exocytosis by increasing the surface expression of LAMP1 (Ayala et al., 2001). Then, LAMP1 was detected at the cell surface following elevated secretion of cytotoxic granules in natural killer (NK) cells (Penack et al., 2005). Later, redistribution of LAMP1 was found in sympathetic neurons, indicating lysosome exocytosis in neurons (Arantes and Andrews, 2006). Although the exact underlying mechanisms of lysosome exocytosis remain unclear, some mechanisms have been described including calcium elicited lysosome exocytosis in sympathetic neurons that may use a calcium sensor synaptotagmin VII through interactions with a t-SNARE molecule, VAMP7 (Arantes and Andrews, 2006). Because it is known that exocytosis of neuronal secretory granules is mediated by calcium and SNARE complex (Galli et al., 1995; Morgan, 1995), the above observations on lysosome exocytosis might suggest a correlation in underlying mechanisms between lysosome exocytosis and secretory granules exocytosis and that they may maintain widespread expression and conservation of a core exocytotic machinery (t-SNARE and v-SNARE). Nevertheless, they may display variations in their regulatory mechanisms related to the specialized functions of particular cell types.

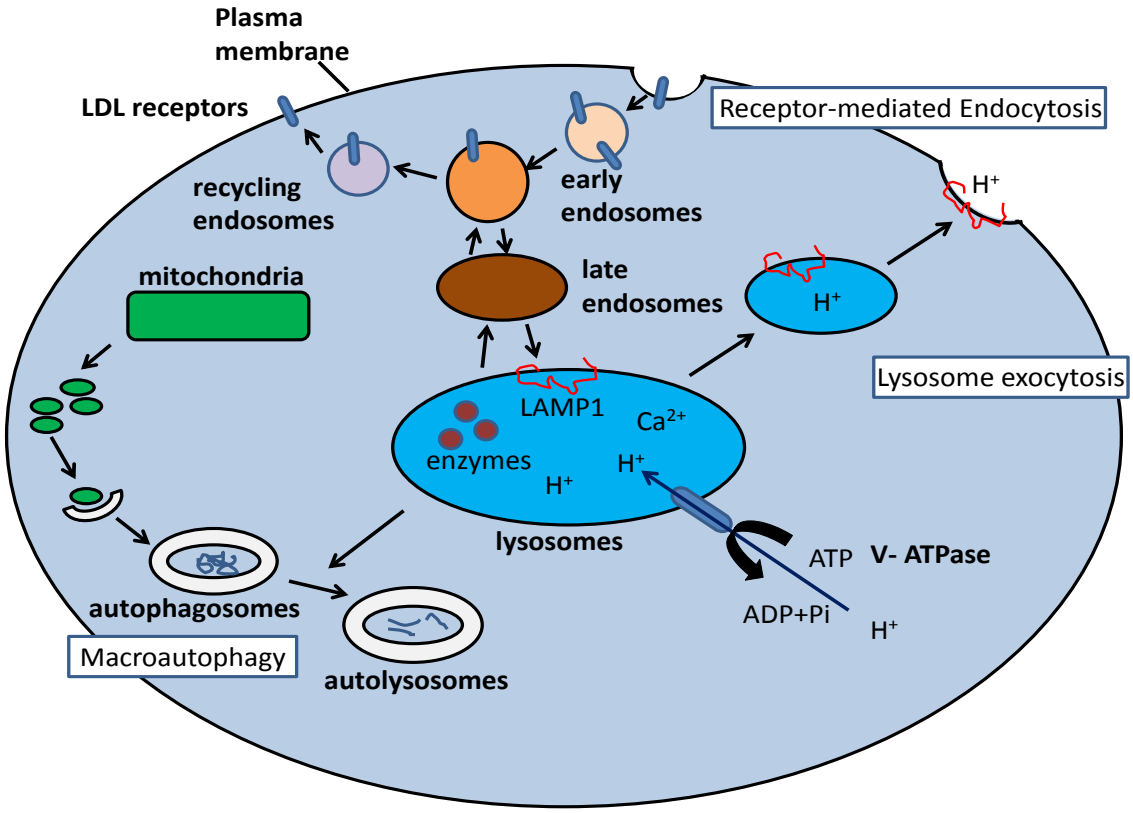

Figure 1. Three Functions of Lysosomes. Lysosomes are a group of central, acidic organelles responsible for degradation through lysosome hydrolases. The basic functions of lysosomes include receptor-mediated endocytosis, macroautophagy and lysosome exocytosis.

CHAPTER III

PATHOLOGICAL FUNCTIONS: LYSOSOMES AND NEURODEGENERATIVE DISORDERS

Lysosomes play an important role in maintaining homeostasis such as cellular degradation. On the contrary, lysosome dysfunction leads to multiple disorders. The association of neurodegeneration with lysosome storage diseases provides the first clue that lysosome dysfunction contributes to neurodegenerative diseases (De Vries et al., 1958). Now, lysosome dysfunction appears to be responsible for a number of neurodegenerative disorders such as sheep scrapie, Lewy body disease (LBD) and Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, Niemann-Pick Type C (NPC) disease, HIV-1 associated neurocognitive disorders and Alzheimer's disease (Bahr and Bendiske, 2002; Gelman et al., 2005; Mayer et al., 1996; Nixon, 2004). In our current dissertation studies, one major aim was to define the role of lysosomes in neurodegeneration, aging and neuronal death. In this Chapter, we want to introduce the development of lysosome study in neurodegeneration, aging and neuronal death.

3.1. Lysosomes, Neurodegeneration and Aging

The presence of acid phosphatase in plaques and dystrophic neurites of AD brains provide the first clue that lysosomes might be involved in the pathogenesis of AD (Suzuki and Terry, 1967; Terry et al., 1964). However, the importance of lysosomes in AD pathogenesis was overlooked until the mid-1980s when it was discovered that the plaques consist of amyloid-β peptide (Aβ), a set of peptides generated from proteolytic cleavage of amyloid-β protein precursor (AβPP). Observations of increased intraneuronal accumulation of lysosome enzymes and increased immunoreactivity and activities of endolysosome enzymes in amyloid plaques in AD brain formed the basis of the notion that lysosome dysfunction promotes amyloidogenic processing of AβPP and contributes to the pathogenesis of AD (Bernstein et al., 1996; Cataldo et al., 1995; Cataldo et al., 1991; Cataldo et al., 1990; Li, 1997; Nakamura et al., 1991). Over the next decade, extensive studies further demonstrated an important role of lysosome dysfunction in the pathogenesis of AD (Nixon and Cataldo, 2006).

AβPP belongs to a family of conserved type I membrane proteins, containing APP-like proteins (APLP1 and APLP2). The exact functions of AβPP are still not clear, but it has been implicated in cell signaling, cell adhesion and trafficking (Hoareau et al., 2008; Reinhard et al., 2005; Zheng and Koo, 2006). AβPP is synthesized in rough ER, modified in the trans-Golgi network (TGN), and then inserted in the plasma membrane. AβPP is processed by cells in two ways; one is the non-amyloidogenic pathway that does not result in amyloid beta

production and is thought not to be related to Alzheimer's disease. In this pathway, AβPP is first cleaved on the plasma membrane by an aspartyl protease, α-secretase (Buxbaum et al., 1998; Lopez-Perez et al., 2001) and thus results in the production of a soluble N-terminal fragment (sAPPα) that is released from cells and a C-terminal fragment (CTF) that is associated with the plasma membrane. The alternative pathway is the amyloidogenic pathway that results in amyloid beta production and this is thought to be related to Alzheimer's disease. In this pathway, AβPP is endocytosed into endosomes and lysosomes, where a β-site-APP cleaving enzyme (BACE, β-secretase), favored by endosome low pH environment (Capell et al., 2000; Vassar et al., 1999; Walter et al., 2001) mediates the cleavage of AβPP at a more distal site along luminal/extracellular domain resulting in the release of a soluble fragment (sAPPβ) and a membrane-associated 99-residue CTF (βCTF) containing Aβ peptide. Further intra-membrane cleavage of βCTF with a γ-secretase enzyme complex yields amyloid beta, the majority of which are a 40-mer peptide ($A\beta_{40}$) and to a lesser extent a 42-mer peptide ($A\beta_{42}$). It is known that γ-secretase consists of presenilin (PS), nicastrin, APH1 and PEN2 (Edbauer et al., 2003; Go et al., 2004; St George-Hyslop, 2000), and is located in plasma membrane and early endosomes (Runz et al., 2002), late endosomes and autophagic vesicles (Yu et al., 2005; Yu et al., 2004) and lysosomes (Cupers et al., 2001; Pasternak et al., 2003; Pasternak et al., 2004).

Indeed, extensive studies have indicated that the endolysosome pathway is tightly involved in the process of the amyloidogenesis. First, AβPP is internalized by receptor-mediated endocytosis or pinocytosis, and is sorted into endosomes for several destinations; recycling back to the plasma membrane, delivering into TGN for packing, trafficking, and targeting late endosomes for lysosome degradation (van der Goot and Gruenberg, 2006). Second, βCTF is produced in early endosomes (Grbovic et al., 2003; Mathews et al., 2002), where BACE (Huse et al., 2000; Vassar et al., 1999) and PS-1 (Lah and Levey, 2000) are located. Third, intracellular levels of soluble Aβ are increased in endolysosome compartments, which precedes extracellularly deposited Aβ (Cataldo et al., 2004; Takahashi et al., 2004) and this correlated with cognitive deficits in AD models, even in the absence of amyloid plaques (Koistinaho et al., 2001; LaFerla et al., 2007). Fourth, some lysosome enzymes such as cathepsin B and cathepsin D may contain β- and γ-secretase activity that results in either Aβ generation (Chevallier et al., 1997; Dreyer et al., 1994; Hook et al., 2007; Ladror et al., 1994; Mackay et al., 1997) or conversely, Aβ degradation (Mueller-Steiner et al., 2006).

In Chapter IV, we will introduce endolysosome involvement in LDL cholesterol-induced Alzheimer's disease-like pathology in primary cultured neurons.

3.2. Lysosomes and Neuronal Cell Death

Understanding the role of lysosomes in neuronal cell death has progressed relatively slowly compared to the rapidly emerging and developing area of apoptosis and involvement of caspases in cell live and death in the last century. It did not receive enough attention until the early 1990s when Clarke described his findings that a form of cell death (autophagic cell death), distinct from apoptosis, was associated with endocytosis/autophagy during brain development, which promoted the study of lysosomes in neuronal cell death (Clarke, 1990). Several reasons delayed the progress of studying involvement of lysosomes in neuronal cell death. First of all, because the metabolism and structure of neurons varies among different species and different developmental stages of the same species, some criteria used to evaluate the contribution of lysosomes to neuronal cell death could not be applied into different neuronal systems. Second, technical limitations also played a role. Because light microscopy failed to discriminate individual components of lysosomes and electron microscopy failed to record dynamics of lysosome biogenesis and trafficking in the pathological process, some lysosome-disrupted events relevant to cell death (lysosomal membrane stability or permeability) were difficult to monitor (Nixon and Cataldo, 1993).

Despite these limitations, researchers began to realize the importance of lysosomes in neuronal cell death because of the multiple observations of abnormalities of autophagy in many human CNS-related disorders (Kegel et al.,

2000; Rubinsztein, 2006; Winslow and Rubinsztein, 2008). In addition, successfully building up CNS-specific autophagy-deficient animal models allow scientists to better understand the protective role of autophagy in neuronal tissues (Marino et al., 2011). Therefore, over the last 15 years, people's understanding to the implication of lysosomes in neuronal cell death has reached an unprecedented level.

As pre-stops of lysosomes, endosomes are responsible for integrating growth factor signaling molecules (Bronfman et al., 2007). Altered endosome function appears to result in neuronal cell death in some inherited neurodegenerative diseases (Gervais et al., 2002; Kholodenko, 2002; Peters et al., 2003; Trushina et al., 2004). Furthermore, some proteins that regulate endocytosis as well as cell survival can couple endosome dysfunction to cell death cascades. For instance, ALIX/AIP (ALG-2 interacting protein X) that controls endocytosis through its binding partners can regulate apoptosis through the calcium binding protein ALG2 (Missotten et al., 1999; Vito et al., 1996; Vito et al., 1999) or type 1A PI3K pathway (Chen et al., 2000; Gout et al., 2000).

Autophagy plays the neuronal cell death game. Based on distinctive morphological characteristics, three types of cell death have been recognized since 1973 (Schweichel and Merker, 1973); Type 1 cell death has features of apoptosis (Kerr et al., 1972), type 3 cell death has features of necrosis (Syntichaki and Tavernarakis, 2002), and type 2 cell death is termed autophagic cell death (Marino and Lopez-Otin, 2004) which is characterized by accumulation of cytoplasmic autophagic vacuoles accompanied by dilation of mitochondria and

enlargement of ER and Golgi apparatus in dying cells. Autophagic cell death found in CNS and other tissues/cultured cells (Uchiyama et al., 2008) could occur in the absence or the presence of necrosis or apoptosis (Borsello et al., 2003; Broker et al., 2005; Edinger and Thompson, 2004; Marino and Lopez-Otin, 2004; Stefanis, 2005).

Although autophagic cell death represents a type of cell death, it is still too simple to say that autophagy mediates cell survival or death. Actually, autophagy can regulate both cell survival and death under different settings (Baehrecke, 2005; Cuervo, 2004; Shintani and Klionsky, 2004). Thus, mild stressors stimulate autophagy to protect against demise while severe stressors induce cell death. In the former situations, autophagy either accelerates breakdown of intracellular substrates to provide energy supply when those essential growth factors and nutrients are scarce, or removes dysfunctional mitochondria and other organelles to prevent apoptosis (Brunk and Terman, 2002; Larsen and Sulzer, 2002; Trushina et al., 2004). In the latter events, either impaired autophagy (failure to clear accumulation of damaged organelles/aberrant proteins) or autophagic cell death (dramatic breakdown of essential components) may interfere with pro-survival mechanisms (Kourtis and Tavernarakis, 2009).

Lysosomes are required for the turnover of mitochondria (mitophagy) (Brunk and Terman, 2002). Mitophagy obviously shares many common steps with macroautophagy (Batlevi and La Spada, 2011). Previous reports suggested that damaged mitochondria can not undergo turnover in lysosome system (Terman et al., 2006). It could be postulated that both dysfunctional organelles

may limit the effectiveness of mitochondrial turnover by lysosomes (those accumulated in autophagy-lysosome system or those maintained in cytosol), which may exaggerate mitochondria and autophagy damage. Eventually damaged mitochondrial would replace normal ones to occupy limited cell volume and lysosome overload would further lose their digestive ability to mitochondria and other substrates, resulting in neuron damage and/or death.

Lysosomes are directly involved in neuronal cell death. Although lysosome responses are absent in some neuronal cell death cases, lysosome rupture and hydrolase leakage are inevitable consequences at the end-stage of cell death (Nixon and Cataldo, 1993). Lysosome compensatory mechanisms attempt to mitigate against the breakdown of non-important components to utilize them for repair instead of causing cell death. Recent studies indicate that Ca^{2+}-induced calpain activation and ROS production concomitantly lead to lysosome membrane rupture (permeabilizaiton) and neuronal cell death (Yamashima and Oikawa, 2009). Indeed, even partial rupture of lysosomes contributes to apoptosis by triggering mitochondrial membrane potential loss or caspase activation (Guicciardi et al., 2004), whereas intense lysosome rupture contributes to necrosis by inducing leakage of cathepsins (Tardy et al., 2006).

To define further the possible roles of endosomes and lysosomes (endolysosomes) in neuronal cell death and neuronal damage, in Chapter V, we will introduce the involvement of endolysosomes in HIV-1 Tat induced neuronal damage.

CHAPTER IV

INVOLVEMENT OF ENDOLYSOSOMES IN LDL CHOLESTEROL-INDUCED ALZHEIMER'S DISEASE-LIKE PATHOLOGY IN PRIMAEY CULTURED NEURONS

4.1. Introduction

Alzheimer's disease (AD) is the most persistent and devastating dementing disorder of old age and has associated with it massive health care costs. AD is characterized clinically by progressive disturbances in memory, judgment, reasoning and olfaction, and pathologically by loss of synaptic integrity, amyloid plaques composed of amyloid beta protein (Aβ), and neuronal tangles composed of hyperphosphorylated tau protein (Blennow et al., 2006; Hardy, 2009; Seabrook et al., 2007). Although a small percentage of AD cases are familial and genetically-based, the vast majority (>90%) of AD cases are sporadic with an unknown etiology. So far, no effective treatments are available for both types of AD. Despite the strong linkage of ApoE4 gene and altered cholesterol homeostasis to the pathogenesis of sporadic AD, the underlying mechanisms are not fully understood.

It is known that people bearing the ApoE4 allele, the strongest genetic risk factor for sporadic AD (Corder et al., 1993; Wisdom et al., 2011), have increased levels of plasma cholesterol (Corder et al., 1993; Marzolo and Bu, 2009), whereas people bearing the ApoE2 allele, a protective factor against sporadic AD

(Corder et al., 1996; Corder et al., 1994; Schachter et al., 1994), have lower levels of plasma cholesterol (Scuteri et al., 2001; Wisdom et al., 2011). Moreover, elevated levels of plasma cholesterol, as an independent factor, increases the risk of developing sporadic AD (Solomon et al., 2009). Therefore, it is very likely that peripherally-derived ApoB-cholesterol, rather than brain *in situ* synthesized ApoE-cholesterol, contributes to the pathogenesis of sporadic AD. Consistent with this notion are findings that ApoB, the essential apolipoprotein transporting circulating cholesterol in peripheral tissue, is not present in normal brain (Pitas et al., 1987) but present in AD brain and co-distributed with amyloid plaques and neuronal tangles (Namba et al., 1992; Takechi et al., 2009). However, little is known about how circulating cholesterol contributes to the pathogenesis of sporadic AD.

The first obstacle that restricts the entrance of circulating cholesterol is the blood-brain barrier (BBB). As a consequence, brain cholesterol is almost completely dependent on *in situ* synthesis by glial cells, predominately astrocytes (Nieweg et al., 2009). Astrocyte-derived ApoE-cholesterol is internalized by neurons through receptor-mediated endocytosis with the assistance of low-density lipoprotein (LDL) receptor, LDL receptor-related protein-1 (LRP1), and apoER2. Once inside the neuron, ApoE-cholesterol is transported to endolysosomes where cholesterol esters are hydrolyzed to free cholesterol, which is then transported out of the endolysosome system via a mechanism involving Niemann-Pick type C proteins and delivered to the plasma membrane and other organelles (Vance et al., 2006). However, when the BBB is leaky, as

occurs in sporadic AD (Kalaria, 1999; Ujiie et al., 2003; Zipser et al., 2007), circulating ApoB-cholesterol could enter brain parenchyma, where ApoB-cholesterol could be internalized and enter neuronal endolysosomes via the same receptor-mediated mechanisms for endocytosis as that of ApoE-cholesterol. Thus, increased brain levels of ApoB-cholesterol may result in enhanced cholesterol endocytosis and increased accumulation of cholesterol in endolysosomes of neurons thereby affecting neuronal endolysosome structure and function, one of the earliest pathological features of AD (Nixon, 2005; Yuyama and Yanagisawa, 2009), and contributing to the pathogenesis of sporadic AD.

Consistent with this notion are our recent findings in a well-developed cholesterol fed rabbit model of sporadic AD that exhibits pathological hallmarks of AD including disrupted synaptic integrity, elevated levels of Aβ, and tau-pathology. First, we demonstrated that the BBB was leaky in the rabbit model of sporadic AD, as evidenced by leakage of Evan's blue dye, extravasation of IgG and fibrinogen, and decreased expression of tight junction proteins (Chen et al., 2008a). Second, we observed increased brain levels of ApoB-cholesterol, which were abnormally accumulated in neuronal endolysosomes (Chen et al., 2010), suggesting that circulating ApoB-cholesterol entered brain parenchyma via the leaky BBB and was internalized by neurons. Third, we demonstrated that the structure and function of neuronal endolysosomes was disturbed in the same rabbit model of sporadic AD (Chen et al., 2010). Fourth, the observed disturbed structure and function of endolysosomes was linked directly to pathological

features of AD, including disrupted synaptic integrity, amyloidosis, and tau-pathology (Chen et al., 2010). Thus, our findings in a sporadic AD animal model suggest strongly that cholesterol coming from the systemic circulation could alter neuronal endolysosome function and contribute to the pathogenesis of sporadic AD.

To further determine the underlying mechanisms whereby elevated levels of circulating cholesterol contributes to the pathogenesis of sporadic AD, the present studies tested the hypothesis that; ApoB-containing LDL cholesterol disturbed neuronal endolysosome structure and function and contributed to the development of AD-like pathology in primary cultured neurons.

The essential findings of the present studies include observations that treatment of neurons with ApoB-LDL cholesterol increased cholesterol accumulation in neurons, enlarged endolysosomes, and elevated endolysosome pH. More importantly, we found that the altered structure and function of endolysosome was directly involved in elevated Aβ production, increased phosphorylation of tau, and disrupted synaptic integrity as evidenced by decreasing levels of the presynaptic protein synaptophysin. Such findings support our hypothesis and suggest strongly that elevated circulating cholesterol could contribute directly to the pathogenesis of sporadic AD by disturbing the structure and function of endolysosomes.

4.2. Methods

4.2.1. Cultured of primary cortical neurons: Primary cortical neurons were cultured from embryonic day 18 rats. Briefly, pregnant Sprague Dawley rats were killed by asphyxiation with CO_2 and the fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto poly-D-lysine-coated glass-bottom 35-mm tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, penicillin/streptomycin/neomycin and B_{27} supplement, and were maintained at 37°C and 5% CO_2 for 7-10 days, at which time they were taken for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by morphology and immunostaining for neurons with NeuN or MAP-2 antibodies and for astrocytes with GFAP antibodies. Neurons treated with ApoB containing LDL cholesterol (Kalein Biomedical) at the concentration of 50 µg/ml for 3 days were subjected to experimental assays with untreated neurons serving as controls. The final concentration of ApoB containing LDL cholesterol used here was derived from a series of concentration-dependent and time-dependent studies (data not shown).

4.2.2. Cholesterol staining: Free cholesterol was stained with filipin (Sigma). Briefly, neurons were fixed with 10% formalin and incubated with PBS containing 1.5 mg/ml of glycine to quench the formalin. Fixed neurons were incubated with filipin working solution for 2 hours at 4°C in dark. The filipin stock solution was prepared by dissolving 5 mg filipin in 1 ml DMSO, and a 100 µg/ml working solution prepared by dissolving the stock solution 1:50 in PBS (pH =

7.2). Neurons were then examined by Zeiss fluorescence microscopy. To further determine the specificity of LDL cholesterol and its intracellular localization, we co-stained for LysoTracker dye (DND-99 from Invitrogen) and Dil-labeled LDL (Kalein Biomedical) in living neurons. Briefly, neurons were loaded with Dil-labeled LDL (10 μg/ml) for 3 days, washed with PBS, and incubated with LysoTracker (50 nM, Invitrogen) for 30 min at 37°C. Neurons were then examined under an Olympus Fluoview 300 Confocal Laser Scanning Microscope.

4.2.3. Measurement of endolysosome pH: according to the previous study (Liu et al., 2008), Endolysosome pH measured using a ratio-metric lysosome pH indicator dye (LysoSensor Yellow/Blue DND-160 from Invitrogen); a dual excitation dye that permits pH measurements in acidic organelles independently of dye concentration. Briefly, neurons were loaded with 2 μM LysoSensor for 5 minutes at 37°C. Light emitted at 520 nm in response to excitation at 340 nm and 380 nm was measured for 20 msec every 30 seconds using a filter-based imaging system (Zeiss). The ratios of light excited at 340/380 nm and emitted at 520 nm were converted to pH using a calibration curve established using 10 μM of the H^+/Na^+ ionophore monensin, and 20 μM of the H^+/K^+ ionophore nigericin dissolved in 20 mM 2-(N-morpholino) ethane sulfonic acid (MES), 110 mM KCl, and 20 mM NaCl adjusted to pH 3.0 to 7.0 with HCl/NaOH.

4.2.4. Immunostaining: Neurons were fixed with cold methanol (-20°C) for 10 min, washed with PBS, blocked with 5% goat serum, and incubated overnight at 4^oC with primary antibodies targeting early endosome antigen-1 (EEA1, 1:500,

rabbit polyclonal, Santa Cruz), lysosome associated membrane protein-1 (LAMP1, 1:500, rabbit polyclonal, Sigma), Aβ (4G8, 1:500, mouse monocolonal, Signet), BACE-1 (1: 500, mouse monoclonal, Milipore), phosphorylated tau (AT8, 1:500, mouse monoclonal, Pierce) or synaptophysin (1:1000, mouse monoclonal, Sigma). After washing with PBS, neurons were incubated with corresponding fluorescence-conjugated secondary antibodies including Alexa 488-conjugated goat anti-mouse antibodies (Invitrogen), and Alexa 546-conjugated goat anti-rabbit antibodies (Invitrogen). Neurons were examined using an Olympus confocal microscope. Controls for specificity included staining neurons with primary antibodies without fluorescence-conjugated secondary antibodies (background controls), and staining neurons with only secondary antibodies – these controls eliminated auto-fluorescence in each channel and bleed-through (crossover) between channels.

