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High resolution approaches for the identification of amyloid fragments in brain

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Abstract

Background: It is now widely recognized that endogenous, picomolar concentrations of the 42 amino acid long peptide, amyloid- β ($A\beta_{42}$) is secreted under normal physiological conditions and exerts important functional activity throughout neuronal intracellular compartments. Transgenic animal models that overexpress $A\beta_{42}$ and its precursor, amyloid precursor protein (APP), have not provided predictive value in testing new treatments for Alzheimer's disease (AD), resulting in failed clinical trials. While these results are discouraging, they underscore the need to understand the physiological roles of $A\beta_{42}$ and APP under normal conditions as well as at early pre-symptomatic stages of AD.

New Method: We describe the use of acrolein-perfusion in immunoelectron microscopy in combination with novel antibodies directed against endogenous murine $A\beta_{42}$ and APP fragments to study abnormalities in the endolysosomal system at early stages of disease. The specific requirements, limitations and advantages of novel antibodies directed against human and murine $A\beta_{42}$, APP and APP fragments are discussed as well as parameters for ultrastructural analysis of endolysosomal compartments.

Results:

—Novel antibodies and a detailed protocol for immunoelectron microscopy using acrolein as a fixative are described. Acrolein is shown to preserve intraneuronal $A\beta_{42}$ species, as opposed to paraformaldehyde fixed tissue, which primarily preserves membrane bound species.

Comparison with Existing Method(s):

—Technology sensitive enough to detect endogenous $A\beta_{42}$ under physiological conditions has not been widely available. We describe a number of novel and highly sensitive antibodies have recently been developed that may facilitate the analysis of endogenous $A\beta_{42}$.

Conclusions: Using novel and highly specific antibodies in combination with electron microscopy may reveal important information about the timing of aberrant protein accumulation, as well as the progression of abnormalities in the endolysosomal systems that sort and clear these peptides.

Introduction

In recent years, the amyloid hypothesis of Alzheimer's disease (AD) has been heavily scrutinized as clinical trials of drugs successful in ameliorating amyloid load in mouse models of AD have failed to show efficacy in humans, and in some cases have had serious adverse side effects (Hardy 2009, Coric, Salloway et al. 2015). The negative outcomes of clinical trials has lead some leaders in the field to re-examine the Amyloid Cascade Hypothesis (Hardy 2009, Selkoe and Hardy 2016), while others believe the problem is a fundamental misunderstanding of the A β peptide and the importance of its physiological function (Puzzo, Gulisano et al. 2015). The latter is rooted in the idea that the complexity of AD, combined with preconceptions of amyloid- β (A β ₄₂) as an exclusively harmful protein have prevented investigators in the field from focusing on important facets of the disease state (Puzzo, Gulisano et al. 2015).

The ability to detect endogenous A β ₄₂ peptides in naïve rats and wildtype mice is a recent advancement enabled by the development of highly specific antibodies that detect the peptide at endogenous picomolar concentrations. Some conventional methods to study AD pathology include genetic models that overexpress human APP and/or presenilin genes, ultimately resulting in abundant production of the A β ₄₂ peptide that leads to rapid accumulation of the protein in the rodent brain. Other investigations have utilized the administration of exogenous A β ₄₂ peptides in high micromolar concentrations and in various conformational states, to recapitulate certain aspects of AD pathology. These methods have been critical in elucidating mechanisms of late disease stages, however, do not yield information regarding early, critical intervention stages of AD. Here, we advocate for the study of endogenous A β ₄₂ peptides, a novel route of investigation that may advance our knowledge regarding the transition from physiological to pathological states. This transition is thought to occur over a prolonged incubation period, termed the prodromal period, during which patients exhibit numerous co-morbid conditions including metabolic disorders such as diabetes, cardiovascular and psychiatric diseases. These disparate co-morbid diseases have an onset of symptoms before the onset of cognitive symptoms of dementia, and during the critical prodromal stage at which neuronal damage leading to AD is thought to occur.

The study of endogenous A β ₄₂ peptides in models of disease states that are co-morbid with Alzheimer's disease may advance our understanding of pathological states that render individuals more susceptible to dementia with age. For example, our laboratory focuses on the locus coeruleus (LC) stress integrative system and has recently localized endogenous A β ₄₂ peptides in the noradrenergic (NE) cell bodies of the LC and axon terminals of the medial prefrontal cortex (mPFC) (see figures 2,3 and 5). The presence of endogenous A β ₄₂ within the LC-NE circuit may therefore have serious implications for conditions of chronic stress in which we hypothesize that aberrant neuronal activity of the LC may result in increased accumulation of the A β ₄₂ peptide.

The physiological and pathological roles of Amyloid Precursor Protein (APP) and the products of its proteolytic cleavage remain poorly understood, thus, the emphasis of this review will be on studying the basic cellular biology of APP and its fragments using novel antibodies directed against APP and the products of its proteolytic cleavage. The use of these

antibodies in combination with electron microscopy may facilitate tracking the location and accumulation of APP fragments in endolysosomal compartments of the cell. Such trafficking studies are emerging as an important area of investigation, as early abnormalities in the endolysosomal system have been identified as a cellular