4.2.5. Immunoblotting: Neurons were lysed with RIPA buffer (Pierce) plus 10 mM NaF, 1 mM Na_3VO_4 and Protease Inhibitor Cocktail (Sigma). After centrifugation (14,000 X g for 10 min at 4°C), supernatants were collected, and protein concentrations were determined with a DC protein assay (Bio-Rad). Proteins (10 µg) were separated by SDS-PAGE (12% gel), and following transfer to polyvinylidene difluoride membranes (Millipore) were incubated overnight at 4°C with antibodies against early endosome antigen-1 (EEA1, 1:1000, rabbit polyclonal, Santa Cruz), lysosome associated membrane protein-1 (LAMP1, 1:1000, rabbit polyclonal, Sigma), acid phosphatase (1:1000,mouse monoclonal, Abcam), cathepsin B (1:500, mouse monoclonal, Sigma), cathepsin D (1:1000,

mouse monocolonal, Sigma), BACE-1 (1:1000, mouse monoclonal, Milipore), phosphorylated tau (AT8, 1:1000, mouse monoclonal, Peirece) or synaptophysin (1:1000, mouse monoclonal, Sigma). GAPDH (1: 10000, mouse monoclonal, Abcam) was used as a gel loading control. The blots were developed with enhanced chemiluminescence, and bands were visualized and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification of results was performed by densitometry and the results were analyzed as total integrated densitometric volume values (arbitrary units).

4.2.6. Quantification of Aβ levels by ELISA: Aβ levels were quantified using human/rat $A\beta_{1-40}$ and $A\beta_{1-42}$ ELISA kits as per the manufacturer's protocol (Wako). Briefly, media from cultured neurons was collected, diluted 1:4 with standard diluent buffer, and quantified by a calorimetric sandwich ELISA method. Each sample was measured in duplicate. Protein concentrations from neurons in each dish were determined by a DC protein assay (Bio-Rad). Aβ levels were normalized to total protein content in each sample.

4.2.7. Measurement of endolysosome enzyme activity: Enzyme activities of acid phosphatase were determined using an Acid Phosphatase Assay kit (Sigma), a luminescence-based assay that uses 4-nitrophenyl phosphate as substrate (Chen et al., 2010). Enzyme activities of cathepsin D and cathepsin B were determined using a cathepsin D activity assay kit and a cathepsin B activity assay kit, respectively (BioVision), fluorescence-based assays that used the preferred cathepsin D and cathepsin B substrate sequence labeled with MCA (Chen et al., 2010). Activities of each endolysosome enzyme were expressed as

optical density per 10 µg of protein. Specific activities of each enzyme were expressed as a ratio of enzyme activity to protein levels as determined by immunoblotting.

4.2.8. Measurement of BACE-1 enzyme activity: Activity assays of BACE-1 were determined with a BACE-1 activity kit (Calbiochem) according to the provided protocol. BACE-1 activity was measured using synthetic peptide substrates containing the BACE-1 cleavage site (MCA-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-(Lys-DNP)-OH) at a 50 mM concentration in reaction buffer (50 mM acetic acid pH 4.1, 100 mM NaCl). Briefly, equal amount of proteins (10 µg) were used from each sample lysate. The fluorescence was measured using a fluorescence microplate reader with an excitation wavelength set at 320 nm and an emission wavelength set at 383 nm. As a control for specificity, BACE-1 activity was tested in the absence and the presence of the BACE-1 inhibitor, H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH, (Calbiochem).

4.2.9. Quantitative RT-PCR measurement of BACE-1 mRNA: Total RNA from treated primary cultured neurons was extracted with TRIzol-Reagent (Invitrogen) and levels were determined spectrophotometrically. Reverse transcription reactions were carried out using a SuperScript® III First-Strand Synthesis supermix (Invitrogen). The primers for BACE-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: f: 5′-TACACCCAGGGCAAGTGG-3′ and r: 5′-GCCTGTGGATGACTGTGA-3′ for BACE-1; and f: 5′-TGCACCACCAACTGCTTAG-3′ and r: 5′-GGATGCAGGGATGATGTTC-3′ for GAPDH. Samples were run with our iCycler

IQ™ Multicolor Real-Time PCR Detection System (Bio-Rad) that monitors fluorescence as a direct indication of PCR product (Chen et al., 2008b). All samples were run in triplicate and the averaged values were used for the relative quantification of gene expression. BACE-1 mRNA expression levels were calculated as the ratio of their expression compared with that of GAPDH.

4.2.10. Statistical analysis: All data were expressed as means ± SEM. Statistical significance was determined by two-tailed Student t-test. $p < 0.05$ was considered to be statistically significant.

4.3. Results

Neuronal cholesterol is dependent mainly on uptake of brain *in situ* synthesized ApoE cholesterol via receptor-mediated endocytosis with the assistance of LDLR, LRP and apoER2, all of which are also receptors for ApoB. Here, we examined the extent to which neurons accumulated ApoB-containing LDL cholesterol. By staining free cholesterol with filipin, we found that LDL cholesterol (50 μg/ml) treatment for 3 days markedly altered the distribution of free cholesterol in primary cultured neurons; cholesterol was distributed along the cell membrane in control neurons, whereas cholesterol was accumulated inside the neurons that were treated with LDL cholesterol (Figure 2A). To further determine the intracellular distribution of LDL cholesterol, we treated neurons with fluorescence-labeled LDL cholesterol (Dil-LDL). We found that Dil-LDL was co-distributed with endolysosomes as identified with LysoTracker (Figure 2B).

Our findings suggest that elevated levels of ApoB containing LDL cholesterol enhance neuronal cholesterol endocytosis and increase accumulation of cholesterol in endolysosomes.

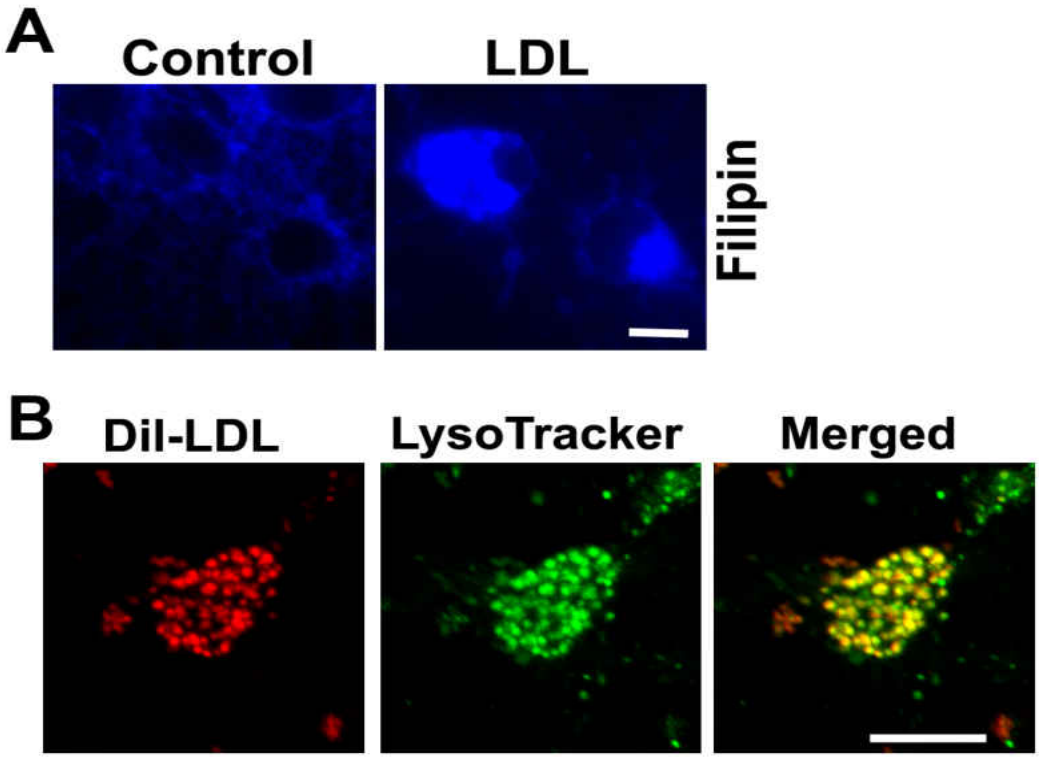

Figure 2. LDL Cholesterol Increased Intraneuronal Accumulation of Cholesterol. (A) LDL cholesterol (50 μg/ml) treatment for 3 days altered free cholesterol (filipin staining) distribution. Whereas cholesterol was mainly present at the cell surface of control neurons, cholesterol was accumulated in the cytosol of LDL cholesterol treated neurons. Bar =10 μm. (B) Dil-labeled LDL (red) co-distributed with endolysosomes (green, LysoTracker). Bar =10 μm.

Because ApoB containing LDL cholesterol is not normally present in brain and not utilized by neurons, increased endolysosome accumulation of ApoB containing LDL cholesterol could affect the structure and function of neuronal endolysosomes. Accordingly, we next determined the extent to which LDL cholesterol treatment affected the morphology of endolysosomes in primary

cultured neurons. First, we identified endolysosomes with LysoTracker in living neurons, and we found that the sizes of the endolysosomes were markedly enlarged in LDL cholesterol treated neurons (Figure 3A). Second, we identified immunohistochemically endosomes with early endosome antigen-1 (EEA1) antibody and lysosomes with lysosome associated membrane protein 1 (LAMP1) antibody in fixed neurons. We found that LDL cholesterol treatment markedly altered the structure of both endosomes and lysosomes; the sizes of endosomes and lysosomes were relatively small, homogeneous, and evenly distributed in control neurons, whereas the sizes of the endosomes and of lysosomes were markedly enlarged and clumped together in neurons treated with LDL cholesterol for 3 days (Figure 3B). Furthermore, we demonstrated that LDL cholesterol treatment significantly increased the protein levels of EEA1 and LAMP1 (Figure 3C). Our findings suggest that ApoB containing LDL cholesterol directly altered the structure of neuronal endolysosomes.

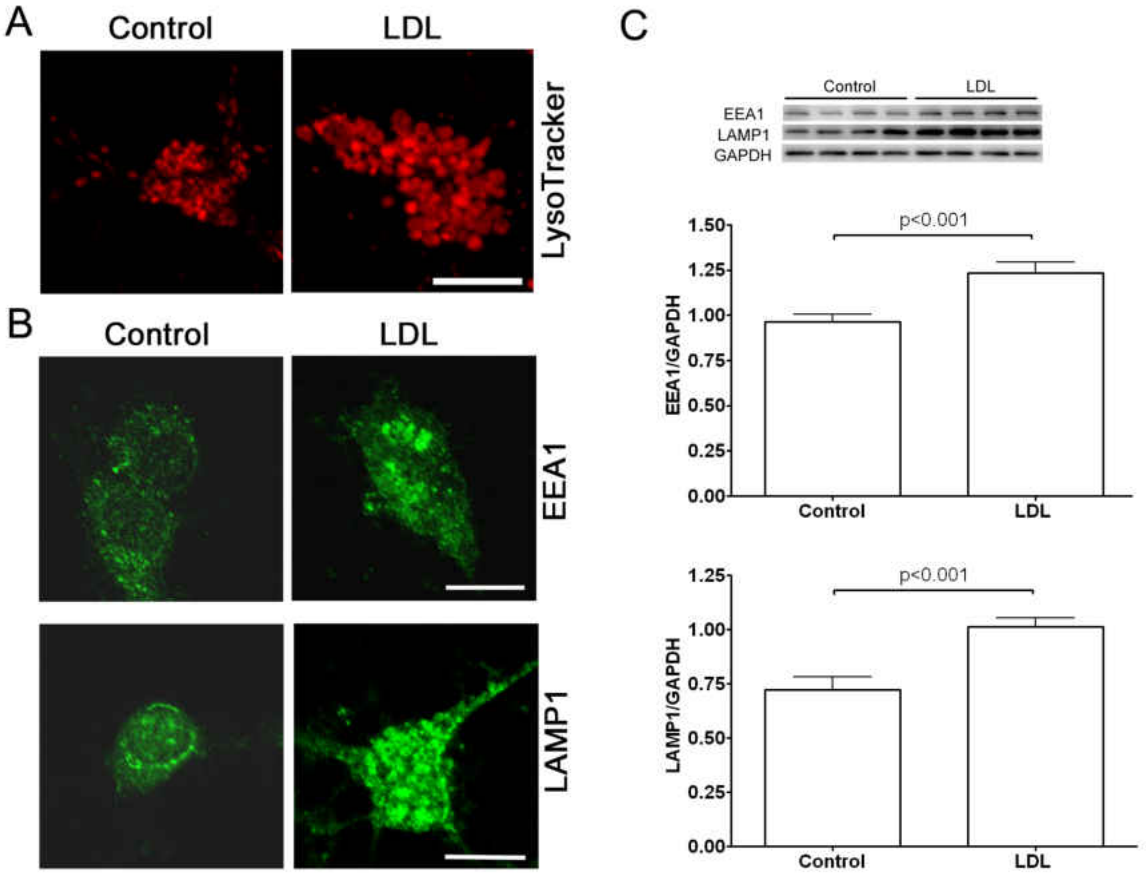

Figure 3. LDL Cholesterol Altered the Structure of Endolysosomes. (A) LDL treatment (50 μg/ml) for 3 days increased the size of endolysosomes (LysoTracker) in living primary cultured cortical neurons. Bar = 10 μm. (B) LDL cholesterol treatment for 3 days markedly altered the structure of both endosomes (EEA1) and lysosomes (LAMP1); the sizes of endosomes and lysosomes were relatively small, homogeneous, and evenly distributed in control neurons, whereas the sizes of endosomes and lysosomes were markedly enlarged and clumped together in neurons treated with LDL cholesterol. (C) LDL cholesterol (50 μg/ml) treatment increased significantly protein levels of EEA1 and LAMP1. GAPDH was used as a loading control (n=4; $p < 0.001$).

Next, we determined the extent to which ApoB containing LDL cholesterol affected the function of endolysosomes. Because pH is of central importance to physiological functions of endolysosomes, we first determined the extent to which LDL cholesterol affected endolysosome pH. Here we measured endolysosome pH using a LysoSensor dye that permits ratiometric assessment of pH changes in

acidic organelles independently of dye concentration. We found that LDL cholesterol (50 μg/ml) treatment for 3 days increased significantly endolysosome pH in primary cultured rat cortical neurons (Figure 4A). Because pH is critical for physiological functions of endolysosome enzymes, we then determined protein expression levels and specific activities of endolysosome enzymes as a measurement of endolysosome function. We found that LDL cholesterol treatment for 3 days significantly increased protein levels of three endolysosome enzymes; acid phosphatase, cathepsin B, and cathepsin D (Figure 4B). However, the specific activities of three endolysosome enzymes were decreased (Figure 4C,D,E). Thus, our findings suggest that ApoB containing LDL cholesterol disturbs the function of neuronal endolysosomes.

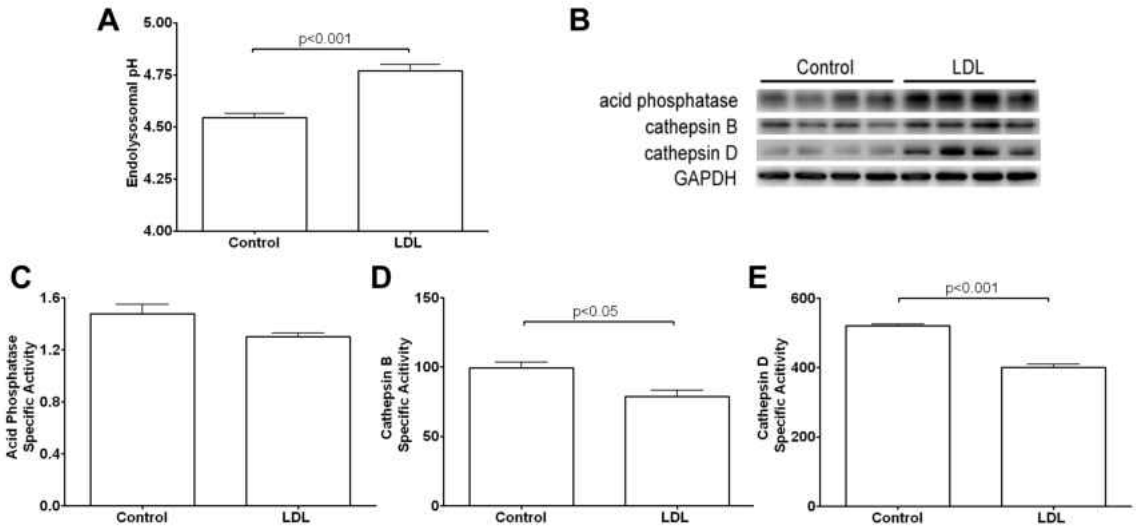

Figure 4. LDL Cholesterol Disturbed the Function of Endolysosomes. (A) Endolysosome pH was measured ratio-metrically with a LysoSensor dye. LDL cholesterol treatment (50 μg/ml) increased significantly neuronal endolysosome pH. (n=8; $p < 0.01$). (B) LDL cholesterol treatment (50 μg/ml) increased protein levels of acid phosphatase, cathepsin B, and cathepsin D. GAPDH was used as a loading control. (C, D, E) LDL cholesterol treatment (50 μg/ml) decreased specific enzyme activity of acid phosphatase, cathepsin B, and cathepsin D when compared with controls (n=6; $p < 0.05$; $p < 0.01$; $p < 0.001$).

Disturbed structure and function of endolysosomes has been recognized as one of the earliest pathological features of sporadic AD, and our previous findings in cholesterol-fed rabbit model of sporadic AD demonstrated that altered structure and function of endolysosomes was linked directly to the development of pathological hallmarks of AD including elevated Aβ production, tau pathology, and disrupted synaptic integrity. Although our *in vivo* studies indicate that elevated levels of circulating cholesterol can alter the structure and function of neuronal endolysosomes and contribute to the pathogenesis of sporadic AD, it is still not clear whether elevated circulating cholesterol exerts its detrimental effects on neurons directly or through a yet unidentified indirect mechanism (Chen et al., 2010). Accordingly, here we determined the extent to which ApoB containing LDL cholesterol directly affected the development of pathological features of AD in primary cultured neurons.

A hallmark of AD is increased levels of Aβ in brain and intraneuronal deposition of Aβ precedes extracellular deposition of Aβ (Gyure et al., 2001; Ohyagi, 2008). Accordingly, we determined the extent to which ApoB-containing LDL cholesterol affected Aβ production. Using ELISA methods, we determined Aβ levels. We found that LDL cholesterol treatment significantly increased levels of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in primary cultured neurons (Figure 5A). Furthermore, we demonstrated that LDL cholesterol treatment increased the accumulation of both Aβ (Figure 5B, two panels in the left) and its precursor protein AβPP in neuronal endolysosomes (Figure 5B, two panels in the right). Our observations are consistent with our previous *in vivo* findings that elevated circulating

cholesterol promoted amyloidogenic processing of AβPP and increased Aβ production (Chen et al., 2010), however, the underlying mechanisms are not fully understood. It is known that Aβ is produced mainly in the endolysosome system by sequential cleavage of its precursor protein AβPP by β-secretase and γ-secretase. Importantly, beta-site APP-cleaving enzyme 1 (BACE-1), the rate-limiting enzyme in Aβ production, is present in endolysosomes and its activity is pH dependent. We showed previously that LDL cholesterol treatment altered the structure of endolysosomes and elevated endolysosome pH. Thus, it is very likely that LDL cholesterol increases Aβ production by affecting BACE-1. Accordingly, we determined the extent to which LDL cholesterol treatment affected levels of expression, enzyme activity, and intracellular distribution of BACE-1 in primary cultured neurons. We found that LDL cholesterol treatment did not affect mRNA levels and total protein levels of BACE-1 (Figure 6A, B). However, LDL cholesterol treatment increased markedly the accumulation of BACE-1 in endolysosomes (Figure 6C). Moreover, LDL cholesterol treatment enhanced significantly enzyme activity of BACE-1 (Figure 6D). Thus, our findings suggest that ApoB-containing LDL cholesterol increases Aβ production by elevating endolysosome pH and enhancing enzyme activity of BACE-1.

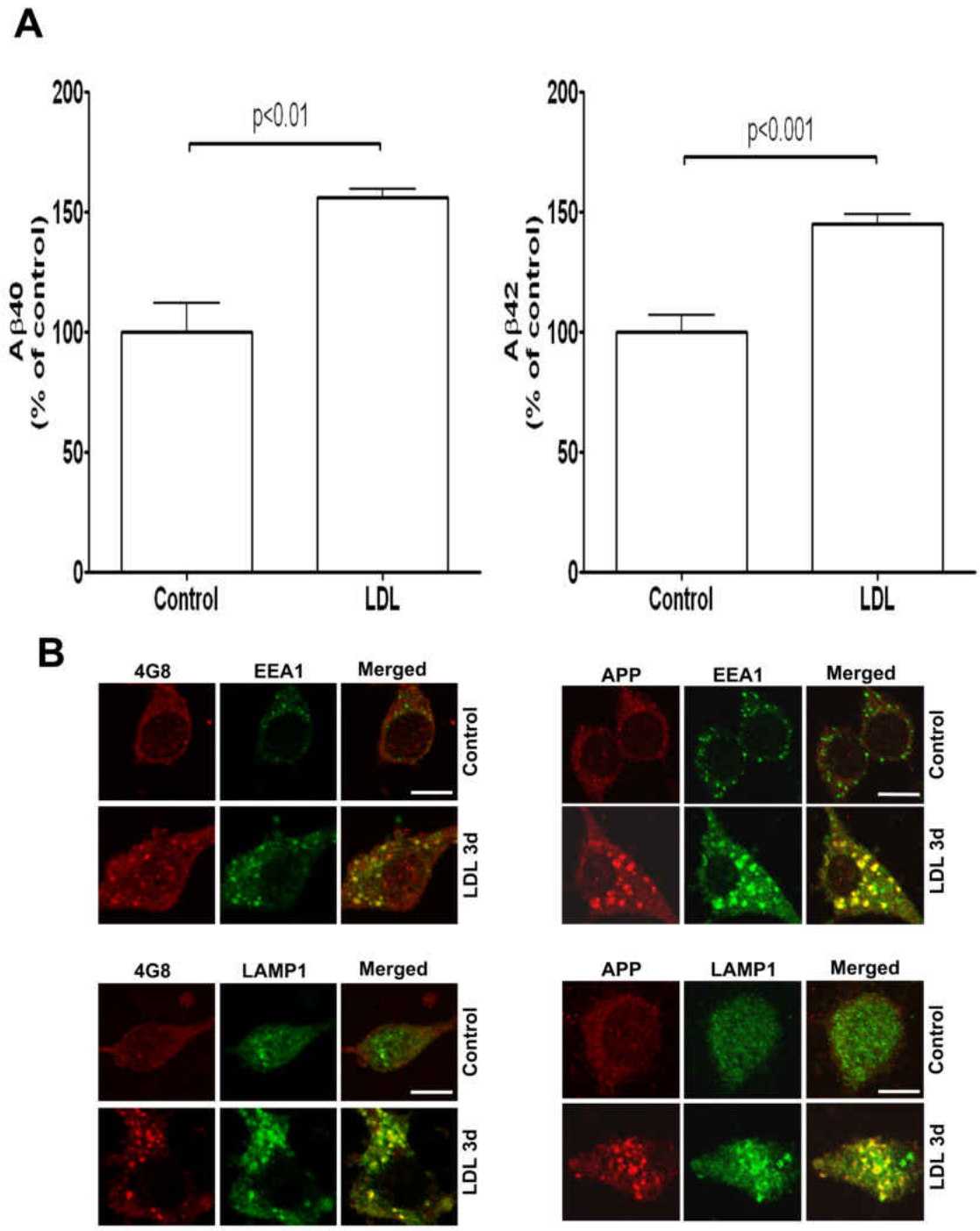

Figure 5. LDL Cholesterol Increased Amyloid Beta Production. (A) LDL cholesterol treatment (50 µg/ml) for 3 days increased significantly levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, when compared with control groups (n=8; $p < 0.01$; $p < 0.001$). (B) (left panel) LDL cholesterol treatment for 3 days increased the co-distribution of Aβ (4G8) with endosomes (EEA1) and lysosomes (LAMP1); (right panel) LDL cholesterol treatment for 3 days increased the co-distribution of APP with endosomes (EEA1) and lysosomes (LAMP1). Bar =10 µm.

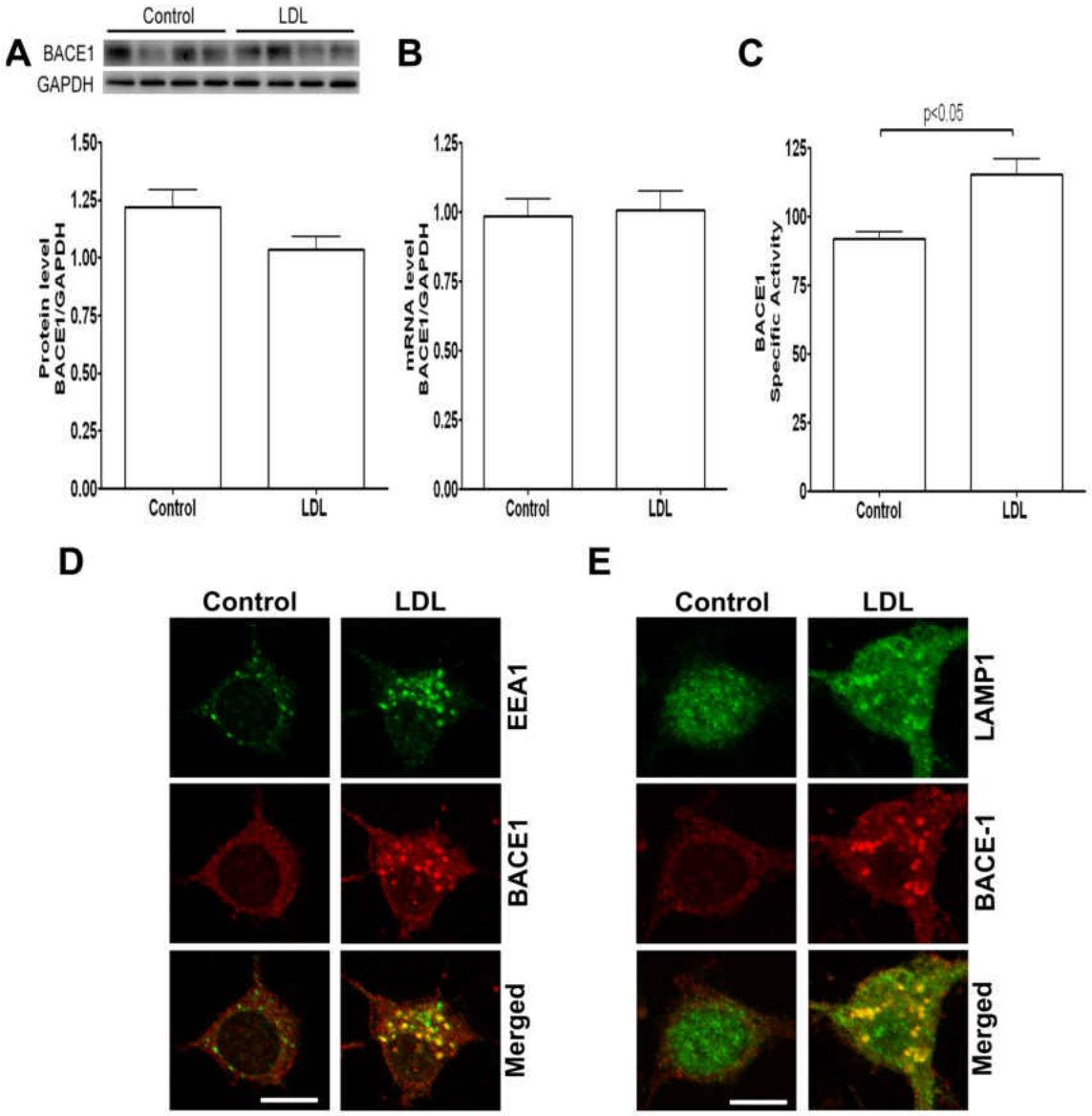

Figure 6. LDL Cholesterol Increased Beta Amyloid Cleavage Enzyme 1 Activity. (A) LDL cholesterol treatment did not change protein levels of BACE-1 (n=6). (B) LDL cholesterol treatment did not change significantly mRNA levels of BACE-1 (n=8). (C) LDL cholesterol treatment (50 µg/ml) increased significantly BACE-1 specific enzyme activity (n=8; $p < 0.05$). (D) LDL cholesterol treatment increased the co-distribution of BACE-1 with endosomes (EEA1) and lysosomes (LAMP1). Bar =10 µm.

Neurofibrillary tangles composed of phosphorylated tau are another pathological feature of AD, and here we determined the extent to which ApoB containing LDL cholesterol directly affected the development of tau-pathology.

LDL cholesterol treatment increased signficantly ($p<0.001$) protein levels of phosphorylated tau in primary cultured neurons (Figure 7A). Furthermore, phosphorylated tau was accumulated by endolysosomes (Figure 7B). Such observations are consistent with reports of others that tau is degraded in lysosomes (Hamano et al., 2008; Oyama et al., 1998; Wang et al., 2009b) and that endolysosome dysfunction induces tau-pathology (Bi and Liao, 2007; Distl et al., 2003; Liao et al., 2007). Thus, our findings suggest that ApoB-containing LDL cholesterol induces tau-pathology possibly by altering endolysosome function.

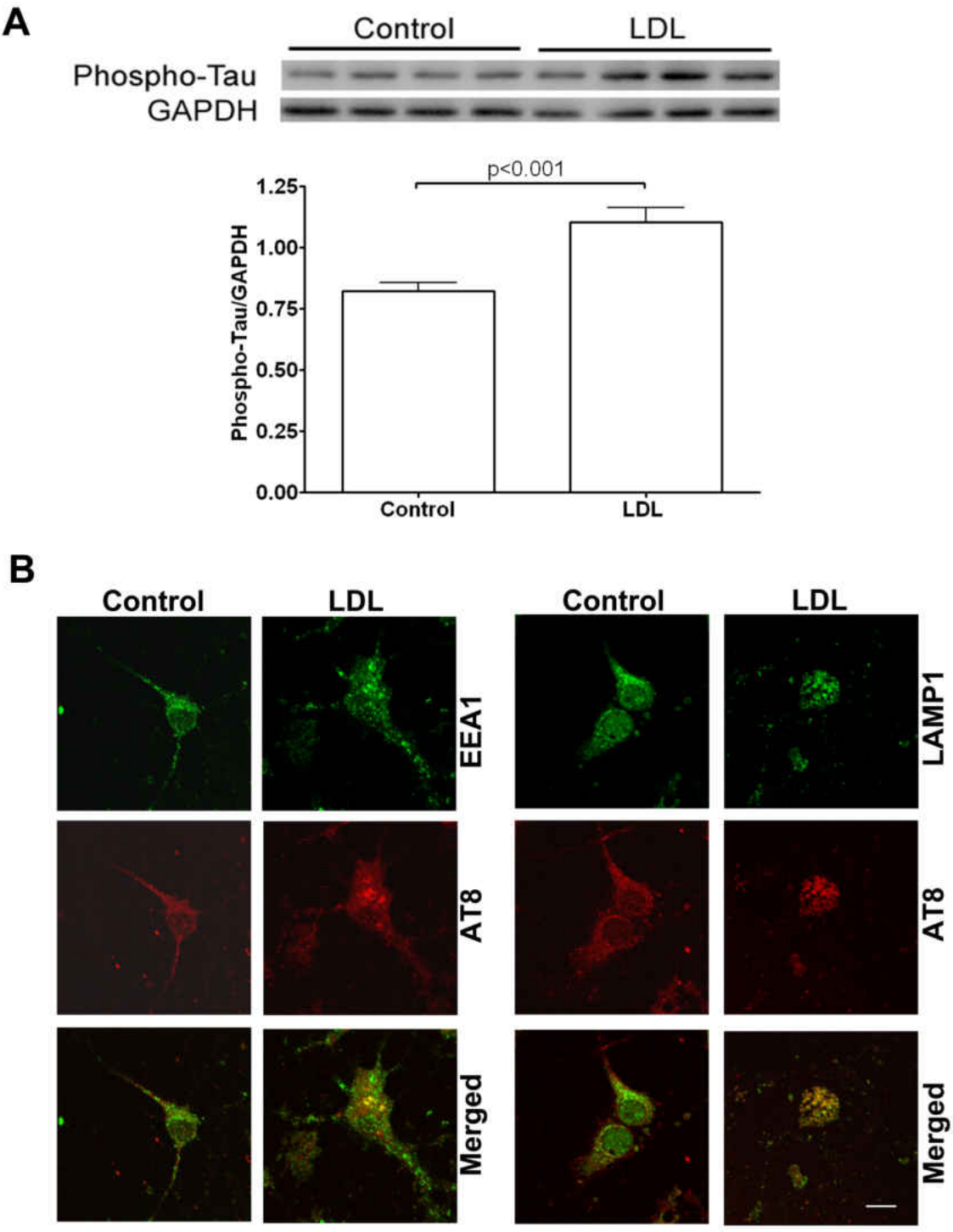

Figure 7. LDL Cholesterol Altered the Expression and Distribution of Phosphorylated Tau. (A) LDL cholesterol treatment (50 µg/ml) increased significantly protein levels of phosphorylated-tau (AT8) (n=6, $p < 0.001$). (B) LDL cholesterol treatment (50 µg/ml) increased the co-distribution of phosphorylated tau (AT8) with endosomes (EEA1) and lysosomes (LAMP1). Bar =10 µm.

Another pathological feature of AD is disruption of synaptic integrity. Accordingly, we determined the extent to which ApoB containing LDL cholesterol affected synaptic integrity, as indicated by protein levels and distribution of presynaptic protein synaptophysin. We found that LDL cholesterol treatment decreased significantly ($p < 0.01$) protein levels of synaptophysin in primary cultured neurons (Figure 8A). Furthermore, we demonstrated that LDL cholesterol treatment markedly increased the accumulation of synaptophysin in endolysosomes (Figure 8B). Our observations are consistent with those findings from others that lysosome dysfunction is linked to synaptic pathology in AD brain (Bahr and Bendiske, 2002; Callahan et al., 1999), and that inhibiting lysosomal function with chloroquine, a lysosomotropic agent that blocks acidification, results in synaptic dysfunction and synaptic loss in hippocampal slices (Bendiske and Bahr, 2003; Bendiske et al., 2002; Kanju et al., 2007). Thus, our findings suggest that ApoB-containing LDL cholesterol disrupts synaptic integrity by altering endolysosome function.

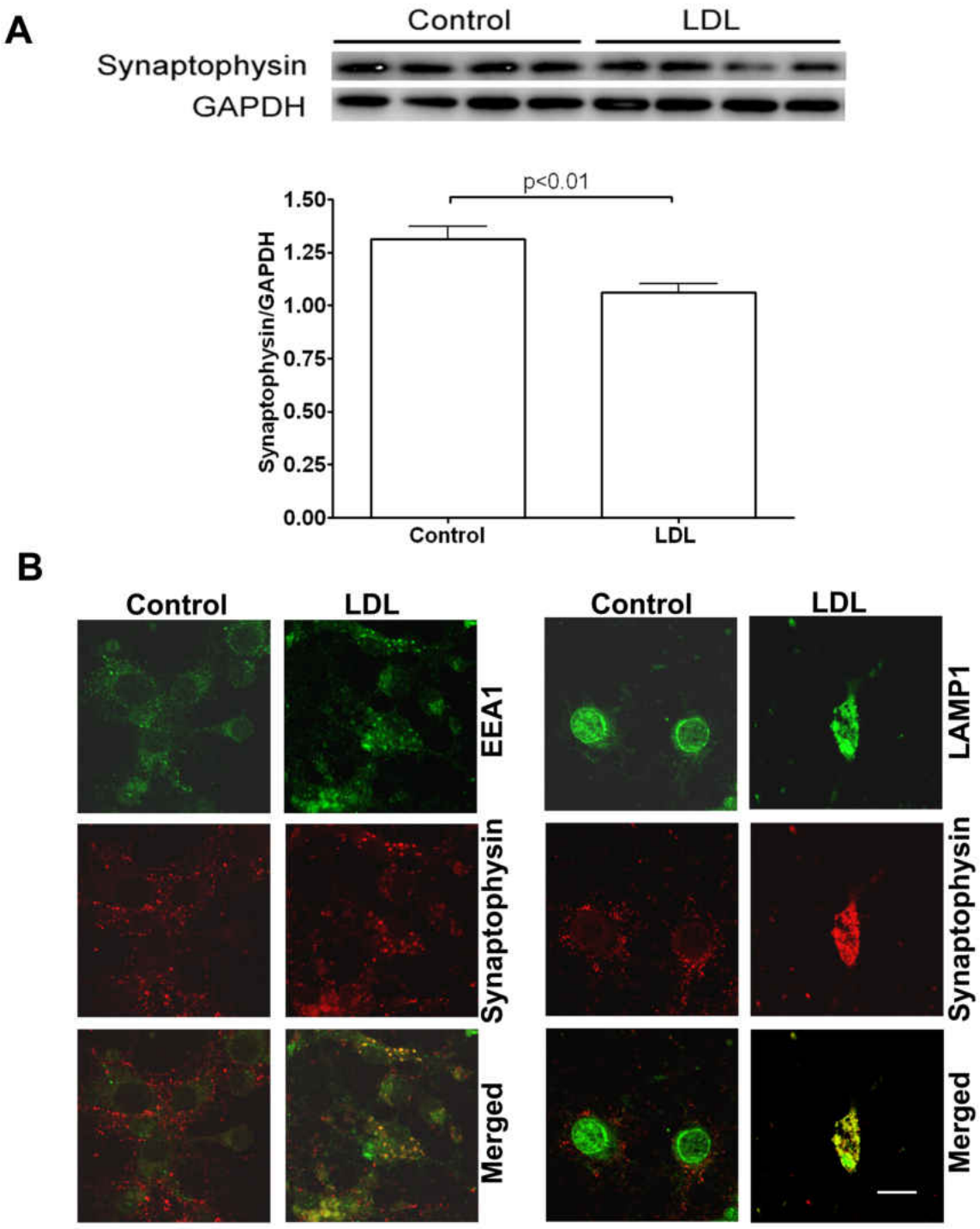

Figure 8. LDL Cholesterol Altered the Expression and Distribution of Synaptophysin. (A) LDL cholesterol treatment (50 µg/ml) decreased protein levels of synaptophysin. (n=6; $p < 0.01$). (B) LDL cholesterol treatment (50 µg/ml) increased the co-distribution of synaptophysin with endosomes (EEA1) and lysosomes (LAMP1). Bar =10 µm.

4.4.Discussion

Several lines of evidence suggest strongly that elevated levels of circulating cholesterol, as an extrinsic factor, contributes to the pathogenesis of sporadic AD (Chen et al., 2010; Crisby et al., 2004; Reiss et al., 2004; Sparks, 2008; Sparks et al., 2000). Epidemiologically, elevated plasma levels of cholesterol during mid-life increases the risk of developing AD later in life (Solomon et al., 2009). Genetically, people carrying the ApoE4 allele, the major genetic risk factor of sporadic AD (Corder et al., 1993; Wisdom et al., 2011), have elevated levels of plasma cholesterol (Corder et al., 1993; Marzolo and Bu, 2009), whereas people carrying the ApoE2 allele, a protective factor against AD (Corder et al., 1996; Corder et al., 1994; Schachter et al., 1994), have decreased levels of plasma cholesterol (Dallongeville et al., 1992). Experimentally, diets enriched in cholesterol induced pathological features of AD in mice, rats and rabbits (Chen et al., 2010; Ghribi et al., 2006; Granholm et al., 2008; Sparks et al., 1994; Thirumangalakudi et al., 2008). Despite such a strong linkage between elevated circulating cholesterol and the pathogenesis of sporadic AD, the underlying mechanisms are still not clear.

Our previous studies demonstrated, in a rabbit model of sporadic AD, that cholesterol-enriched diet disrupted the BBB integrity, increased ApoB accumulated in neuronal endolysosomes and disturbed the structure and function of endolysosomes, which was linked to the development of pathological features of AD including disrupted synaptic integrity, increased Aβ levels, and tau

pathology (Chen et al., 2010). ApoB is the main apolipoprotein of LDL and VLDL that transports cholesterol to peripheral tissues. Because ApoB is not present in normal brain (Pitas et al., 1987) but is present in AD brain (Namba et al., 1992; Takechi et al., 2009), our findings suggest that elevated circulating cholesterol *per se* contributes to the pathogenesis of sporadic AD. To extend further our *in vivo* findings and determine the underlying mechanisms whereby elevated levels of circulating cholesterol contribute to the development of AD-like pathology, here we determined the extent to which and mechanisms by which ApoB containing LDL cholesterol contributed to the development of AD-like pathology in primary cultured hippocampal neurons. Consistent with our *in vivo* observations, we have now demonstrated that ApoB containing LDL cholesterol treatment increased endolysosome accumulation of cholesterol, induced enlargement of endolysosomes and elevated endolysosome pH, enhanced production of Aβ, increased levels of phosphorylated tau, and decreased levels of the presynatpic protein synaptophysin. Thus, our findings from both *in vivo* and *in vitro* studies suggest strongly that altered structure and function of endolysosomes underlies elevated levels of circulating cholesterol-induced development of AD-like pathology.

One of the major sources of neuronal cholesterol is uptake of cholesterol containing lipoproteins through receptor-mediated endocytosis, a process where lipoproteins bound to its receptors are internalized, transported to endolysosomes, hydrolyzed to free cholesterol, and from where free cholesterol is transported to various intracellular compartments via a mechanism involving

the Niemann-Pick type C (NPC) proteins (Maxfield and Tabas, 2005; Sleat et al., 2004; Vance et al., 2006). Under normal conditions, circulating ApoB containing cholesterol is largely excluded from the brain due to the intact BBB, and secreted ApoE cholesterol from glial cells, predominately astrocytes, supplies the needed cholesterol for neurons. However, under pathological conditions when the BBB is leaky, as occurs in sporadic AD, circulating ApoB cholesterol can enter brain parenchyma and can be transported to neuronal endolysosomes via the same receptor-mediated endocytosis mechanisms as that of ApoE cholesterol. Consistent with this notion are our previous *in vivo* findings in a cholesterol-fed rabbit model of sporadic AD that the BBB is leaky and ApoB cholesterol is accumulated in neuronal endolysosomes, and our current *in vitro* findings that ApoB containing LDL cholesterol treatment increases neuronal internalization of LDL cholesterol.

Because ApoB containing LDL cholesterol is not normally present in brain and not utilized by neurons, internalized ApoB containing LDL cholesterol could disrupt the structure and function of neuronal endolysosomes. Indeed, we demonstrated that LDL cholesterol treatment increased intraneuronal accumulation of LDL cholesterol and enlarged markedly neuronal endolysosomes. Our observation is consistent with that of others that increased intraneuronal loading of cholesterol increased the sizes of endosomes (Cossec et al., 2010). However, it is not known exactly how ApoB containing LDL cholesterol induces enlargement of endolysosomes. It could be that increased levels of LDL cholesterol enhances receptor-mediated endocytosis of cholesterol,

which exceeds the ability of neurons to export cholesterol out of endolysosomes, or alternatively, internalized ApoB containing LDL cholesterol specifically suppresses the ability of endolysosomes to export cholesterol. Under both circumstances, there probably exists a traffic jam in intraneuronal transportation of cholesterol, which could alter the structure of endolysosomes. Consistent with this notion are findings that lysosome accumulation of cholesterol in Niemann-Pick type C diseased brain is associated with an altered structure of lysosomes (Bi and Liao, 2007; Distl et al., 2003; Liao et al., 2007). It is also known that endolysosomes are very complicated structures composed of different sub-groups of vesicles including early endosomes, late endosomes, recycling endosome, lysosomes, and autophagosomes, all of which are involved in intracellular transportation of cholesterol. Thus, another possibility is that ApoB containing LDL cholesterol could be transported into different sub-groups of endolysosomes than that of ApoE cholesterol thus disturbing the normal intraneuronal cholesterol transportation. Further research addressing such possibilities is warranted.

In addition to an altered structure of endolysosomes, we demonstrated that ApoB containing LDL cholesterol treatment disturbed the function of endolysosomes, as evidenced by elevated endolysosome pH. Since pH is of central importance to physiological functions of endolysosomes, elevated endolysosome pH could affect dramatically endolysosome function. Indeed, we demonstrated that LDL cholesterol treatment decreased enzyme activities of three different endolysosome enzymes including acid phosphatase, cathepsin B,

and cathepsin D, all of which are pH-sensitive enzymes with an optimal pH value around 4. Although, it is not known how increased LDL cholesterol affects endolysosome pH, it is possible that increased endolysosome accumulation of LDL cholesterol suppresses the activity of vacuolar H^+-ATPase, which maintains the low pH of endolysosome by pumping H^+ into endolysosomes (Cox et al., 2007).

Neurons are long-lived post mitotic cells that possess an elaborate endolysosome system for quality control. It is known that altered morphological and functional features of endolysosomes are one of the earliest pathological features of AD (Boland et al., 2008; Tate and Mathews, 2006). It was also shown that endosome enlargement was apparent in brains of AD patients and non-demented patients with early signs of AD, in Down's syndrome individuals, and in patients bearing the ApoE4 allele (Arriagada et al., 2007; Cataldo et al., 2004). The hypothesis that endosome enlargement is an early pathogenic event in AD is supported by findings that endosome enlargement largely preceded extracellular deposition of Aβ in brain (Cataldo et al., 2000). Abnormalities of lysosomes have been noted also in AD; lysosomal components are present in amyloid plaques (Boland et al., 2008; Cataldo et al., 1990), increased numbers of neuronal lysosomes have been observed, there is increased expression and synthesis of all classes of lysosomal hydroxylase enzymes (Nixon, 2007), and residual bodies accumulate as an indicator of lysosome dysfunction (Cataldo et al., 1994). Clearly, a disturbed structure and function of endolysosomes appears to play an important and early role in the pathogenesis of sporadic AD. Given our

observations, both *in vivo* (Chen et al., 2010) and *in vitro*, that elevated levels of ApoB containing LDL cholesterol disturbed the structure and function of neuronal endolysosomes, it is very likely that elevated levels of cholesterol contributes to the pathogenesis of sporadic AD by disturbing neuronal endolysosomes.

Recent studies suggest strongly that pathological changes in endolysosomes contribute to Aβ production, a pathological hallmark of AD, as evidenced by the following findings; AβPP and its cleavage products are present in clathrin-coated vesicles that are part of the endocytic pathway (Ferreira et al., 1993; Harris and Milton, 2010). Aβ production is decreased in cultured cells that were stably transfected with an AβPP construct where the C-terminal endocytic targeting signal was removed (Perez et al., 1999; Soriano et al., 1999); Aβ production is decreased in cells transfected with dominant negative dynamin, which prevents endocytosis (Chyung and Selkoe, 2003); BACE-1, a key enzyme for amyloidogenesis, is localized in endosomes and its activity is optimal at a pH of about 5.0 (Rajendran et al., 2008; Shimizu et al., 2008; Vassar et al., 1999); and Aβ is accumulated in endolysosomes of neurons from AD brain (Cataldo et al., 2004). Consistent with this notion, we demonstrated that ApoB containing LDL cholesterol treatment elevated endolysosome pH, disturbed the function of endolysosomes, and increased Aβ production. Importantly, we demonstrated that LDL cholesterol treatment increased the accumulation of BACE-1 in endolysosomes and increased significantly BACE-1 enzyme activity. Because the activity of BACE-1, the rate limiting enzyme in the production of Aβ, is pH-dependent with an optimal pH around 5 (Rajendran et al., 2008; Shimizu et al.,

2008; Vassar et al., 1999), the observed elevation of endolysosome pH could be responsible for enhanced BACE-1 enzyme activity and increased Aβ production. In addition, BACE-1 is degraded in lysosomes under more acidic conditions (pH < 4) (Koh et al., 2005). Thus, the observed elevation of endolysosome pH could lead to decreased degradation of BACE-1 and to increased accumulation of BACE-1 in endolysosomes, which also resulted in increased Aβ production. On the other hand, Aβ can be degraded in lysosomes by endolysosome enzymes such as cathepsin D, whose activity is also pH dependent and with an optimal pH around 4 (Hamazaki, 1996; Higaki et al., 1996; Ladror et al., 1994; Saftig et al., 1996). Collectively, observation of elevated endolyosome pH and decreased cathepsin D activity could contribute to increased levels of Aβ. In addition, although not examined in the present studies, γ-secretase, consisting of presenilin, nicastrin, Aph-1, and Pen-2, is also present in endolysosomes (Frykman et al., 2010; Pasternak et al., 2003; Refolo et al., 1995; Vetrivel et al., 2004), its activity is also sensitive to pH (Pasternak et al., 2003; Pasternak et al., 2004) and it can be degraded in lysosomes (He et al., 2007). Therefore, ApoB containing LDL cholesterol induced elevation of endolysosome pH could also increase the production of Aβ by affecting the activity and protein levels of γ-secretase. Thus, our results suggest that LDL cholesterol induced elevation of endolysosome pH plays a key role in over-production of Aβ. Our results also suggest that LDL cholesterol disrupts the function of endolysosomes and subsequently affects Aβ production (Jin et al., 2004).

In addition to Aβ production, endolysosomes have been implicated in the development of tau-pathology, another pathological hallmark of AD. Although the underlying mechanisms are not yet clear, it has been shown that tau is degraded by cathepsin D in the autophagy-lysosome system (Hamano et al., 2008; Kenessey et al., 1997; Oyama et al., 1998; Wang et al., 2009b), and that cholesterol storage in lysosomes induces lysosome dysfunction and tau-pathology in Niemann-Pick type C diseased brain (Bi and Liao, 2007; Bu et al., 2002; Distl et al., 2003; Liao et al., 2007; Sawamura et al., 2001; Vance, 2006). Consistent with this notion and our previous *in vivo* findings (Chen et al., 2010), here we demonstrated that ApoB containing LDL cholesterol disturbed the structure and function of endolysosomes, decreased enzyme activity of cathepsin D, and increased endolysosome accumulation of phosphorylated tau. Thus, it is possible that the observed LDL cholesterol induced elevation of endolysosome pH decreases the degradation of phosphorylated tau and contributes to the development of tau pathology in AD.

Besides Aβ and tau-pathology, endolysosomes are also involved in the development of disrupted synaptic integrity, another pathological hallmark of AD that correlates best with dementia (Selkoe, 2002; Terry et al., 1991). Endolysosomes are responsible for recycling synaptic proteins (Blumstein et al., 2001; Kuromi and Kidokoro, 1998; Murthy and Stevens, 1998), lysosome dysfunction is linked to synaptic pathology in AD brain (Bahr and Bendiske, 2002; Callahan et al., 1999), and that inhibiting lysosome function with chloroquine, a lysosomotropic agent that blocks acidification, results in synaptic dysfunction and

synaptic loss in hippocampal slices (Bendiske and Bahr, 2003; Bendiske et al., 2002; Kanju et al., 2007). Consistent with this notion and our previous *in vivo* findings (Chen et al., 2010), we have now demonstrated that ApoB containing LDL cholesterol disturbed the structure and function of endolysosomes, increased the endolysosome accumulation of presynaptic protein synaptophysin, and decreased total protein levels of synaptophysin. Thus, our results suggest that altered endolysosome structure and function contributes to LDL cholesterol induced development of disrupted synaptic integrity in AD.

In summary, we demonstrated, in primary cultured neurons, that ApoB containing LDL cholesterol treatment increased endolysosome accumulation of cholesterol, induced enlargement and the elevated pH of endolysosomes, enhanced production of Aβ, increased levels of phosphorylated tau, and decreased levels of the presynaptic protein synaptophysin. Such findings suggest strongly that altered structure and function of endolysosomes plays an important role in the pathogenesis of sporadic AD, and that increased levels of circulating ApoB containing cholesterol directly contribute to the pathogenesis of sporadic AD, which occurs mechanistically because of alteration in the structure and function of endolysosomes.

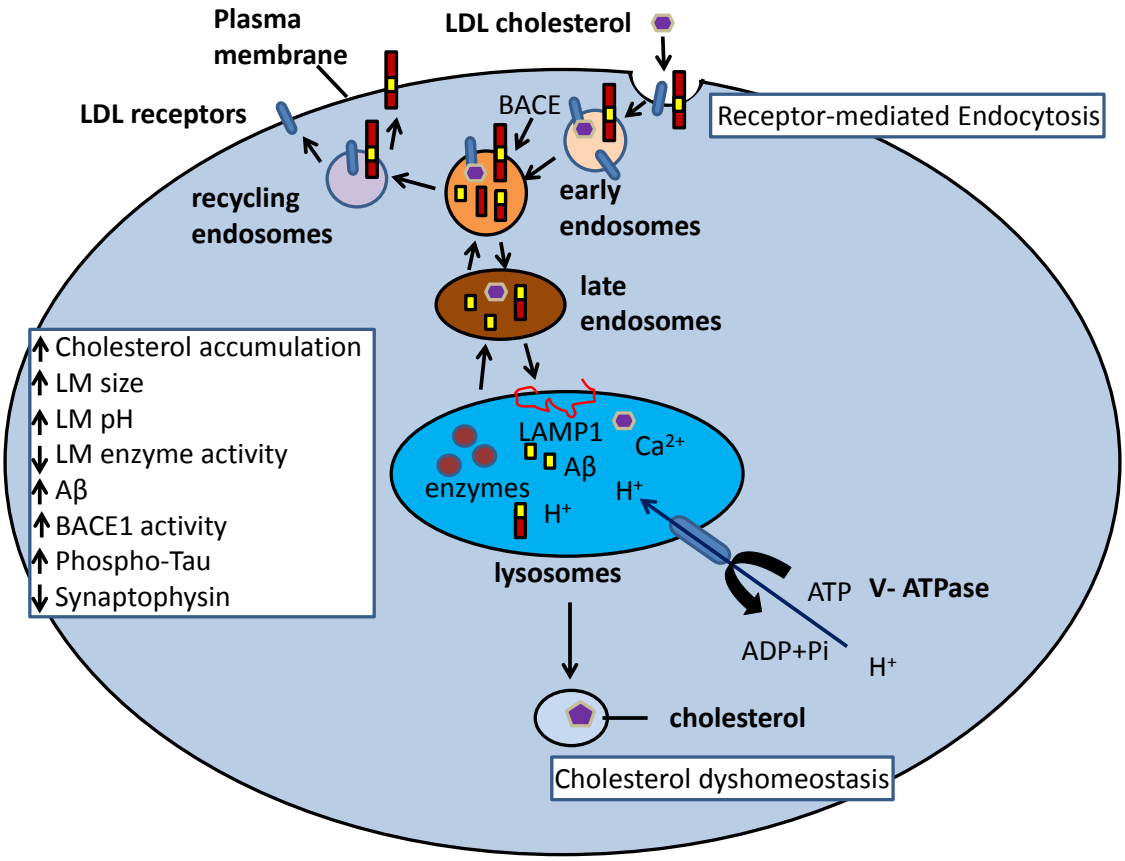

Figure 9. Model Summarizing Involvement of Endolysosomes in LDL Cholesterol-Induced Alzheimer's Disease-Like Pathology in Primary Cultured Neurons. Treating neurons with ApoB containing LDL cholesterol increases endolysosomal accumulation of cholesterol, enlarges endolysosomes and elevates endolysosome pH. In addition, ApoB containing LDL cholesterol increases endolysosome accumulation of beta amyloid converting enzyme 1, BACE-1, enhances BACE-1 activity and increases production of Aβ levels.

CHAPTER V

INVOLVEMENT OF ENDOLYSOSOMES IN HIV-1 Tat-INDUCED TOXICITY AND AMYLOID BETA GENERATION IN PRIMARY CULTURED NEURONS

5.1. Introduction

Greater than 40 million people worldwide are infected with the human immunodeficiency virus-1 (HIV-1) and combined antiretroviral therapeutic drugs have effectively increased the life span of people living with HIV-1 infection. Increased as well is the prevalence of HIV associated neurocognitive disorders (HAND) with recent epidemiological studies indicating that the prevalence of HAND in the USA is greater than 50% of HIV-1 infected people (Ellis et al., 2010; Heaton et al., 2010). Clinically, HAND represents a set of conditions ranging from subtle neuropsychological impairments to profoundly disabling HIV-associated dementia. Although the underlying mechanisms for HAND pathogenesis are not fully understood, soluble factors including HIV-1 viral products and pro-inflammatory mediators released from infected glia and monocytes have been implicated (Ances and Ellis, 2007; Ghafouri et al., 2006; King et al., 2006; Wallace, 2006). Among the viral products, HIV-1 transactivator of transcription protein (Tat) has been shown to be neuroexcitatory, neurotoxic, and it continues to be implicated as a causative agent in HAND (Agrawal et al., 2012; Buscemi et al., 2007; Haughey et al., 1999; King et al., 2006; Nath et al., 2000; Perez et al.,

2001; Sabatier et al., 1991; Weeks et al., 1995). Interestingly, emerging evidences have revealed that there is a significant incidence of AD-like pathology, such as increased amyloid beta (Aβ) deposition, in aged HIV patients (Achim et al., 2009; Clifford et al., 2009; Esiri et al., 1998; Gelman and Schuenke, 2004; Green et al., 2005; Nebuloni et al., 2001; Pulliam, 2009; Xu and Ikezu, 2009). Recently, HIV-1 transactivator of transcription (Tat) has been shown to increase neuronal Aβ generation (Aksenov et al., 2010; Giunta et al., 2009; Rempel and Pulliam, 2005). Thus, Tat, a HIV-1 viral protein that continues to be implicated as a causative agent in HAND (Agrawal et al., 2012; Buscemi et al., 2007; Haughey et al., 1999; King et al., 2006; Nath et al., 2000; Perez et al., 2001; Sabatier et al., 1991; Weeks et al., 1995) also contributes to the development of AD-like pathology in HIV-1 infected individuals, but the underlying mechanisms are not fully understood.

HIV-1 Tat is a nonstructural transcriptional regulator essential for the replication of HIV-1. The first exon of HIV-1 Tat encodes for the first 72 amino acids and the second exon encodes for another 14 to 32 amino acids. Tat_{1-72} is sufficient for transactivation, which requires the arginine rich domain of Tat between amino acid residues 49 and 57. Nanomolar concentrations of HIV-1 Tat have been reported in sera of HIV-1 infected patients, but these levels are almost certainly underestimated given how avidly HIV-1 Tat binds to proteins and cells (Westendorp et al., 1995; Xiao et al., 2000). HIV-1 Tat can be transported across the blood-brain barrier from the systemic circulation (Banks et al., 2005; Kim et al., 2003), can be secreted by infected macrophages and microglia, and has

been detected in brains of patients with HIV-1 associated dementia (Ellis et al., 2000; Nath, 2002; Westendorp et al., 1995).

HIV-1 Tat enters neurons via receptor-mediated endocytosis involving CD26 (Gutheil et al., 1994), CXCR4 (Xiao et al., 2000), heparin sulfate proteoglycans (Tyagi et al., 2001), and low-density lipoprotein receptor-related proteins (Deshmane et al., 2011; King et al., 2006; Liu et al., 2000; Vendeville et al., 2004). This very rapid and early event results in the accumulation of HIV-1 Tat in endolysosomes with its subsequent release into the cytoplasm and uptake into the nucleus (Caron et al., 2004; Liu et al., 2000; Vives et al., 1997) most likely through mechanisms involving the high H^+ gradient maintained by vacuolar H^+-ATPase (Vendeville et al., 2004). The endolysosome system is very dynamic, and lysosomes and other acidic subcellular compartments are involved in endocytosis and autophagy (Jeyakumar et al., 2005; Nixon and Cataldo, 2006). Endosomes and lysosomes process proteins and other materials that are endocytosed, while autophagy and autophagosomes predominantly process cytosolic proteins. Because neurons are highly polarized long-lived post-mitotic cells, they possess an elaborate endolysosome system that is critical for the maintenance of neuronal function (Nixon and Cataldo, 1995; Nixon and Cataldo, 2006).

Increasingly, endolysosome dysfunction has been implicated in neuronal damage and in the pathogenesis of a variety of neurological disorders including AD, PD and HAND (Gelman et al., 2005; Spector and Zhou, 2008; Zhou and Spector, 2008). For example, as one of the earliest pathological features of AD,

endolysosome dysfunction precedes extracellular deposition of Aβ in brain (Cataldo et al., 2000) has been implicated in the pathogenesis of AD (Boland et al., 2008; Cataldo et al., 2004; Tate and Mathews, 2006), especially the generation of Aβ. Several lines of evidence indicate that Aβ is mainly generated in the endocytic pathway, when AβPP is internalized to endolysosomes (Chyung and Selkoe, 2003; Ferreira et al., 1993; Perez et al., 1999; Soriano et al., 1999) where BACE-1, the rate-limiting enzyme for Aβ generation is located (Rajendran et al., 2008; Shimizu et al., 2008; Vassar et al., 1999).

Here we tested the hypothesis that; HIV-1 Tat induces neuronal damage and contributes to Aβ generation by affecting the structure and function of endolysosomes.

We observed that prior to HIV-1 Tat induced neuronal cell death, this HIV protein enlarged endolysosomes, elevated endolysosome pH, decreased specific activities of endolysosome enzymes, disrupted endolysosome membrane integrity, and inhibited autophagy. These findings suggest that disturbed structure and function of endolysosomes play an early and important role in HIV-1 Tat-induced neuronal damage.

Additionally, we found that HIV-1 Tat-induced increases in Aβ generation, increased endolysosome accumulation of AβPP and Aβ and increased endolysosome accumulation of BACE-1 and enhanced BACE-1 activities. Such findings suggest that HIV-1 Tat increases neuronal Aβ generation and contributes to the development of AD-like pathology in HIV-1 infected individuals by disturbing endolysosome structure and function.

5.2 Methods

5.2.1. Hippocampal neuron primary cultures: Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rats as described previously (Buscemi et al., 2007). Pregnant dams (embryonic day 18) were sacrificed by asphyxiation with CO_2. The fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto 35-mm poly-D-lysine-coated glass-bottom tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, antibiotic/antimycotic and B_{27} supplement, and were maintained at 37°C and 5% CO_2 for 10-14 days at which time they were used for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by neuronal immunostaining with mouse anti-NeuN or goat anti-MAP2 antibodies (Millipore), and for astrocytes with a mouse anti-GFAP antibody (Sigma). Neurons were treated either with HIV-1 Tat_{1-72} (100 nM), mutant Tat ($Tat_{\Delta 31-61}$, 100 nM), or phosphate-buffered saline (PBS) as vehicle.

5.2.2. Neuronal cell viability assay: Neuronal cell viability was determined using a triple staining method as described previously (Buscemi et al., 2007). Neurons were stained with Hoechst 33342 (10 μg/ml), ethidium homodimer-1 (4 μM), and calcein (1 μg/ml). Hoechst 33342, which labels DNA, was used as a marker for identifying condensed nuclei characteristic of apoptotic cell death. Cells dead or dying as a result of loss of membrane integrity were unable to exclude ethidium homodimer dye. Cells were considered viable when cytoplasm

was stained with green fluorescence after the cleavage of the non-fluorescent calcein acetoxymethyl ester to calcein. Fields were chosen at random and at least five images from five separate fields of culture dishes for every experimental condition were taken with our Axiovert 200M fluorescence microscope (Zeiss) and filter-based imaging system. The number of dead or dying neurons (ethidium-labeled red nuclei and blue-condensed nuclei without green cytoplasmic staining) and total neuron numbers were counted manually. More than 900 neurons were counted per experimental condition. Neuronal viability was reported as a percentage of total neurons.

5.2.3. Measurement of endolysosome pH: Details were described in Chapter 4.2.3.

5.2.4. Live cell imaging: The morphology of endolysosomes in living neurons was determined using a LysoTracker dye. After treatments, neurons were loaded with LysoTracker Red DND-99 (50 nM, Invitrogen) and calcein AM (1 μg/ml, Invitrogen) for 30 min at 37°C. Fields were chosen at random and at least five images from every experimental condition were acquired by confocal microscopy (Olympus). For measurement of HIV-1 Tat endocytosis, neurons were incubated with FITC-Tat_{47-57} (100 nM, AnaSpec) for 1 day at 37°C followed by loading of LysoTracker Red DND-99 (50 nM) for an additional 30 min. Images were taken with an Axiovert 200M fluorescence microscope (Zeiss).

5.2.5. Immunostaining: Details were described in Chapter 4.2.4. Here we used primary antibodies against early endosome antigen-1 (EEA1, 1:500, rabbit polyclonal, Santa Cruz), lysosome-associated membrane protein-1 (LAMP1,

1:500, rabbit polycolonal, Sigma), APP (1:500, mouse monoclonal, Milipore), Aβ (4G8, 1:500, mouse monocolonal, Signet) or BACE-1 (1: 500, mouse monoclonal, Milipore), and two secondary antibodies-Alexa 488-conjugated goat anti-mouse antibodies (Invitrogen) and Alexa 546-conjugated goat anti-rabbit antibodies (Invitrogen).

5.2.6. Endolysosome membrane permeability: Endolysosome membrane permeability was determined by measuring the leakage of endolysosome fluorescent dye Lucifer Yellow CH (Invitrogen). Neurons were incubated with Lucifer Yellow (100 μg/ml) for 16 h followed by incubation with Tat at 37°C for 1 and 2 days. Levels of dye inside of neurons were detected by confocal microscopy (Olympus).

5.2.7. Immunoblotting: Details were described in Chapter 4.2.5. Western blots were conducted using the following primary antibodies; anti-EEA1 (1:1000, rabbit polyclonal, Santa Cruz), anti-LAMP1 (1:1000, rabbit polyclonal, Sigma), anti-acid phosphatase (1:1000, mouse monoclonal, Abcam), anti-cathepsin B (1:500, mouse monoclonal, Sigma), anti-cathepsin D (1:1000, mouse monoclonal, Sigma), anti-LC3b (1:1000, rabbit polyclonal, Abcam), anti-Atg5 (1:2000, mouse monoclonal, Millipore), anti-P62 (1:1000, rabbit polyclonal, Sigma) antibodies or anti-BACE-1 (1:1000, mouse monoclonal, Milipore). Anti-β-actin (1:10000, mouse monoclonal, Abcam) antibody was used for a gel loading control.

5.2.8. Measurement of activities of endolysosome enzymes: Details were described in Chapter 4.2.7.

5.2.9. Quantification of Aβ levels by ELISA: Details were described in Chapter 4.2.6.

5.2.110. Measurement of BACE-1 enzyme activity: Details were described in Chapter 4.2.8.

5.2.9. Statistical analysis: All data were expressed as means ± SEM. Statistical significance for multiple comparisons was determined by one-way ANOVA plus a Tukey post hoc test. $p < 0.05$ was considered to be statistically significant.

5.3. Results

In order to compare the effects of HIV-1 Tat on neuronal damage and the structure and function of endolysosomes, we needed to first determine the time course and extent to which HIV-1 Tat decreased neuronal viability. HIV-1 Tat_{1-72} induced significant amounts of neuronal cell death starting from 48 hours of treatment ($p < 0.05$) with a maximum of 50% neuronal cell death ($p < 0.001$) after treatment for 96 hours. (Figure 10B). These results are consistent with previous studies that have shown similar neurotoxic effects of HIV-1 Tat (Aksenov et al., 2003; Bonavia et al., 2001; Buscemi et al., 2007; Eugenin et al., 2007; Haughey et al., 1999; Kruman et al., 1998). No statistically significant increases in neuronal cell death were observed with either mutant $Tat_{\Delta 31-61}$, or PBS, consistent with previous reports that this deletion mutant of HIV-1 Tat is not overtly toxic to neurons (Buscemi et al., 2007).

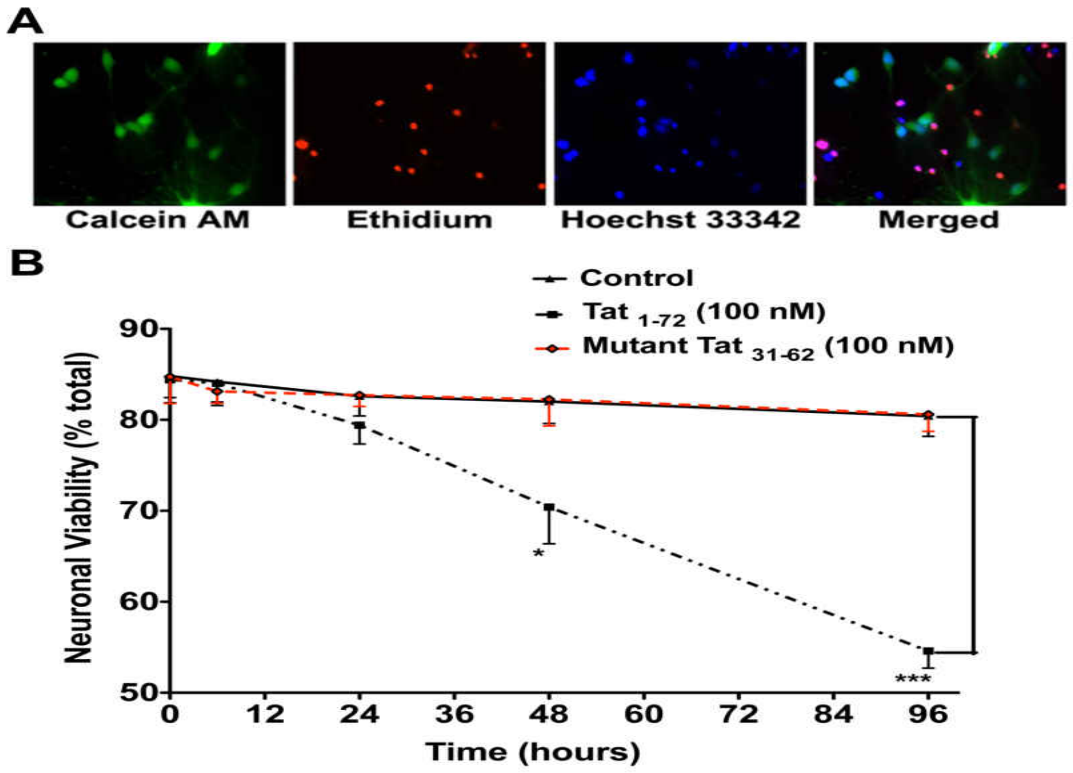

Figure 10. HIV-1 Tat Decreased Neuronal Viability in A Time-Dependent Manner. (A) Neuronal viability was determined by a triple staining method with calcein AM (green, live cells), ethidium homodimer-1 (red, dead cells) and Hoechst 33342 (blue, nuclei). Cells were considered viable when cytoplasm was stained with green fluorescence. Cells were considered dead or dying when cells were stained with red fluorescence or had condensed nuclei characteristic of apoptosis. (B) Significant amounts of neuronal cell death were observed after 2 days of incubation with HIV-1 Tat (100 nM) and reached a maximal level of 50% cell death by the fourth day. No significant neuronal cell death was observed in neurons treated with either mutant Tat or PBS (* $p < 0.05$, and *** $p < 0.001$).

In neurons and other cells, HIV-1 Tat uses receptor-mediated endocytotic mechanisms (King et al., 2006; Liu et al., 2000; Vendeville et al., 2004) to enter cells where HIV-1 Tat accumulates first in endolysosomes. Because the basic region of amino acids 49 to 57 of HIV-1 Tat is required for binding to membrane receptor proteins (Sabatier et al., 1991) and exerting its neurotoxic effects (Weeks et al., 1995), we used a fluorescence (FITC)-labeled Tat_{47-57} to determine

first the extent to which HIV-1 Tat was internalized into neuronal endolysosomes. After incubating neurons with the FITC-labeled HIV-1 Tat_{47-57} (FITC-Tat, green), we observed significant intracellular residence of FITC-Tat, which was compartmentalized in endolysosomes identified with LysoTracker dye (red, Figure 11).

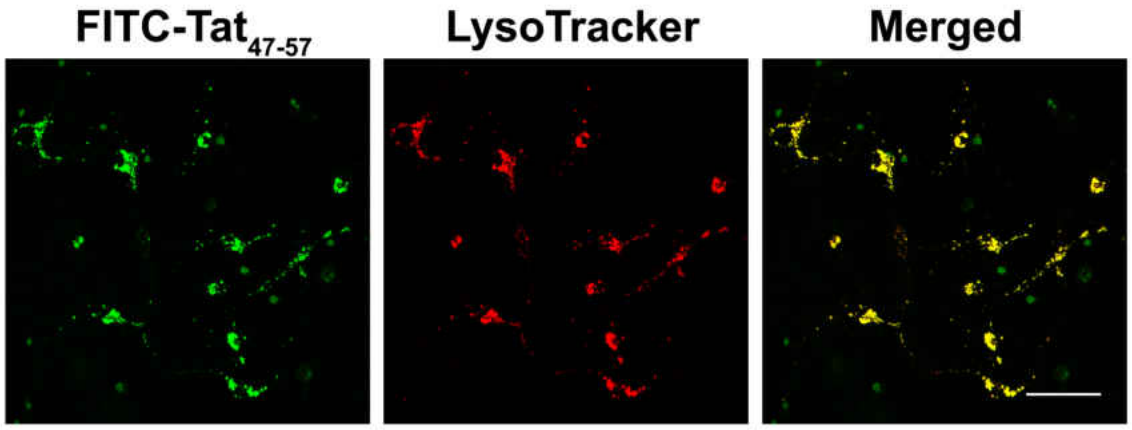

Figure 11. HIV-1 Tat was Internalized into Endolysosomes of Primary Cultured Neurons. Fluorescence (FITC) labeled Tat_{47-57} peptide (100 nM, green) co-localized with endolysosomes (LysoTracker). Bar = 50 μm.

Because alterations in the structure and function of endolysosomes have been implicated in the neuropathogenesis of a number of neurological disorders, we next determined the extent to which HIV-1 Tat affected endolysosome morphology. In living neurons, we identified endolysosomes with LysoTracker, and we found that treatment with HIV-1 Tat for 1 and 2 days increased significantly (both $p < 0.05$) the size of endolysosomes (Figure 12 A, B). Treatments with mutant Tat did not affect endolysosome morphology (data not shown). Using immunocytochemistry methods, we found that endosomes labeled with EEA1 antibody and lysosomes labeled with LAMP1 antibody were relatively

small and evenly distributed in neurons treated with PBS or mutant Tat, but were markedly enlarged and clumped together in HIV-1 Tat-treated neurons (Figure 11 A, middle and bottom). To determine if the enlarged endolysosomes expressed higher levels of their marker proteins, immunoblots were performed and we found that treatment of neurons with HIV-1 Tat for 1 or 2 days increased significantly protein levels of EEA1 ($p < 0.05$ at 2 days) and LAMP1 ($p < 0.01$ at 1 day; $p < 0.05$ at 2 days) (Figure 12 C, D)

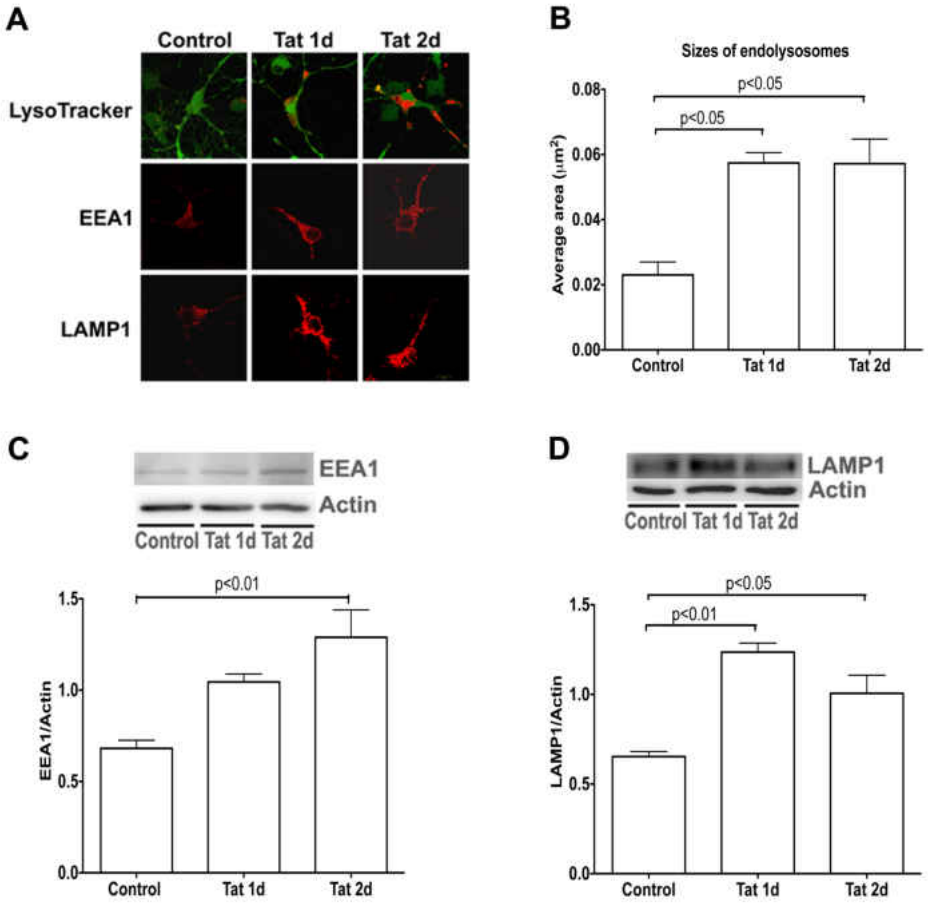

Figure 12. HIV-1 Tat Altered the Structure of Neuronal Endolysosomes. (A) Live cell imaging showed that HIV-1 Tat (100 nM) treatment increased the size of neuronal endolysosomes. LysoTracker (red) was used to identify endolysosomes and calcein AM (green) was used to stain live cells (bar = 10 μm, top panel). HIV-1 Tat enlarged endosomes as identified with EEA1 staining (bar = 10 μm, middle

panel). HIV-1 Tat enlarged lysosomes as identified with LAMP1 staining (bar = 10 μm, bottom panel). (B) Quantification of the top panel of Figure 3A showed that HIV-1 Tat (100 nM) treatment for 1 and 2 days increased significantly the size of neuronal endolysosomes. The sizes of endolysosomes were quantified with Image J software. $p < 0.05$. (C) HIV-1 Tat increased significantly protein levels of EEA1. $p < 0.01$. (D) HIV-1 Tat increased significantly the protein levels of LAMP1. $p < 0.05$; $p < 0.01$.

The observations that HIV-1 Tat altered endolysosome morphology led us to determine next the extent to which HIV-1 Tat affected endolysosome function. Because pH is critical for endolysosome function, we determined the extent to which HIV-1 Tat affected endolysosome pH using lysoSensor dye that permits ratio-metric assessment of pH changes in acidic organelles. We found that HIV-1 Tat, but not mutant Tat treatment, for 1 or 2 days elevated significantly (both $p < 0.001$) endolysosome pH in cultured hippocampal neurons (Figure 13). Because endolysosome pH affected endolysosome enzyme activity, we next determined the protein levels and activity of endolysosome enzymes as evaluations of endolysosome function. Treatment of neurons with HIV-1 Tat for 1 or 2 days increased significantly protein levels of the endolysosome enzymes acid phosphatase (Figure 14A, $p < 0.01$ at 1 day and $p < 0.05$ at 2 days), cathepsin B (Figure 14C, $p < 0.05$), and cathepsin D (Figure 14E, $p < 0.01$ at 1 day and $p < 0.05$ at 2 days). However, specific activity levels of acid phosphatase (Figure 14B), cathepsin B (Figure 14D), and cathepsin D (Figure 14F) were decreased significantly ($p < 0.001$) in HIV-1 Tat treated cultures.

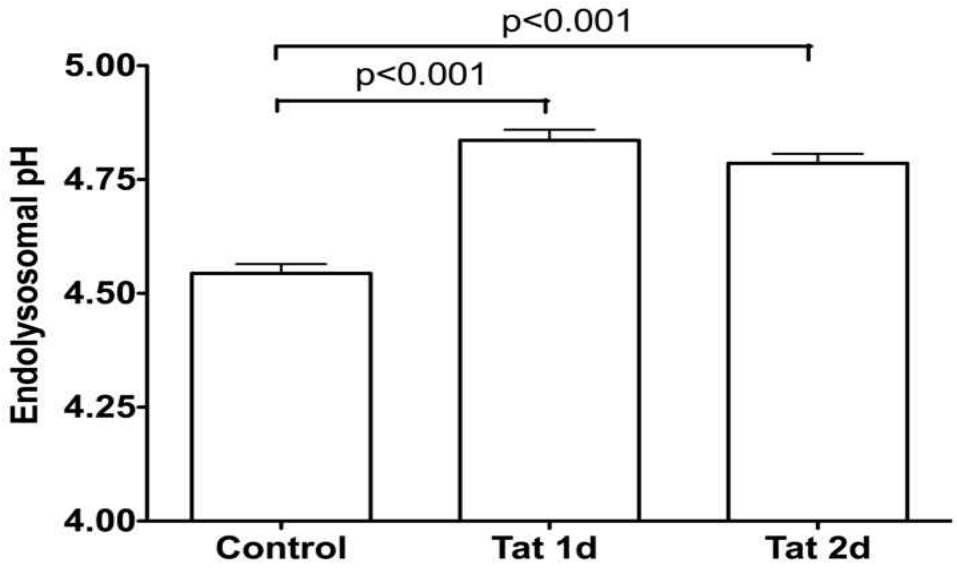

Figure 13. HIV-1 Tat Elevated Endolysosome pH in Primary Cultured Neurons. Endolysosome pH was measured ratio-metrically using LysoSensor dye. HIV-1 Tat (100 nM) treatment for 1 and 2 days elevated significantly endolysosome pH. $p < 0.001$.

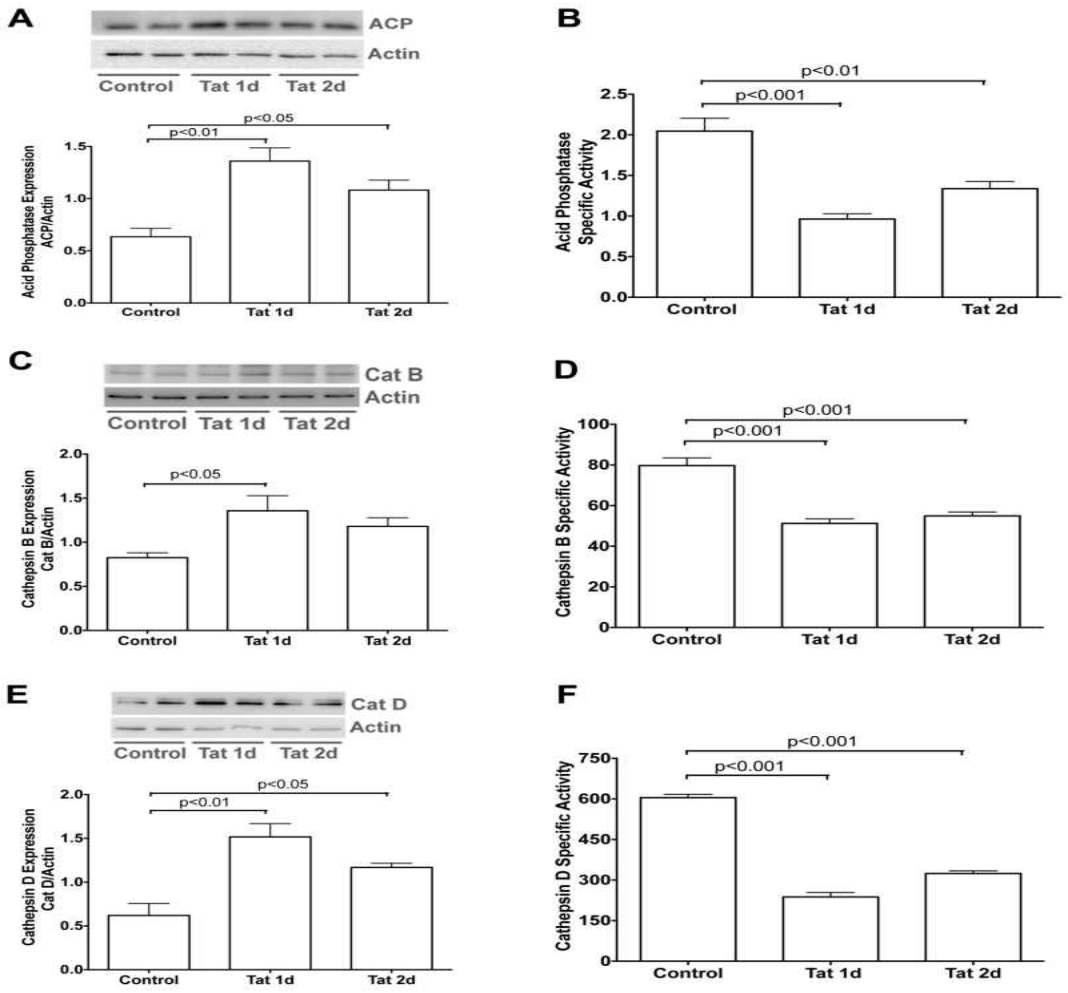

Figure 14. HIV-1 Tat Altered the Expression and Activity of Endolysosome Enzymes. (A. C. E) HIV-1 Tat (100 nM) increased protein levels of acid phosphatase (ACP), cathepsin B (Cat B), and cathepsin D (Cat D). Representative western blots and quantitative data from each of the enzymes were shown. Actin was used as a loading control. $p < 0.05$; $p < 0.01$. (B. D. F) HIV-1 Tat (100 nM) decreased significantly specific enzyme activity of acid phosphatase, cathepsin B, and cathepsin D. $p < 0.01$; $p < 0.001$.

Endolysosome dysfunction has been implicated in initiating stress pathways that lead to cellular dysfunction and death (Guicciardi et al., 2004; Kroemer and Jaattela, 2005; Kurz et al., 2008; Roberg and Ollinger, 1998; Turk et al., 2002). Here, we determined the extent to which HIV-1 Tat affected endolysosome membrane integrity using Lucifer Yellow dye (Yang et al., 1998).

We found that while control neurons displayed a discrete punctuated pattern of perinuclear fluorescent staining (Figure 15, left), HIV-1 Tat-treated neurons displayed an increased endolysosome membrane leakage as evidenced by diffuse fluorescent staining (Figure 15, right) in the cytoplasm.

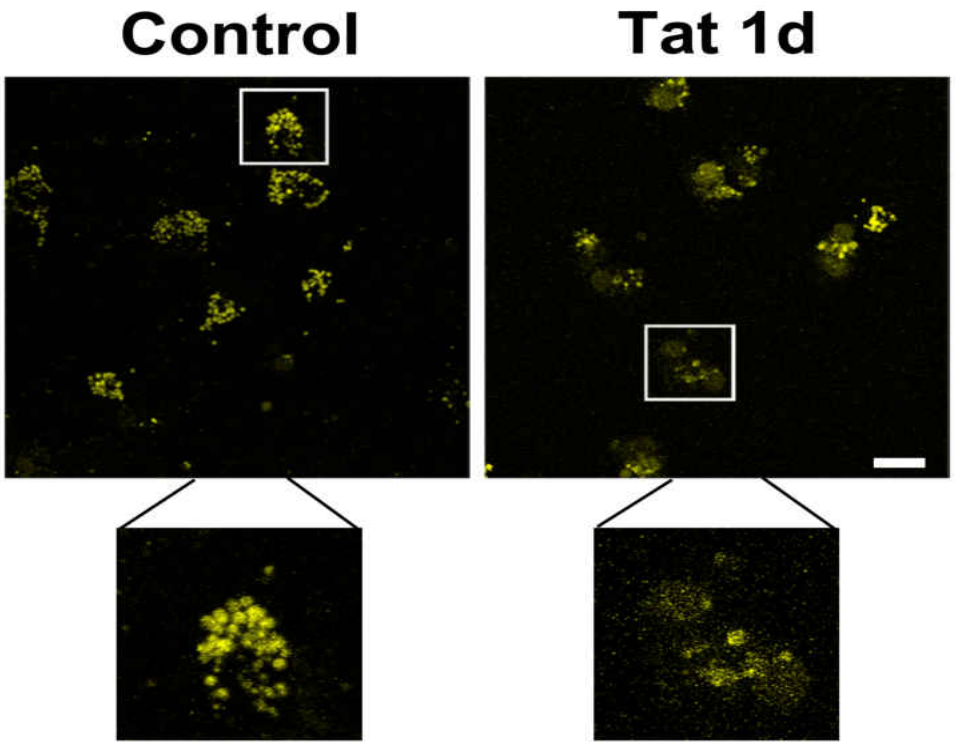

Figure 15. HIV-1 Tat Disrupted Endolysosome Membrane Integrity. Endolysosome membrane integrity was evaluated by measuring the leakage of Lucifer Yellow dye. Control neurons displayed a discrete punctuated fluorescent staining pattern in perinuclear regions with no fluorescence in cytoplasm (left panel), whereas neurons treated with HIV-1 Tat for 1 day displayed endolysosome membrane leakage as indicated by diffusion of fluorescence into cytoplasm (right panel), bar =10 μm.

Endolysosomes also function to control autophagy, a process important for normal physiological functions of neurons. Dysfunctions in autophagy have been implicated in the pathogenesis of a variety of neurodegenerative disorders

(Wong and Cuervo, 2010) including HAND (Alirezaei et al., 2008a; Alirezaei et al., 2008b; Spector and Zhou, 2008; Zhou et al., 2011; Zhou and Spector, 2008; Zhu et al., 2009). Based on findings that HIV-1 Tat disrupts autophagy in immune cells (Van Grol et al., 2010), we determined the extent to which HIV-1 Tat affected autophagy in primary cultured hippocampal neurons. Three markers for the formation of autophagosomes were used to evaluate the status of autophagy; microtubule-associated protein 1 light chain 3 (LC3) that regulates the initiation of autophagosomes, autophagy related gene-5 (Atg5) that regulates the elongation of autophagosomes, and P62 that inhibits the formation of autophagosomes. We found that HIV-1 Tat treatment decreased significantly protein levels of LC3 (Figure 16A, $p < 0.05$ at 2 day treatment) and Atg5 (Figure 16B, $p < 0.05$ at 1day and $p < 0.01$ at 2 day treatment), but increased significantly protein levels of P62 (Figure 16C, $p < 0.05$ at 1 and 2 day treatment).

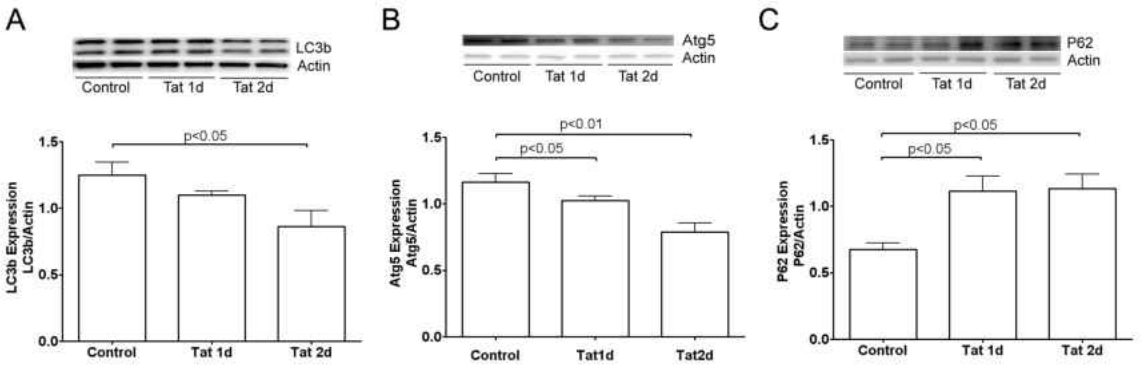

Figure 16. HIV-1 Tat Inhibited Macroautophagy. The formation of autophagosomes was estimated by measuring protein levels of LC3, Atg5, and p62. (A) HIV-1 Tat (100 nM) decreased significantly protein levels of LC3. $p < 0.05$. (B) HIV-1 Tat (100 nM) reduced significantly protein levels of Atg5. $p < 0.05$; $p < 0.01$. (C) HIV-1 Tat (100 nM) increased significantly protein levels of P62. Representative western blots and quantitative data from each of proteins were shown and actin was used as a loading control. $p < 0.05$.

Recently, increased amyloid beta (Aβ) deposition has been observed in aged HIV-1 infected individuals. Although the underlying mechanisms remains unclear, HIV-1 Tat, especially Tat_{1-86}, has been shown to increase neuronal Aβ generation (Aksenov et al., 2010; Giunta et al., 2009; Rempel and Pulliam, 2005). Here, we determined the extent to which a shorter form of Tat, Tat_{1-72}, affects Aβ generation in primary cultured neurons. Using an ELISA method, we demonstrated that HIV-1 Tat_{1-72} treatment at the concentration of 100 nM for 2 days increased significantly levels of both $Aβ_{1-40}$ and $Aβ_{1-42}$ (Figure 17 A,B), but treatment with HIV-1 Tat_{1-72} for 1 day did not increase neuronal Aβ generation (Figure 17A,B).

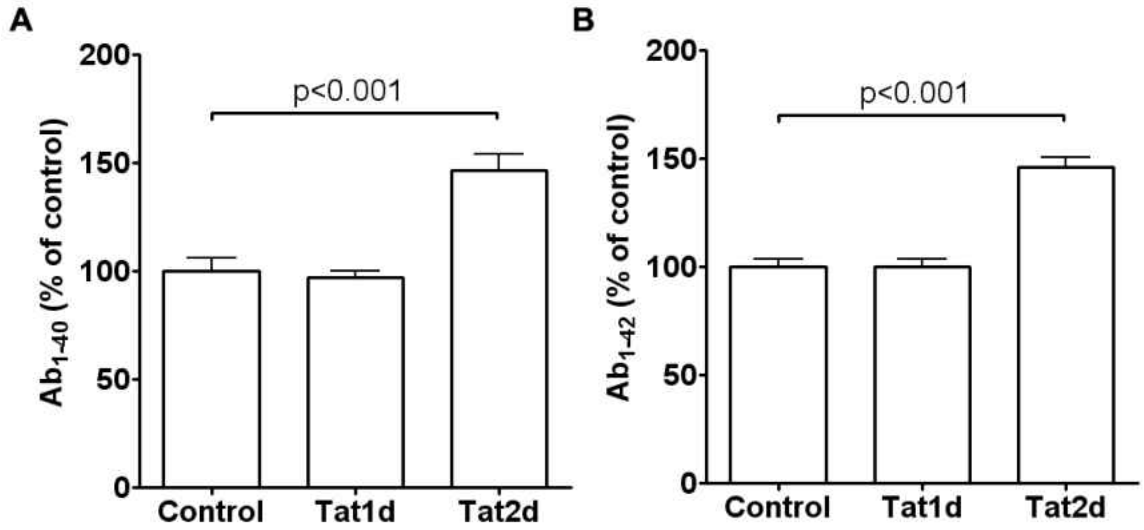

Figure 17. HIV-1 Tat Increased Neuronal Amyloid Beta Generation. (A) HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased significantly levels of $Aβ_{1-40}$, when compared with control groups (n=5; $p < 0.001$). (B) HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased significantly levels of $Aβ_{1-42}$, when compared with control groups (n=5; $p < 0.001$).

Aβ is the result of proteolytic processing of its precursor protein AβPP by β-secretase and γ-secretase, and it is known that Aβ is produced mainly in the endolysosome system. Importantly, it is shown that there is increased

intraneuronal Aβ production, especially in endolysosomes, in HIV-1 infected individuals (Achim et al., 2009). Thus, we determined the extent to which HIV-1 Tat affected endolysosome accumulation of AβPP and Aβ in primary cultured neurons with double fluorescent staining. We found that there was some accumulation of AβPP (Figure 18A) and Aβ as identified with 4G8 antibodies (Figure 18C) in neuronal endosomes as identified with EEA1 antibodies, and there was no accumulation of either AβPP (Figure 18B) or Aβ (Figure 18D) in neuronal lysosomes as identified with LAMP-1 antibodies. However, HIV-1 Tat_{1-72} (100 nM) treatment for 2 day increased dramatically endosome and lysosome accumulation of both AβPP and Aβ (Figure 18).

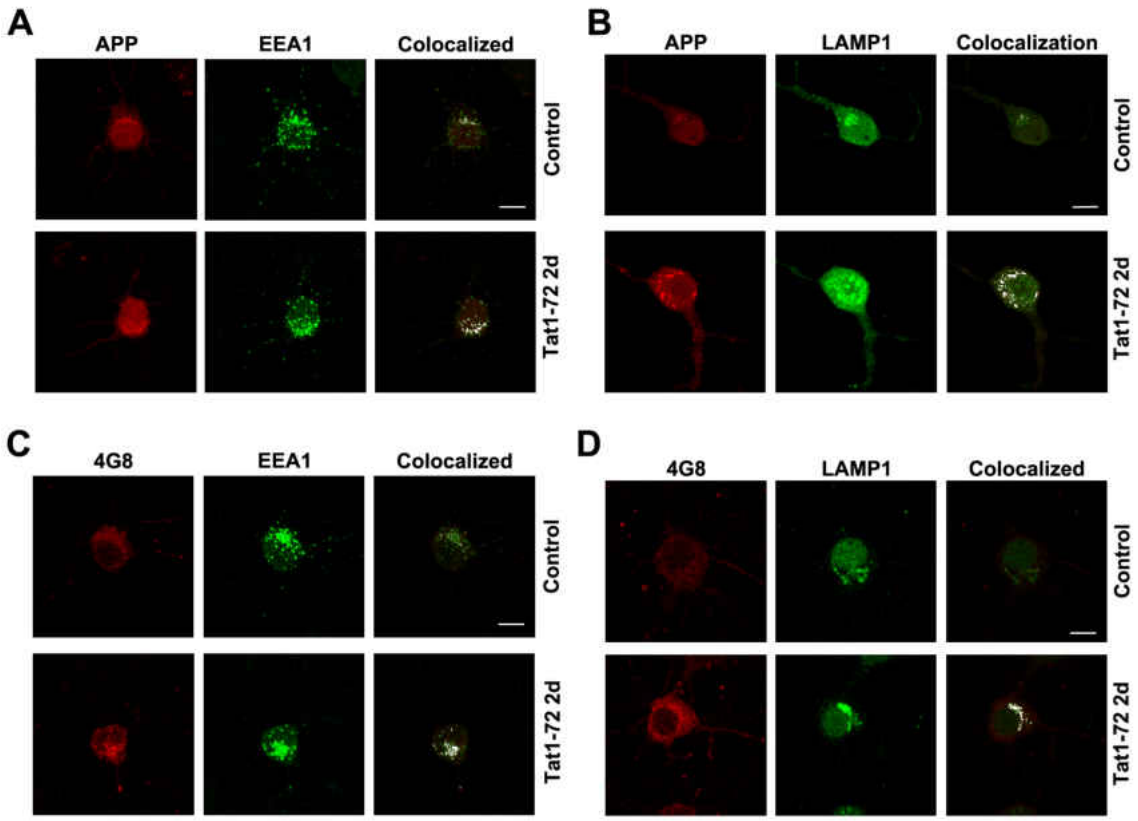

Figure 18. HIV-1 Tat Increased Endolysosome Accumulation of AβPP and Amyloid Beta. (A) Although there was some extent co-localization of AβPP with endosomes (EEA1) in control neurons, HIV-1 Tat_{1-72} treatment (100 nM) for 2

days increased markedly the co-colocalization of AβPP with endosomes (EEA1). Bar = 10 μm. (B) There was little co-localization of AβPP with lysosomes (LAMP1) in control neurons, but HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased markedly the co-colocalization of AβPP with lysosomes (LAMP1). Bar = 10 μm. (C) Although there was some extent co-localization of Aβ (4G8) with endosomes (EEA1) in control neurons, HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased markedly the co-colocalization of AβPP with endosomes (EEA1). Bar = 10 μm. (D) There was little co-localization of Aβ (4G8) with lysosomes (LAMP1) in control neurons, but HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased markedly the co-colocalization of AβPP with lysosomes (LAMP1). Bar = 10 μm.

It is known that the rate-limiting enzyme in Aβ production, beta-site APP-cleaving enzyme 1 (BACE-1), is present in endolysosomes and its activity is pH dependent (Rajendran et al., 2008; Shimizu et al., 2008; Vassar et al., 1999). Given our observations that HIV-1 Tat altered the structure of endolysosomes and elevates endolysosome pH, it is likely that HIV-1 Tat increases Aβ production by affecting BACE-1. Accordingly, we determined the extent to which HIV-1 Tat treatment affected levels of expression, enzyme activity, and intracellular distribution of BACE-1 in primary cultured neurons. We found that HIV-1 Tat treatment increased protein levels of BACE-1 (Figure 19A), and enhanced significantly enzyme activity of BACE-1 (Figure 19B). When analyzing the intracellular distribution of BACE-1, we found that there were some levels of accumulation of BACE-1 in endosomes as identified with EEA1 antibodies, but no accumulation BACE-1 in lysosomes as identified with LAMP1 antibody in control neurons. However, HIV-1 Tat treatment for 2 days increased markedly accumulation of BACE-1 in both endosomes (Figure 19C) and lysosomes (Figure 19D).

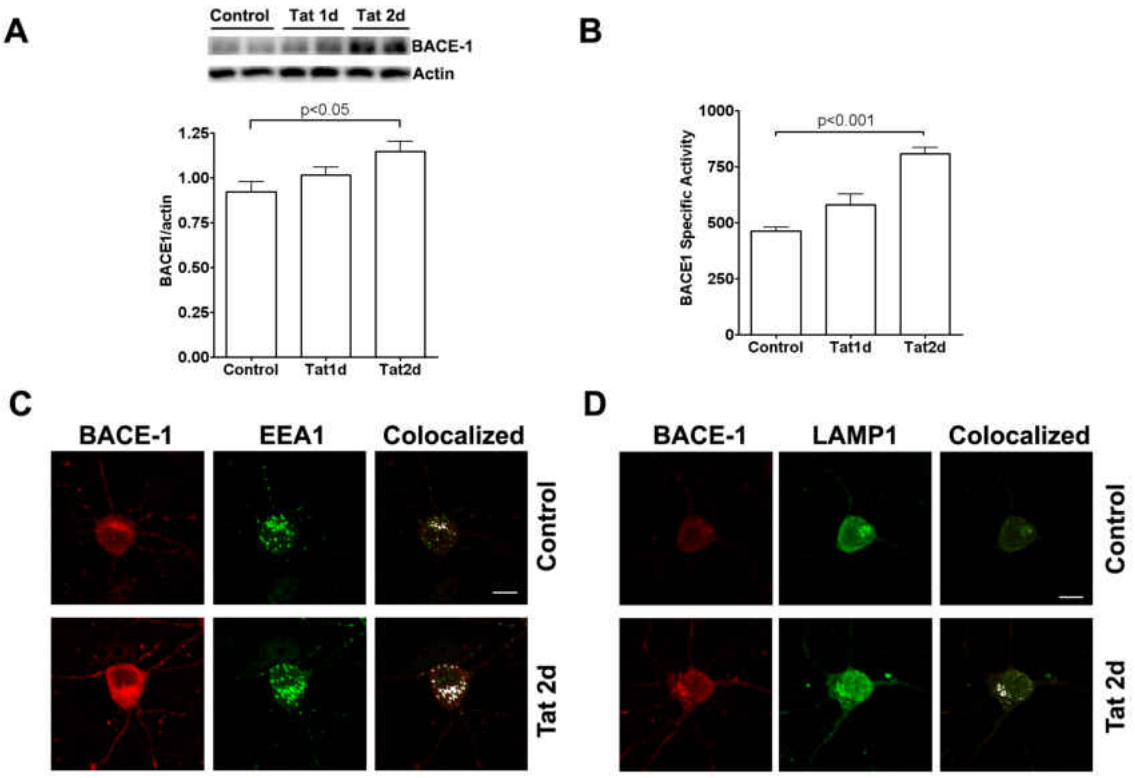

Figure 19. HIV-1 Tat Increased Endolysosome Accumulation of Beta Amyloid Cleavage Enzyme 1 and Enhanced Beta Amyloid Cleavage Enzyme 1 Activity. (A) HIV-1 Tat1-72 treatment (100 nM) for 2 days increased significantly protein levels of BACE-1. (n=6, $p<0.05$). (B) HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased significantly specific activity of BACE-1. (n=8; $p<0.001$). (C) There was some extent co-localization of BACE-1 with endosomes (EEA1) in control neurons, and HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased markedly the co-colocalization of BACE-1 with endosomes (EEA1). Bar = 10 μm. (D) There was little co-localization of BACE-1 with lysosomes (LAMP1) in control neurons, but HIV-1 Tat_{1-72} treatment (100 nM) for 2 days increased markedly the co-colocalization of BACE-1 with lysosomes (LAMP1). Bar = 10 μm.

5.4. Discussion

Combined highly active antiretroviral therapeutic drugs have increased dramatically the length of time people are now living with AIDS. However, this increased life span is accompanied by increased prevalence of HAND that ranges up to 50% of people with HIV-1 infection (Ellis et al., 2010; Heaton et al.,

2010). The underlying mechanisms for HAND pathogenesis are not fully understood but one mechanism that appears to be important yet relatively understudied is the involvement of endolysosomes. Disturbed endolysosomes have been noted in brains of HIV-1 infected individuals (Gelman et al., 2005; Spector and Zhou, 2008; Zhou and Spector, 2008), but the mechanisms for these pathological observations are not known. Because neurons are long-lived post-mitotic cells with extreme polarity they possess an elaborate endolysosome system containing hydrolases that degrade macromolecules, high concentrations of readily releasable calcium (Christensen et al., 2002; Moreno and Docampo, 2009; Patel and Docampo, 2010), and high concentrations of potentially redox-active iron (Brun and Brunk, 1970; Kidane et al., 2006). When dysfunctional, endolysosomes can contribute to altered calcium homeostasis (Korkotian et al., 1999; Lloyd-Evans et al., 2008; Pelled et al., 2005) and increased oxidative stress (Pivtoraiko et al., 2009). Disruptions in endolysosome functions perturb numerous cellular functions and can ultimately result in the initiation of cell death pathways (Kroemer and Jaattela, 2005; Kurz et al., 2008). Therefore, it is potentially significant in terms of furthering our understanding of the pathogenesis of HAND that we found that HIV-1 Tat enlarged endolysosomes, elevated endolysosome pH, decreased specific activities of endolysosome enzymes, disrupted endolysosome membrane integrity, and inhibited autophagy; all of which occurred prior to significant increases in HIV-1 Tat-induced neuronal cell death. Thus, the altered structure and function of endolysosomes could underlie, at least in part, the pathogenesis of HAND.

HIV-1 Tat protein continues to be implicated in the pathogenesis of HAND, in part, because there is significant neuronal dysfunction even though neurons are not infected by HIV-1 virus (Merino et al., 2011; Nuovo et al., 1994). HIV-Tat has been shown to activate NMDA receptors (Eugenin et al., 2003; Haughey et al., 2001; Nath et al., 2000), alter calcium homeostasis (Bonavia et al., 2001; Haughey et al., 1999; Kruman et al., 1998), and increase oxidative stress (Aksenov et al., 2001; Kruman et al., 1998; Perry et al., 2005). HIV-1 Tat is actively secreted by infected glial cells and following binding to neuronal cell surface receptors it enters the endolysosome systems following receptor-mediated endocytosis (Liu et al., 2000; Mann and Frankel, 1991). Although we did not determine the extent to which previously identified receptors mediate the endocytosis of HIV-1 Tat in neurons including CD26 (Gutheil et al., 1994), CXCR4 (Xiao et al., 2000), heparin sulfate proteoglycans (Tyagi et al., 2001), and the low density lipoprotein receptor-related protein (Deshmane et al., 2011; Liu et al., 2000) we did observe the presence of HIV-1 Tat in neuronal endolysosomes.

Because HIV-1 Tat can accumulate in neuronal endolysosomes, the observed changes in the morphology and function of endolysosomes could result from directly disruptive effects of the HIV-1 Tat protein. Central to the observed changes might be the ability of HIV-1 Tat to elevate endolysosome pH. Although the underlying mechanisms are unknown, the arginine rich domain of HIV-1 Tat between amino acid residues 49 and 57 could be responsible for HIV-1 Tat induced elevation of endolysosome pH because a series of other arginine rich

peptides including penetratin, an amino acid domain from the Antennapedia protein (sequence 43-58) of Drosophila, a flock house virus coat peptide (sequence 35-49), and oligoarginines (R9) all have the ability to elevate endolysosome pH (unpublished observations). It has been shown that most of these arginine rich peptides have the ability to escape endolysosomes using the high proton gradient (Drin et al., 2003; Fischer et al., 2004; Henriques et al., 2006; Magzoub et al., 2005; Potocky et al., 2003) and here we postulate that an unidentified proton-dependent peptide transporter might be present on endolysosome membranes. Such a peptide transporter could transport arginine rich peptides such as HIV-1 Tat using the arginine rich domain as a signal, and during the transporting process protons leak out and endolysosome pH is elevated. Low pH is important for the degradation of internalized materials, the trafficking and fusion of endolysosomes, and the formation of autophagosomes (Marshansky and Futai, 2008; Ravikumar et al., 2010; Williamson et al., 2010). Therefore, the elevation of endolysosome pH that we observed could result in alterations in the digestive capability of endolysosomes as evidenced by decreased specific activity of three different endolysosome enzymes, increased accumulation of internalized material thus altering the structure and size of endolysosomes, and inhibition of autophagy thereby exaggerating neuronal injury and degeneration (Wong and Cuervo, 2010). There exists three types of autophagy in cells; macroautophagy, microautophagy and chaperon-mediated autophagy. As the best-studied type of autophagy, macroautophagy includes three stages including autophagosome membrane origination, autophagosome

formation, and autolysosome formation. Accordingly, we focused our studies of the effects of HIV-1 Tat on macroautophagy using three markers; LC3 that mediates the initiation of (Winslow and Rubinsztein, 2008), Atg5 that drives the elongation of (Mizushima, 2007), and p62 that inhibits the formation of autophagosomes (Bjorkoy et al., 2005; Ichimura and Komatsu, 2010; Pankiv et al., 2007). Thus, future experiments are needed to address the extent to which HIV-1 Tat affects other stages of macroautophagy as well as other subtypes of autophagy.

It is still unclear how HIV-1 Tat elevates endolysosome pH. One possibility is that HIV-1 Tat increases pH by directly disrupting the membrane integrity of endolysosomes and this hypothesis is consistent with our present observations of increased leakage of Lucifer Yellow dye into cytosol. Consistent with these findings are previous reports that low pH induces the exposure of a very conserved tryptophan residue and allows the insertion of HIV-1 Tat into endolysosome membranes (Yezid et al., 2009). Furthermore, disrupted endolysosome membrane integrity *per se* could lead to neuronal dysfunction and ultimately cell death because increased endolysosome membrane permeability occurs in several models of apoptosis (Guicciardi et al., 2004; Kroemer and Jaattela, 2005; Kurz et al., 2008; Roberg and Ollinger, 1998; Turk et al., 2002) and is an early event in the apoptotic cascade that precedes destabilization of mitochondria and caspase activation (Kroemer and Jaattela, 2005; Kurz et al., 2008).

Increased life span of HIV-1 infected individuals is accompanied by increased prevalence of HAND (Ellis et al., 2010; Heaton et al., 2010) and increased incidence of AD-like pathology, such as increased neuronal Aβ deposition (Achim et al., 2009). Recent studies indicate that one of the viral proteins, HIV-1 Tat, which has continued to be implicated in the pathogenesis of HAND (Merino et al., 2011; Nath et al., 1996; Nuovo et al., 1994), also contributes to increased neuronal Aβ generation (Aksenov et al., 2010; Giunta et al., 2009; Rempel and Pulliam, 2005). However, the underlying mechanisms remain unclear. The principle finding of the present study is that HIV-1 Tat-induced incretion in neuronal Aβ generation is accompanied by altered endolysosome structure and function, increased endolysosome accumulation of AβPP, Aβ and BACE-1, and enhanced BACE-1 activity. Our findings suggest that HIV-1 Tat-induced endolysosome dysfunction underlies the development of AD-like pathology in HIV-1 infected individuals.

It is well known that ones of the earliest pathological features of AD are altered morphological and functional features of endolysosomes (Boland et al., 2008; Tate and Mathews, 2006), as evidenced by the findings that endosome enlargement was apparent in brains of AD patients and non-demented patients with early signs of AD, in Down's syndrome individuals, and in patients bearing the ApoE4 allele (Arriagada et al., 2007; Cataldo et al., 2004), and that endosome enlargement largely precedes extracellular deposition of Aβ in brain (Cataldo et al., 2000). Recent studies also suggest strongly that pathological changes in endolysosomes contribute to Aβ production, a pathological hallmark

of AD, as evidenced by the following findings; AβPP and its cleavage products are present in clathrin-coated vesicles that are part of the endocytic pathway (Ferreira et al., 1993; Harris and Milton, 2010). Aβ production is decreased in cultured cells that were stably transfected with an AβPP construct where the C-terminal endocytic targeting signal was removed (Perez et al., 1999; Soriano et al., 1999); Aβ production is decreased in cells transfected with dominant negative dynamin, which prevents endocytosis (Chyung and Selkoe, 2003); BACE-1, a key enzyme for amyloidogenesis, is localized in endosomes and its activity is pH dependent (Rajendran et al., 2008; Shimizu et al., 2008; Vassar et al., 1999); Aβ is accumulated in endolysosomes of neurons from AD brain (Cataldo et al., 2004).

Disturbed endolysosomes have been noted in brain of HIV-1 infected individuals (Gelman et al., 2005; Spector and Zhou, 2008; Zhou and Spector, 2008), and a recent finding that increased Aβ accumulation in neuronal endolysosomes in HIV-1 infected individuals (Achim et al., 2009) suggest that endolysosome dysfunction contributes to increased Aβ generation in HIV-1 infected patients. Consistent with this notion, we demonstrated that HIV-1 Tat disturbed endolysosome structure and function, promoted neuronal Aβ production, and increased endolysosome accumulation of both AβPP and Aβ. Although detailed molecular underlying mechanisms were not explicitly explored, we propose that HIV-1 Tat increased neuronal Aβ generation in two-ways. First, HIV-1 Tat promotes AβPP internalization. As mentioned earlier, HIV-1 Tat enters neuron endolysosomes via receptor-mediated endocytosis with the assistance of

LRP-1 (Deshmane et al., 2011; Liu et al., 2000) and it is known that LRP-1 interacts directly with AβPP (Klug et al., 2011; Waldron et al., 2008; Waldron et al., 2006). Thus, the binding of HIV-1 Tat with LRP-1 and subsequent receptor-mediated endocytosis promotes AβPP internalization. Our observation that HIV-1 Tat increases endolysosome accumulation of AβPP seems to support this premise. Second, BACE-1, the rate limiting enzyme in the production of Aβ, is present in endosomes and its activity is pH-dependent with an optimal pH around 5 (Rajendran et al., 2008; Shimizu et al., 2008; Vassar et al., 1999), the observed elevation of endolysosome pH could be responsible for enhanced BACE-1 enzyme activity and increased Aβ production. In addition, BACE-1 is degraded in lysosomes under more acidic conditions (pH < 4) (Koh et al., 2005). Thus, the observed elevation of endolysosome pH could lead to decreased degradation of BACE-1 and increased accumulation of BACE-1 in endolysosomes, which also results in increased Aβ production. In addition, our observations that HIV-1 Tat-induced endolysosome dysfunction occurs prior to increased Aβ production suggest that HIV-1 Tat disrupts endolysosome function and subsequently affects Aβ production (Jin et al., 2004). Collectively, elevated endolyosome pH could contribute to HIV-1 Tat-induced increases in neuronal Aβ generation.

In summary, our finding that HIV-1 Tat disturbed the structure and function of endolysosomes in primary cultured neurons prior to any significant increase in HIV-1 Tat-induced neurotoxicity suggests that the effects of HIV-1 Tat on endolysosomes may cause considerable neuronal dysfunction. Furthermore, we demonstrated that HIV-1 Tat enhanced production of Aβ, increased

endolysosome accumulation of AβPP, Aβ and BACE-1, and enhanced BACE-1 activity in primary cultured hippocampal neurons. Such findings suggest strongly that altered structure and function of endolysosomes plays an important role in HIV-1 Tat-induced neuronal Aβ generation and contribute directly to the development of AD-like pathology in HIV-1 infected individuals.

Further elucidation of the involvement of endolysosomes in HAND might lead to the design of novel therapeutic agents capable of blocking HIV-1 Tat endocytosis and improving endolysosome function.

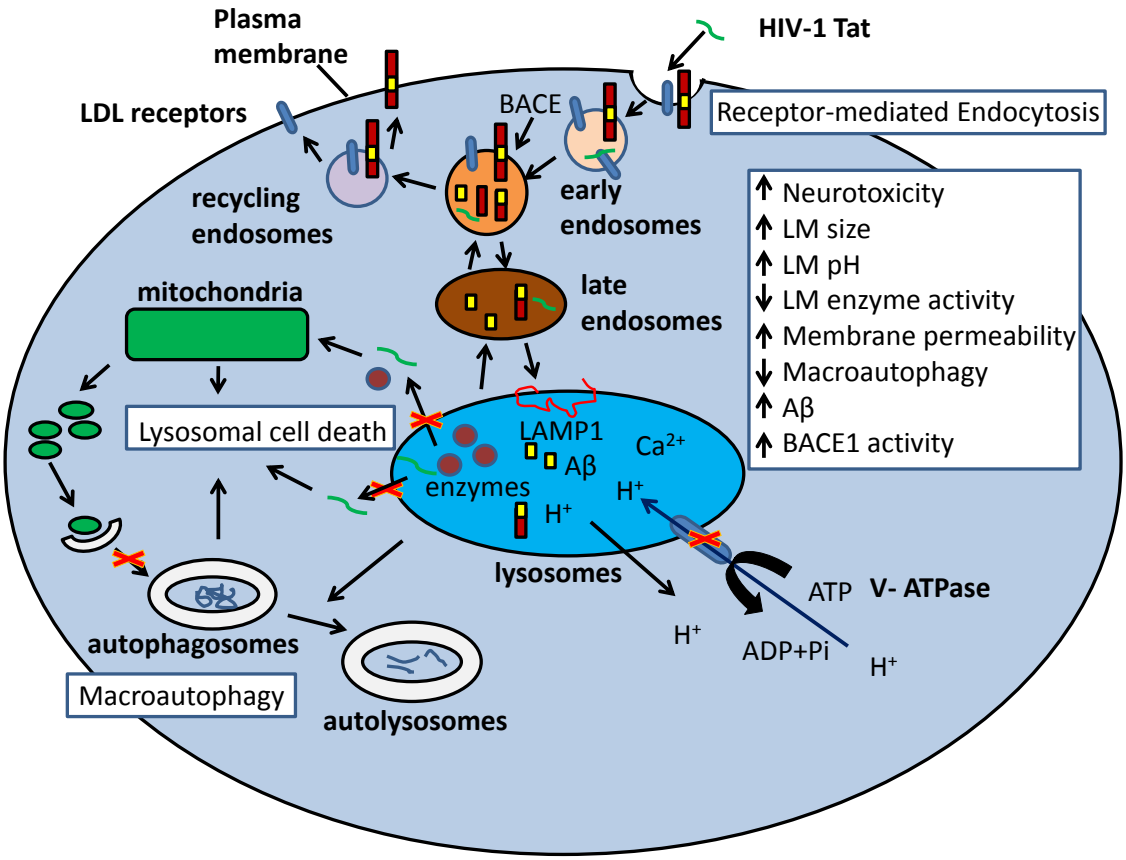

Figure 20. Model Summarizing Involvement of Endolysosomes in HIV-1 Tat-Induced Toxicity and Amyloid Beta Generation in Primary Cultured Neurons. Prior to statistically significant changes in HIV-1 Tat-induced neuronal cell death (48 hours), as early as 24 hours after HIV-1 Tat treatment, HIV-1 Tat

accumulates in endolysosomes, increases endolysosome sizes, raises endolysosome pH, decreases specific activities of endolysosome enzymes, disruptes endolysosome membrane integrity and inhibits the formation of autophagosomes. In addition, following endolysosome dysfunction, HIV-1 Tat elevates Aβ generation, increases endolysosome accumulation of AβPP, Aβ and BACE-1 and enhances BACE-1 activity.

CHAPTER VI

ACIDIC STORE-OPERATED CALCIUM ENTRY IN PRIMARY CULTURED NEURONS

6.1. Introduction

Calcium is a highly versatile intracellular signaling molecule that plays a pivotal role in regulating the physiology and biochemistry of cells (Berridge, 1998; Berridge et al., 2000). In the central nervous system, calcium is essential for neurotransmitter release, neuronal excitability, synaptic plasticity and neuronal viability (Berridge, 1998; Berridge et al., 2003). Calcium signals are tightly regulated temporally and spatially through a coordinated interplay between calcium release from the intracellular stores and calcium influx across the plasma membrane. One such coordinated interplay is store-operated calcium entry (SOCE), in which depleting calcium in endoplasmic reticulum (ER) activates store-operated channels and triggers calcium entry from extracellular space. The machinery of SOCE remained unidentified for many years until the disclosure of two pivotal molecular components of SOCE following recent RNAi screening studies. STIM (stromal interacting molecule) functions as calcium sensor in the ER (Liou et al., 2005; Roos et al., 2005), and Orai proteins comprise the CRAC channel pore forming subunit (Prakriya et al., 2006; Vig et al., 2006; Zhang et al., 2006b). Depletion of ER calcium results in STIM oligomerization and

translocation to ER junctions close to the plasma membrane, and this STIM translocation induces Orai channels and/or TRP channels to cluster in the adjacent plasma membrane and allow calcium entry (Cahalan, 2009; Deng et al., 2009; Putney, 2009; Wang et al., 2009a).

Over the last 15 years, endolysosome compartments have been recognized as intracellular calcium stores that contain readily releasable pools of calcium. Because of the low pH in their lumen, these endolysosome calcium stores are often referred to as “acidic calcium stores”. Acidic calcium stores have a high concentration of calcium ranging from 400 - 600 μM (Christensen et al., 2002). This high concentration of calcium is maintained by the pH gradient across these acidic organelles, where vacuolar H^+-ATPase pumps H^+ into the lumen and drives Ca^{2+} uptake by Ca^{2+}/H^+ exchanger (Moreno and Docampo, 2009; Patel and Docampo, 2010). Like other intracellular calcium stores, calcium within acidic calcium stores is readily releasable. Nicotinic acid adenine dinucleotide phosphate (NAADP) is able to mobilize calcium release specifically from acidic calcium stores in a variety of cell types including neurons (Brailoiu et al., 2009b; Churchill et al., 2002; Pandey et al., 2009; Pitt et al., 2010; Thai et al., 2009; Tugba Durlu-Kandilci et al., 2010; Zhang et al., 2006a). Subsequent studies demonstrated that two pore channels (TPCs) and possible members of the TRP channel family like TRPM2 mediate NAADP-evoked calcium release from acidic calcium stores (Brailoiu et al., 2009a; Brailoiu et al., 2009b; Calcraft et al., 2009; Lange et al., 2009; Ruas et al., 2010; Schieder et al., 2010; Zhang et al., 2009; Zhu et al., 2010; Zong et al., 2009). In addition to NAADP, elevation of

endolysosome pH with a selective vacuolar H^+-ATPase inhibitor bafilomycin or alkaline lysosomotrophic agents like NH_4Cl can also induce calcium release from acidic calcium stores (Camacho et al., 2008; Christensen et al., 2002; Machado et al., 2009), and TRPM2 channels might be responsible for calcium release from acidic stores induced by elevation of endolysosome pH (Starkus et al., 2010). Because NAADP has been shown to elevate luminal pH of acidic calcium stores (Morgan and Galione, 2007a; Morgan and Galione, 2007b), it is possible that NAADP could also induce calcium release from acidic calcium stores, in part, by elevating endolysosome pH.

Calcium released from acidic calcium stores appears to be relatively small and highly localized. However, given the dynamic properties of these acidic calcium stores, the released calcium from acidic stores could result in complex communications with other intracellular calcium stores and plasma membranes. Currently, three models of acidic calcium store-induced calcium signaling mechanisms have been implicated. First, acidic calcium stores have the ability to communicate with ER calcium stores. In many types of cells, localized calcium release from acidic stores can trigger larger calcium release from ER (Calcraft et al., 2009; Cancela et al., 1999; Macgregor et al., 2007). Calcium released from acidic stores might enhance calcium loading of ER (Macgregor et al., 2007) and/or trigger calcium-induced calcium release from ER (Calcraft et al., 2009; Ruas et al., 2010). Second, locally released calcium from a subgroup of acidic calcium stores could communicate with other acidic calcium stores. Regulation of local cytoplasmic calcium and luminal calcium and/or pH of endolysosome

compartments can affect vesicular fusion of late endosomes and lysosomes (Galione et al., 2009; Ruas et al., 2010). Third, acidic calcium stores have the ability to communicate with plasma membrane calcium channels. In many types of cells including neurons, calcium released from acidic calcium stores depolarizes plasma membrane, evokes calcium-dependent currents, and stimulates calcium influx across the plasma membrane (Beck et al., 2006; Brailoiu et al., 2009b; Galione, 2011; Moccia et al., 2006a; Moccia et al., 2003; Moccia et al., 2006b; Naylor et al., 2009; Santella et al., 2000). To date, little is known about how acidic calcium stores communicate with plasma membrane calcium channels, and understanding such underlying mechanisms is the main objective of this aspect of our research work.

We demonstrated, in primary cultured neurons, that calcium released from acidic calcium stores induced by either selective inhibition of vacuolar H^+-ATPase with bafilomycin or selective disruption of endolysosome membranes with GPN triggered calcium influx across the plasma membrane; a phenomenon we termed 'acidic store-operated calcium entry (aSOCE)'. Further studies indicated that lysosome exocytotic insertion of N-type calcium channels into the plasma membrane was responsible for aSOCE.

6.2. Methods

6.2.1. Primary neuronal culture: Details were described in Chapter 5.2.1.

6.2.2. Intracellular calcium concentration measurement: Intracellular Ca^{2+} was determined using the Ca^{2+}-specific fluorescent probe fura-2/AM (Invitrogen) as described previously (Haughey et al., 1999). Neurons were incubated with 2 μM fura-2/AM for 40 min at 37°C, washed with different calcium buffers (as listed below) to remove extracellular fura-2, and incubated at 37°C for another 10 min to allow complete de-esterfication of fura-2. Neurons were excited at 340 and 380 nm, and emission was recorded at 510 nm with our filter-based calcium imaging system (Zeiss, Germany). Images were acquired every 2 s and at this acquisition rate we were able to measure baseline as well as peak increases in levels of free intracellular calcium. The ratio of F340/F380 was used as a measurement of intracellular calcium levels. Locke's buffer contained 154 mM NaCl, 3.6 mM $NaHCO_3$, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and 10 mM D-glucose, pH=7.4. Calcium free buffer contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.2 mM EGTA and 10 mM HEPES, pH=7.4. Nominally calcium-free buffer contained 154 mM NaCl, 3.6 mM $NaHCO_3$, 5.6 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES and 10 mM D-glucose, pH=7.4.

6.2.3. Surface immunostaining: Treated neurons as required were incubated with primary antibodies (30 min, 4 °C) including anti-Cav2.2 (N-type VOCC) (1:100, rabbit polyclonal, Millipore) or anti-LAMP1 (1:100, rabbit polyclonal, Sigma) primary antibodies, washed with PBS twice, fixed with paraformaldehyde (4%, 5 min), and incubated for 40 minutes with fluorescently labeled Alexa 546-conjugated goat anti-rabbit secondary antibody (Invitrogen) at

room temperature in the absence of ambient light. Images were taken under a confocal microscopy (Olympus).

6.2.4. Biotinylation of neuronal surface proteins: Biotinylation of neuronal surface proteins was conducted using methods described before (Cayouette et al., 2004) with slight modification. Briefly, primary cultured neurons grown in 60-mm dishes were washed with nominal calcium buffer 3 times and treated with bafilomycin (100 nM), GPN (2 pM) or thapsigargin (2 μM) for 10 min with DMSO treatment serving as the control. Following the treatments, $CaCl_2$ at a final concentration of 1 mM was added. 10 min later, neurons were incubated with Sulfo-NHS-SS-Biotin (0.5 mg/ml, 4°C, 30 min, Pierce) and washed with a quenching buffer (20 mM Tris and 120 mM NaCl, pH=7.4) for 3 times to remove un-reacted biotin. After washing with ice-cold PBS twice, neurons were lysed with 200 μl RIPA buffer containing proteinase inhibitor cocktail (Sigma) followed by sonication. After centrifugation (100,000×g for 2 min), supernatants were collected, and protein concentrations were determined with a DC protein assay (BioRad). Equal amounts of protein were transferred to new sets of tubes, and 100 μl streptavidin-agarose beads (Pierce) were added and incubated for 16 hours at 4°C. The biotin-streptavidin-agarose complexes were washed with RIPA buffer 5 times and collected by centrifugation (14,000×g, 30 sec, 4°C). The biotin-streptavidin-agarose complexes were suspended in 1x Laemmli buffer (containing 50 mM DTT and 5% beta-mecapthoethonal) and heated at 95°C for 5 min before being taken for analysis with immunoblotting.

6.2.5. Immunoprecipitation: Neurons were lysed with 200 µl of ice-cold RIPA buffer containing proteinase inhibitors (Sigma). After centrifugation (14,000 × g for 10 min at 4°C), supernatants were collected, and protein concentrations were determined with a DC protein assay (Bio-Rad). After pre-clearing with protein A/G, protein samples (400 µg) were incubated (4°C overnight) with 5 µl of antibodies against target proteins including anti-Cav2.2 (N-type VOCC, mouse monoclonal, Santa Cruz) or anti-LAMP1 (rabbit polyclonal, Sigma) with irrelevant antibodies serving as controls. The immune complexes were incubated (4°C, 4 hours) with 100 µl of protein A/G (Santa Cruz) while being rotated on a belly dancer platform. The immunoprecipitated complexes were washed with RIPA buffer 4 times, collected by centrifugation at 14,000 × g for 30 sec at 4°C, resuspended in 120 µl 1x Laemmli buffer containing 50 mM DTT and 5% beta-ME, and heated at 95°C for 10 min before being analyzed with immunoblotting.

6.2.6. siRNA Transfection: Targeted proteins were knocked down with specific siRNAs at a final concentration of 60 nM (invitrogen); negative control siRNAs (Invitrogen) were used as controls. Before siRNA transfection, fresh neuronal culture media was added to 60 mm dishes 10 days after plating. The transfection cocktail containing 300 µl of transfection buffer (SignaGen), 12 µl of siRNA stock (15 µM) for each target protein, and 9 µl of GenMute^{TM} reagent was added drop wise to each dish. After incubation in a CO_2 incubator for 5 hours, the transfection media was replaced with fresh neurobasal media and cells were incubated for 48 hours before further experiments. Knockdown efficiency for each targeted protein was measured by immunobloting.

6.2.7. Immunoblotting: Details were described in Chapter 5.2.5. Equal amounts of proteins (10 μg) or equal volumes of samples (15 μl, for biotinylated or immunoprecipitated protein sample) were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). The primary antibodies used were anti-Cav2.2 (1: 200, rabbit polyclonal, Millipore), anti-Cav2.2 (1:500, mouse monoclonal, Santa Cruz), anti-LAMP1 (1:1000, rabbit polyclonal, Sigma), anti-Na^+/K^+ ATPase (1:1000, mouse monoclonal, Millipore), and anti-β-actin (1:10000, mouse monoclonal, Abcam). Secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (1:2,000) and HRP-conjugated goat anti-mouse IgG (1:2000).

6.2.8. Statistics: Details were described in Chapter 5.2.9.

6.3. Results

Endolysosomes are acidic calcium stores that contain high concentrations of readily releasable calcium. These stores can be induced to release calcium by inhibition of vacuolar H^+-ATPase with bafilomycin (BAF) or selective disruption of endolysosome membranes with Gly-Phe-β-naphtylamide (GPN) (Lopez et al., 2005; Ramos et al.; Singaravelu and Deitmer, 2006). However, it is only recently appreciated that acidic calcium stores contribute to neuronal calcium signaling (Dickinson et al., 2010; Haas et al., 2009; Pandey et al., 2009). Here, we determined the extent to which BAF and GPN induced the release of calcium from endolysosomes in primary cultured neurons. First, we determined the

involvement of the ER calcium stores in BAF- and GPN- induced calcium release. Under calcium free conditions (zero calcium plus 10 mM EGTA), thapsigargin, a selective SERCA inhibitor that depletes ER calcium stores, induced a rise in intracellular calcium (Figure 21). After calcium levels returned towards baseline levels, both BAF (Figure 21A) and GPN (Figure 21B) were still able to induce a transient rise in intracellular calcium levels. Additionally, we determined the involvement of mitochondria in BAF- and GPN- induced calcium release. We found that after depleting calcium in mitochondria with a mitochondria calcium pump inhibitor CCCP, BAF and GPN were still able to induce a rise in intracellular calcium levels (data not shown). These observations indicate that BAF and GPN induced calcium release is independent of calcium stores in ER and mitochondria.

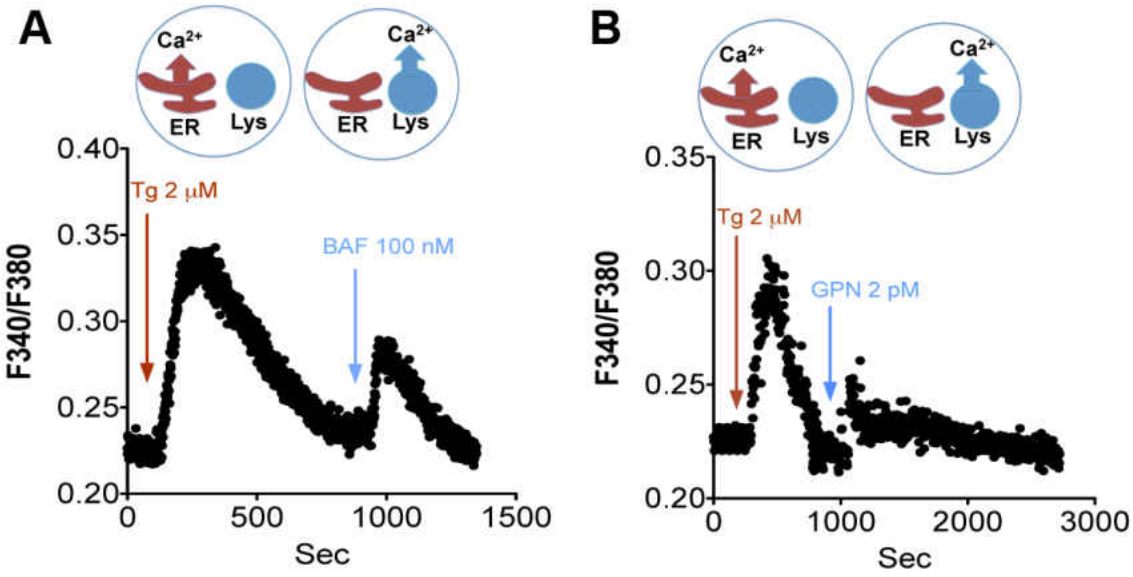

Figure 21. Endolysosomes Contributed to Homeostatic Control of Intracellular Calcium Levels. (A) Under calcium free conditions (zero calcium plus 10 mM EGTA), bafilomycin (BAF, 100 nM) released calcium from endolysosome (Lys) compartments after depleting the ER calcium store with a selective SERCA inhibitor thapsigargin (Tg 2 μM). (B) Under calcium free conditions (zero calcium

plus 10 mM EGTA), GPN (2 pM) released calcium from endolysosome (Lys) compartments after depleting the ER calcium store with thapsigargin (Tg, 2 μM).

Previously, NAADP that could induce calcium released from acidic stores was also found to activate cell surface calcium channels to stimulate calcium influx in neurons as well as other types of cells (Beck et al., 2006; Brailoiu et al., 2009b; Galione, 2011; Moccia et al., 2006a; Moccia et al., 2003; Moccia et al., 2006b; Naylor et al., 2009; Santella et al., 2000). Here, we examined the extent to which BAF and GPN induced calcium influx. Under calcium free conditions, both BAF and GPN induced a small transient rise in levels of intracellular calcium (Figure 22, grey lines). However, in the presence of extracellular calcium, both BAF and GPN dramatically increased levels of free intracellular calcium (Figure 22, black lines). These observations indicate that BAF and GPN not only release calcium from acidic stores, but also trigger calcium influx across the plasma membrane.

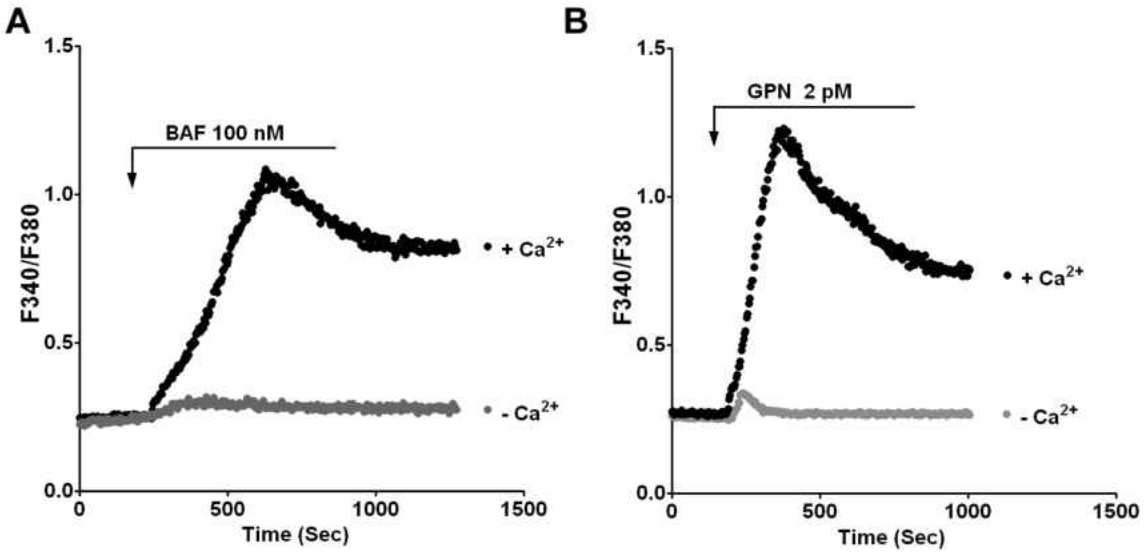

Figure 22. BAF and GPN Affected Influx of Extracellular Calcium. (A) Under calcium free conditions (zero calcium plus 10 mM EGTA), 100 nM bafilomycin

(BAF) induced a small and transient rise in levels of intracellular calcium (grey line). By contrast, in the presence of extracellular calcium, BAF markedly increased levels of intracellular calcium (black line). (B) Under calcium free conditions (zero calcium plus 10 mM EGTA), 2 pM GPN induced a small and transient rise in intracellular calcium (grey line). By contrast, in the presence of extracellular calcium, GPN markedly increased levels of intracellular calcium (black line).

It is well known that depleting ER calcium stores with a selective SERCA inhibitor thapsigargin (Tg) triggers larger amounts of calcium influx across plasma membrane, a phenomenon termed store-operated calcium entry (SOCE). First we confirmed the functional presence of SOCE in our primary cultured neurons (Figure 23A). Next, using similar calcium-imaging techniques, we determined the extent to which BAF and GPN affected extracellular calcium influx. Under nominally calcium free conditions, BAF induced a small transient rise in levels of intracellular calcium. When calcium was re-added to the media, a much larger rise in levels of intracellular calcium was observed (Figure 23B), which indicated calcium influx across the plasma membrane. Moreover, a very similar profile of calcium entry was observed when endolysosome membranes were selectively disrupted with GPN (Figure 23C). Thus, both BAF and GPN induced a profile of calcium entry similar to Tg-induced store-operated calcium entry. Accordingly, we have termed this novel calcium influx mechanism as “acidic store-operated calcium entry (aSOCE)”.

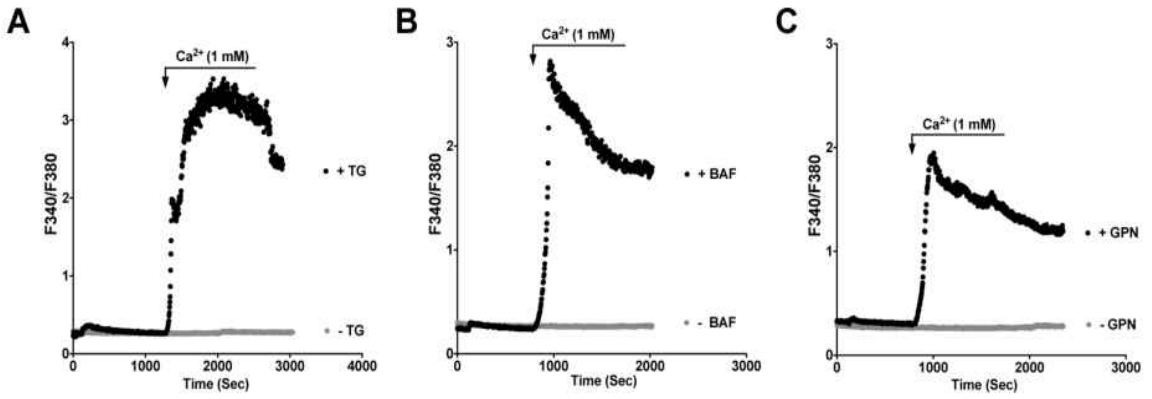

Figure 23. Similar to Store-Operated Calcium Entry, Endolysosome Calcium Release Led to Increased Influx of Extracellular Calcium. (A) Under nominally calcium free conditions (no calcium and no EGTA), emptying ER calcium stores with 2 μM thapsigargin (Tg) induced a small and transient rise of intracellular calcium, which was followed by a large calcium influx as calcium was re-added to the medium. (B) Under nominally calcium free conditions (no calcium and no EGTA), calcium release from endolysosomes with a selective vacuolar H^+-ATPase inhibitor, BAF, induced a small and transient rise in levels of intracellular calcium, which was followed by a large calcium influx as calcium was re-added to the medium. (C) Under nominally calcium free conditions (no calcium and no EGTA), calcium released from endolysosomes with a lysosomotropic agent, GPN, induced a small and transient rise in levels of intracellular calcium, which was followed by a large calcium influx as calcium was re-added to the medium.

To determine further the involvement of cell surface calcium channels in aSOCE, we pharmacologically blocked different calcium channels. We found that BAF-induced calcium entry was not blocked by the classic store-operated calcium entry inhibitors SKF-96365 or 2-APB, the L-type calcium channel inhibitor nimodipine, or the P/Q-type calcium channel inhibitor ω-agatoxin (Figure 24A). However, a selective N-type calcium channel blocker ω-conotoxin attenuated significantly BAF-induced calcium entry (Figure 24A). Similar results were obtained with GPN-induced calcium entry; only the N-type calcium channel blocker ω-conotoxin attenuated significantly GPN-induced calcium entry (Figure 24B). In contrast, blocking N-type calcium channels did not affect thapsigargin-

induced calcium entry, which was attenuated by the classic store-operated calcium entry inhibitors including SKF-96365 and 2-APB (Figure 24C). Thus, our pharmacological data indicates that N-type calcium channels are involved in this novel acidic store-operated calcium entry. To confirm our pharmacological findings, we knocked down the expression of N-type calcium channels with a specific siRNA (Figure 24D) and found that when expression levels of N-type calcium channels were reduced, BAF- and GPN-induced calcium entry was significantly attenuated. However, Tg-induced calcium entry was not affected (Figure 24E). Together, our findings indicate that N-type calcium channels are responsible for acidic store-operated calcium entry in primary cultured neurons.

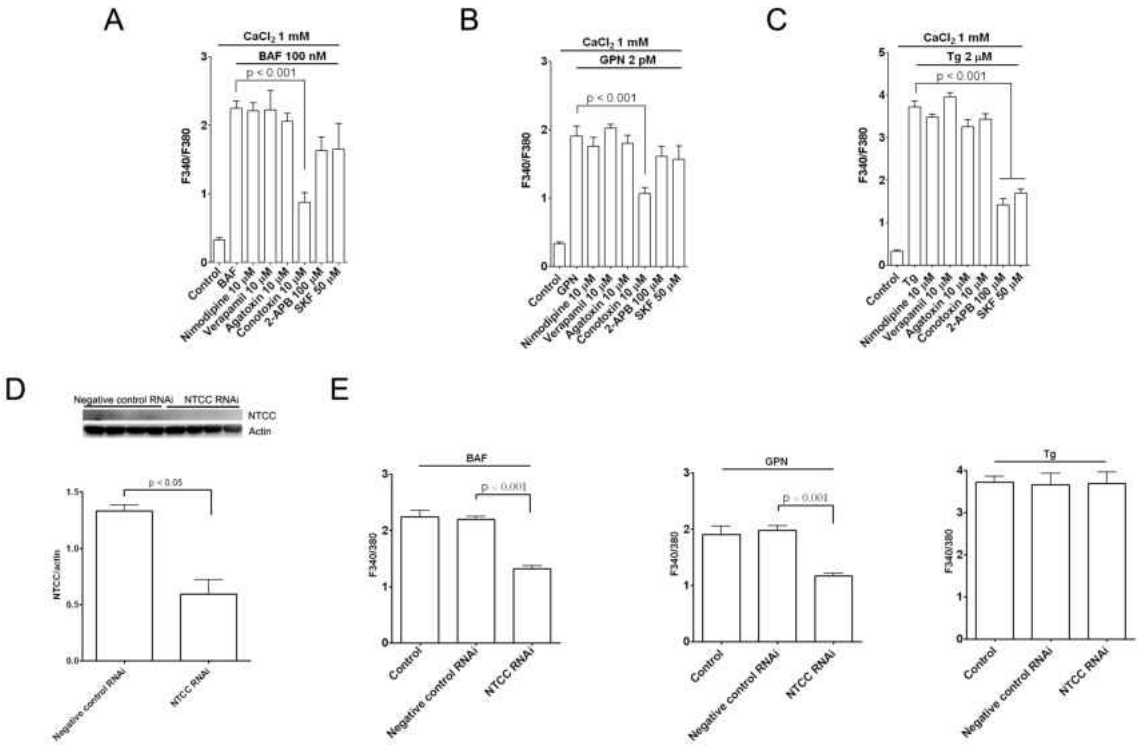

Figure 24. N-Type Calcium Channels Mediated BAF- and GPN- Induced Calcium Influx. Neurons were pretreated with L-type calcium channel blockers (nimodipine 10 μM or verapamil 10 μM), P/Q-type calcium channel blocker (ω-agatoxin 10 μM), N-type calcium channel blocker (ω-conotoxin 10 μM) or SOC blockers (2-APB 100 μM or SKF 50 μM) and then incubated with Fura-2/AM (2

μM, 37°C, 20 min), rinsed with nominal calcium buffer. Free intracellular calcium levels were measured under a filter based calcium-imaging system (Axiovert 200 miciroscopy, Zeiss). After pretreatment, neurons were further treated with BAF, GPN or Tg and then $CaCl_2$ (1 mM) was re-introduced. Free cytosolic calcium concentrations were measured as a ratio of 340/380. (A.B) Only the N-type calcium channel blocker, ω-conotoxin significantly attenuated BAF- or GPN-induced calcium entry (n=15). (C) In contrast, SOC channel blockers significantly attenuated Tg-induced calcium entry (SOCE) (n=18). $p < 0.001$. (D) siRNA of NTCC (60 nM) significantly downregulated the expression of NTCC. $p < 0.05$. (E) siRNA of NTCC significantly reduced BAF- or GPN- but not Tg-induced calcium influx. $p < 0.001$.

To determine further the mechanisms whereby BAF- and GPN- induced the entry of extracellular calcium via N-type calcium channels we conducted immunostaining studies for N-type calcium channels and LAMP1. Using a surface immunostaining method, we demonstrated that both BAF and GPN, but not thapsigargin, increased surface expression of N-type calcium channels and LAMP1 in primary cultured neurons (Figure 25A). Meanwhile, using a biotinylation of surface proteins assay, we demonstrated that both BAF and GPN increased the surface expression of N-type calcium channels and LAMP1 (Figure 25B). Furthermore, using immunoprecipitation methods, we demonstrated that N-type calcium channels physically interacted with LAMP1, and that BAF and GPN increased the interaction of N-type calcium channels with LAMP1 (Figure 26). It is known that LAMP1, a specific marker protein for lysosomes, is critical for lysosome exocytosis (Arantes and Andrews, 2006). Our observations that BAF and GPN increased the neuronal surface expression of LAMP1 indicated that calcium released from endolysosomes induced lysosome exocytosis. Given the physical interaction between N-type calcium channels and LAMP1, such a BAF- and GPN- induced lysosome exocytotic process results in exocytotic insertion of

N-type calcium channel into the plasma membrane, which underlies the novel aSOCE. Such a notion is consistent with several recent findings that lysosome exocytosis is able to insert calcium channels into plasma membrane (Gerasimenko et al., 2001; Schmidt et al., 2009; Smith et al., 2000; Tuck and Cavalli, 2010; Vogel, 2009). To further confirm this notion, we knocked down the expression of LAMP1 with a specific siRNA (Figure 27A), and we found that siRNA knockdown of LAMP1 significantly attenuated BAF and GPN, but not thapsigargin, induced calcium entry (Figure 27B).

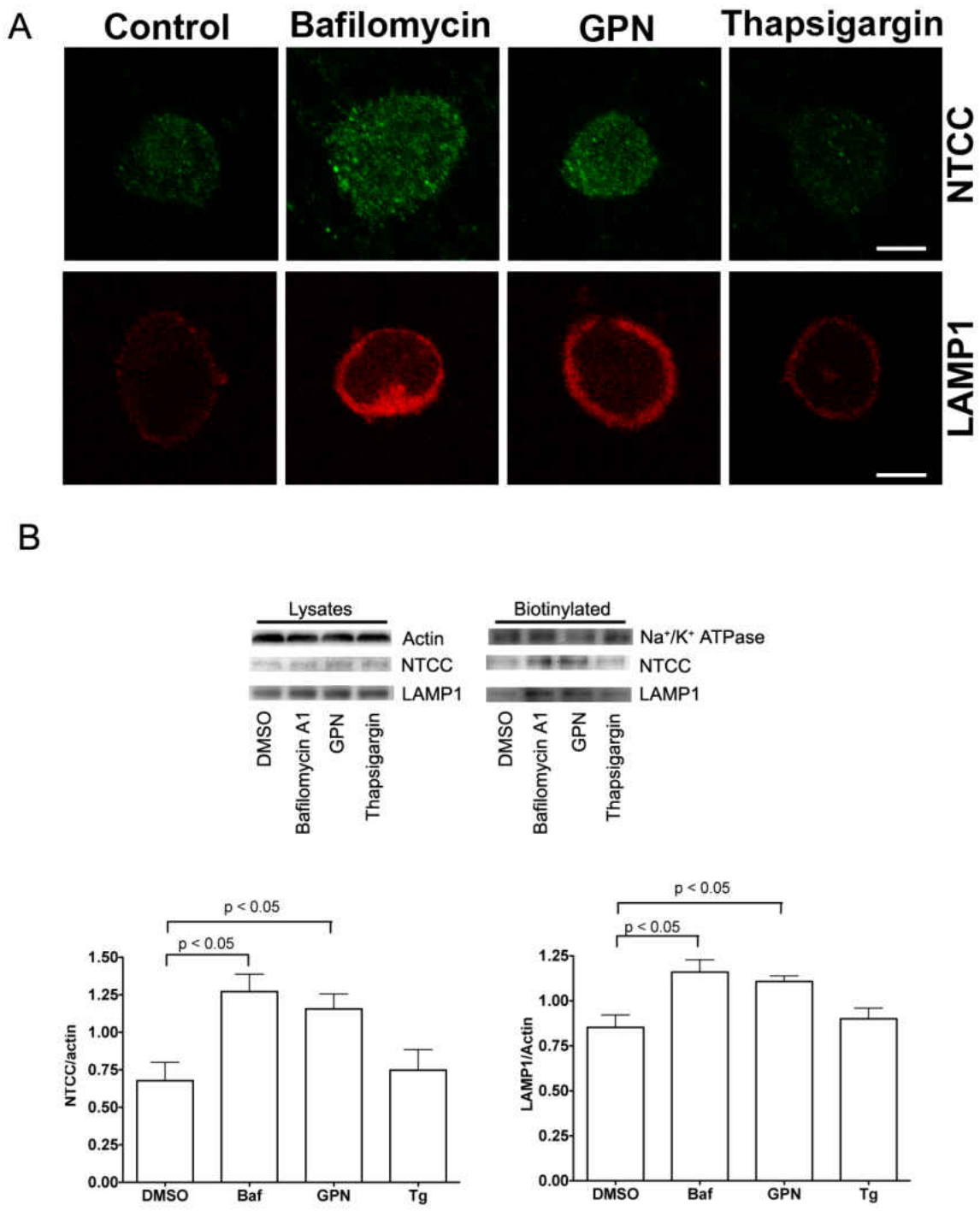

Figure 25. BAF and GPN Increased the Cell Surface Expression Levels of N-Type Calcium Channels. In Addition, BAF and GPN Redistributed LAMP1 Into the Plasma Membranes. (A) Surface staining showed that 100 nM BAF or 2 pM GPN increased the surface expression of N-type calcium channels (NTCC) and relocated the lysosome marker (LAMP1) onto the plasma membrane. (B) 100 nM BAF or 2 pM GPN did not affect expression levels of NTCC and LAMP1 in whole cell lysates (lane 2 and 3 of lysates sample, left panel of blots data). However,

BAF or GPN increased expression levels of N-type calcium channels (NTCC) and LAMP1 on the plasma membrane ($p < 0.05$), but 2 μM thapsigargin failed to affect the surface expression of both proteins (lane 2 and 3 of biotinylated samples, right panel of blots data). β-actin, a cytoskeletal marker, was used as a loading control for the whole cell lysates (lane1, left panel). Na^+/K^+ ATPase, a cell membrane marker, was used as a positive control of plasma membrane (lane1, right panel). Immunoblots of Na^+/K^+ ATPase and β-actin were obtained by reprobing the same immunoblotting membrane as that for NTCC and LAMP1 with corresponding antibodies. Similar results were obtained from at least four repeated experiments. Quantification of immunbloting data of NTCC and LAMP1 was obtained from biotinylated samples.

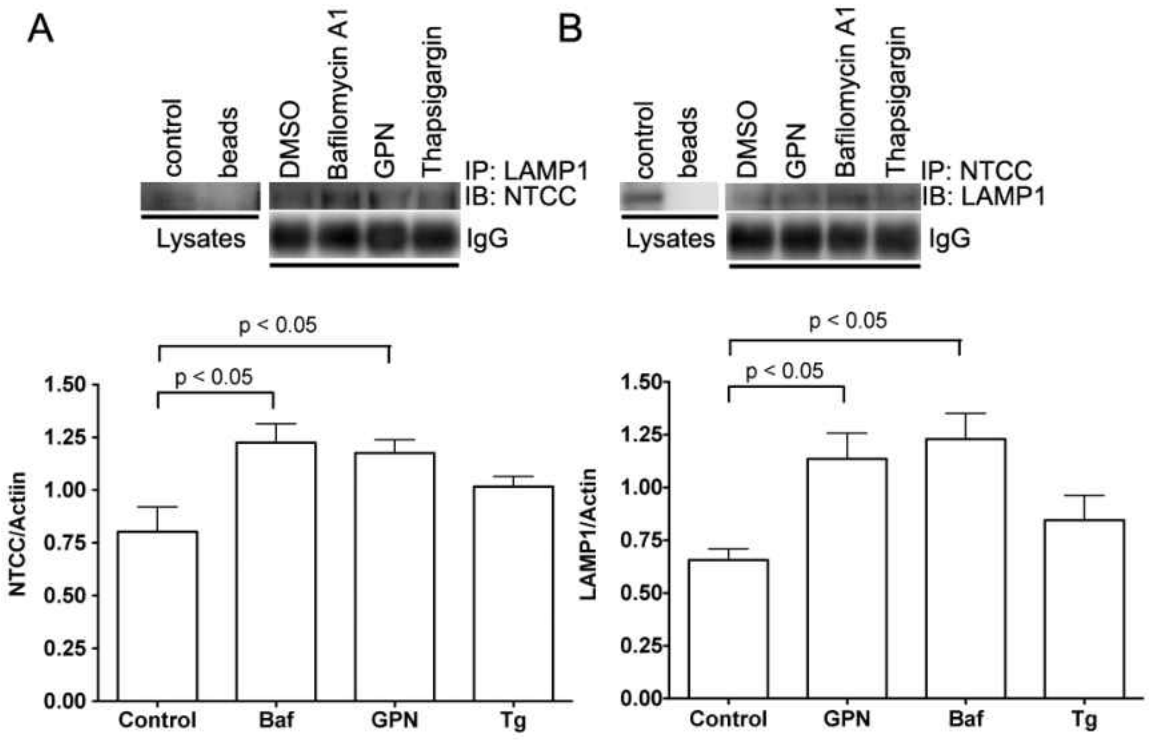

Figure 26. LAMP1 Interacted Physically with N-Type Calcium Channels When Neurons were Treated with BAF or GPN but not Tg. (A) With BAF or GPN treatment, LAMP1 pulled down N-type calcium channel protein (NTCC) (top); Immunobloting data of NTCC from immunoprecipitation samples were quantified using a Gel-doc system (bottom). $p < 0.05$. (B) With BAF or GPN treatment, NTCC pulled down LAMP1 (top); Immunbloting data of LAMP1 from immunoprecipitation data were quantified using a Gel-doc system (bottom). $p < 0.05$.

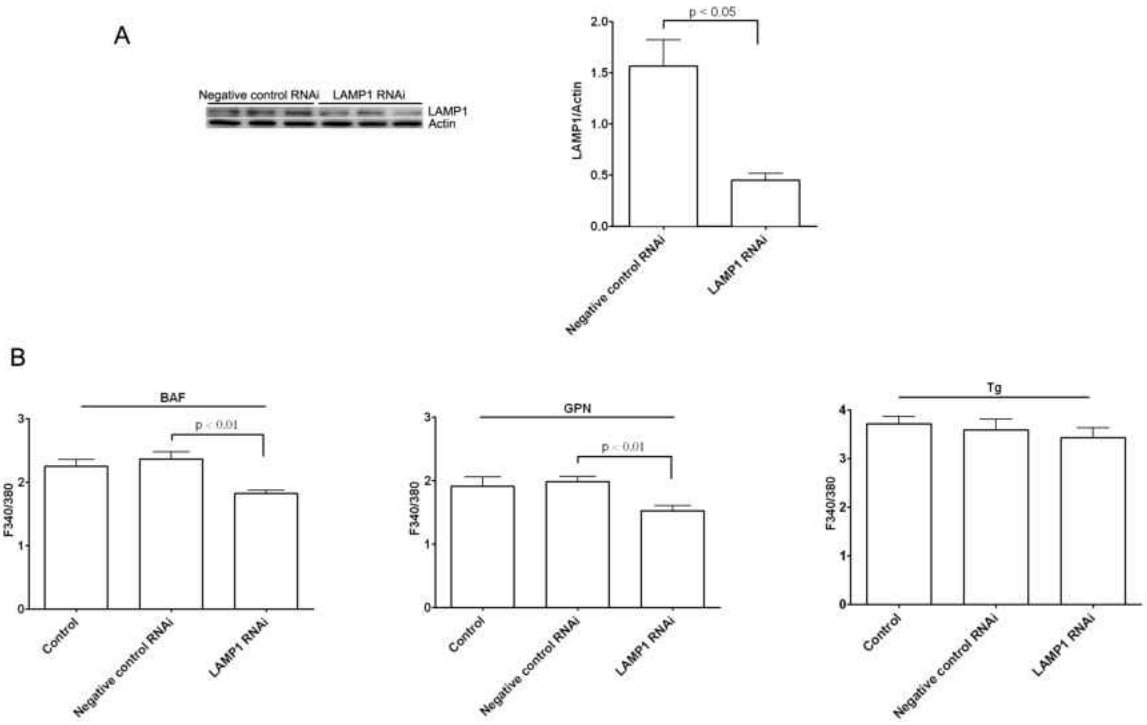

Figure 27. Involvement of LAMP1 in BAF- and GPN- Induced Calcium Influx. (A) siRNA of LAMP1 significantly reduced protein expression levels of LAMP1. $p < 0.05$. (B) siRNA of LAMP1 significantly decreased BAF- or GPN- ,but not Tg-induced calcium influx. $p < 0.01$.

6.4. Discussion

Calcium is an indispensable second messenger responsible for integrating cell signaling in neurons. Free levels of intracellular calcium can translate extracellular stimuli into intracellular signaling events such as regulation of gene expression and neurotransmitter release from presynaptic axon terminals (Gleichmann and Mattson, 2011). Integration of the neuronal information is maintained by neuronal calcium homeostasis, which is precisely regulated by intracellular calcium stores and the plasma membrane (Verkhratsky and

Petersen, 1998). Among those long known intracellular calcium stores capable of shaping calcium signals, endolysosomes are less well characterized and their roles in calcium homeostasis are much less appreciated. However, current studies have suggested strongly that endolysosomes match several essential criteria for being considered significant calcium stores: endolysosomes contain higher concentrations of calcium (400-600 μM), endolysosomes mediate calcium uptake by proton gradients maintained by V-ATPase and/or H^+/Ca^{2+} exchangers on their membranes; and endolysosomes regulate calcium release via two-pore channels (TPC) and possible TRPM channel (Lloyd-Evans et al., 2010). In our current study, we found that altering endolysosome pH with the inhibitor of V-ATPase by BAF or lysis of endolysosome membrane by GPN induced transient increases in levels of intracellular calcium even after depleting calcium from ER and mitochondrial stores. These findings suggest that BAF or GPN can specifically induce neuronal endolysosome calcium release to cause transient calcium increases in $[Ca^{2+}]_i$. In contrast to the above pharmacological modulation of endolysosome calcium release, one intracellular second messenger, NAADP, has been found to discharge endolysosome calcium via TPC. Therefore, detection of the effect of NAADP on endolysosome calcium will be an attractive direction in the future.

ER is so far the best-characterized intracellular calcium store. ER integrates its ability to accumulate and store calcium with extracellular calcium influx via a mechanism referred to as store operated calcium entry (SOCE) whereby emptying ER calcium stores leads to enhanced influx of extracellular

calcium. SOCE was first described in non-excitable cells and later in excitable cells including neurons, and it has been implicated into multiple physiological and pathological functions of neurons (Hoke et al., 2009; Selvaraj et al., 2009; Wu et al., 2004; Yao et al., 2009). Using calcium imaging techniques, we confirmed the presence of Tg-initiated SOCE in our cultured neurons. However, when we replaced Tg with BAF or GPN, we observed that calcium released from endolysosomes also promoted extracellular calcium influx in cultured neurons. Because this SOCE-like phenomenon occurred in endolysosomes, a group of acidic stores, we termed it as acidic store operated calcium entry (aSOCE). Several relevant questions might be answered to extend the current work. First, in contrast to SOCE that has been identified as a ubiquitous phenomenon in multiple cell types, this novel aSOCE has so far only been described in neurons and therefore it will be important to test its functional presence in other cell types. Second, it will be important to determine the role of aSOCE in various physiological and pathological states. Third, it will be important to determine the extent to which aSOCE is regulated by depleting calcium from acidic stores.

The possible mechanisms underlying aSOCE are unclear at present. However, it is certainly possible that aSOCE shares similar modes of integrating calcium signal with SOCE. Currently, two major areas are focused on with SOCE; identifying the involved channels on the plasma membrane and the mechanisms that couple ER depletion to SOC channels' opening. The cell surface channels identified belong mainly to the TRP family (Parekh and Putney, 2005). However, the mechanisms by which the stores communicate with the

plasma membrane channels are more complicated. So far, several hypotheses have been proposed (Parekh and Putney, 2005). Reduction in ER calcium concentration generates a diffusible message that can open SOC in the plasma membrane. Components of ER physically contact with plasma membrane, and ER may move closely to the plasma membrane using actin as a binder. A ER sensor, STIM1, may interact with the calcium channel protein ORAI1 on the plasma membrane (Klejman et al., 2009). Store-operated channels do not appear to be present in the plasma membrane under basal conditions, but may insert into the plasma membrane following store depletion. Store operated channels may be in an inactivated state under basal conditions, while store depletion removes the inhibition by upregulating SERCA pump activity.

Here, uncovering the mechanisms underlying aSOCE may start with answering the below questions. First, what kind of calcium channels might be involved in this acidic SOCE? Second, how is endolysosome calcium release connected with corresponding calcium influx? For the first question, we screened different voltage- and store- operated calcium channel blockers and our data demonstrated that N-type voltage operated calcium channels (VOCC) were involved in acidic store operated calcium entry. Meanwhile, our studies on gene knockdown of relevant calcium channels supported this result. Besides, we also observed that TRP channels mediated store operated calcium entry by using two blockers of TRP channels, which was consistent with previous findings in SOCE (Bennett et al., 1995; Berridge, 1995). These results suggested that aSOCE and SOCE both initiate calcium entry by discharging calcium from organelles, but

they might have distinctive mechanisms. Thus, endolysosome signaled calcium influx occurs through N-type calcium channels, whereas ER induced calcium entry happens via TRP channels.

Several things need to be addressed in any future studies. Because N-type calcium channels are mainly expressed in postsynaptic terminals and are functionally involved neurotransmitter release (Miller, 1990; Murakoshi and Tanabe, 1997), by driving more vesicles to be fused into plasma membrane the involvement of N-type VOCC in aSOCE might give us insights into the role of aSOCE in potential neuronal functions such as regulation of neurotransmitter release. Therefore, further studies are warranted to evaluate the role of aSOCE in neurotransmission and other possible neuronal functions. Furthermore, it is possible that either increased expression of voltage operated calcium channels or elevated activation of N-type VOCC could contribute to the increased calcium current. We have described the possibility of the increased expression of N-type VOCC on plasma membrane contributing to calcium influx, but we can not neglect the fact that altered activation of N-type VOCC could affect aSOCE as well. Therefore, further investigation is needed to determine the activation state of N-type VOCC in aSOCE. Moreover, there is a possibility that other regulatory mechanisms are involved in aSOCE in addition to voltage-operated calcium channels such as plasma membrane Na^{+}-Ca^{2+} exchanger (NCX) (Parekh and Putney, 2005). It is known that NCX1, one of three isoforms (NCX1, NCX2 and NCX3), is present in neurons (Sakaue et al., 2000). Further investigations are

needed to examine whether other possible mechanisms are also participating in aSOCE.

We were next concerned with how acidic store calcium release was coupled to calcium influx through opening relevant channels on the plasma membrane. Previous evidence indicated that calcium triggers the exocytosis of lysosomes onto plasma membranes (Jaiswal et al., 2002; Li et al., 2008) and that N-type calcium channels undergo endocytosis, degradation in endolysosomes, and reinsertion into plasma membrane (Jarvis and Zamponi, 2007). Our combined findings in aSOCE demonstrated that a lysosome membrane protein, LAMP1 was redistributed onto plasma membrane, there was a physical link between LAMP1 and N-type VOCC, and that knockdown of LAMP1 reduced calcium influx. Together, these results support the hypothesis that endolysosome calcium release could change lysosome behavior (exocytosis) resulting in lysosome exocytosis of N-type calcium channels on the plasma membrane. Increased levels of $[Ca^{2+}]_i$ drives exocytosis of secretory granules in many cell types including neurons (Easom, 2000; Morgan, 1995; von Gersdorff and Matthews, 1994) and that secretory granules have intracellular N-type calcium channel pools that can transport this VOCC to plasma membrane within minutes (Passafaro et al., 1996). Our biochemical observation that there was an increased expression of N-type calcium channels in plasma membranes during aSOCE might support this hypothesis that calcium release from endolysosomes triggered movement of secretory granules containing N-type calcium channels onto the plasma membrane.

Taken together, current observation on endolysosome calcium behavior is very similar to SOCE that calcium release from endolysosomes by increasing endolysosome pH or lysis of endolysosome membrane can induce extracellular calcium entry (aSOCE). However, the involved calcium channels of this acidic store operated calcium influx are distinctive from SOCE that aSOCE is mediated by N-type voltage-operated calcium channels. In addition, the possible mechanism coupling of endolysosome calcium release to N-type calcium channel is that endolysosome calcium discharge driving exocytosis of lysosomes and/or secretory vesicles to bring more N-type calcium channels to the plasma membrane, thus resulting in tremendous calcium influx.

Scientists are increasingly realizing that endolysosome calcium plays key roles in physiology and pathology. Physiologically, endolysosome calcium participates in formation of endosome-lysosome hybrids and reformation of lysosomes in addition to their involvement in membrane repair for wound healing by driving exocytosis of endolysosomes. Pathologically, disturbed endolysosome calcium has been associated with several lysosome storage diseases (LSD) which are characterized by mutation in endolysosome membrane proteins, e.g Niemann-Pick type C1 disease, mucolipidosis type IV and Chediak-Higashi syndrome (Lloyd-Evans et al., 2010). However, our current knowledge of these acidic calcium stores is still limited compared to other calcium stores.

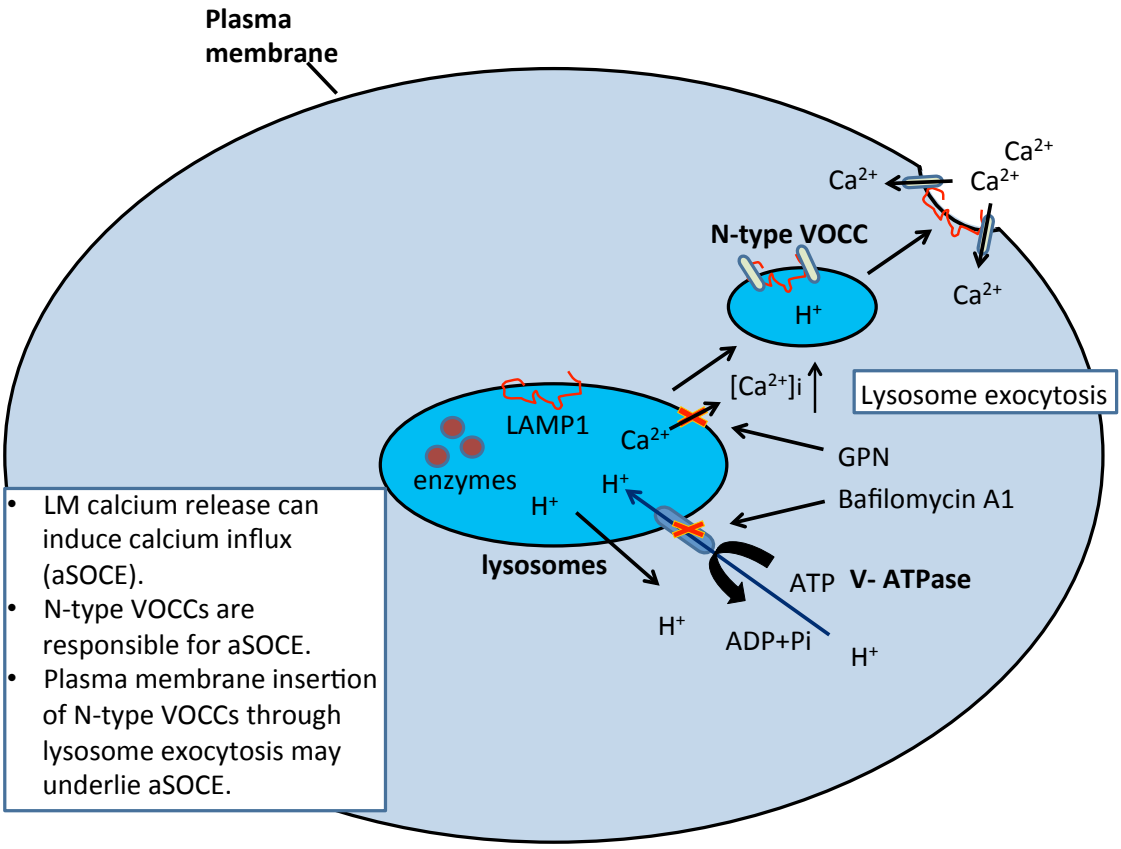

Figure 28. Model Summarizing Acidic Store-Operated Calcium Entry in Primary Cultured Neurons. Similar to classical store-operated calcium entry (SOCE), calcium releases from acidic calcium stores triggers calcium influx, which we term 'acidic store-operated calcium entry' (aSOCE). Here, the aSOCE is mediated by lysosome exocytotic insertion of N-type calcium channels in neurons.

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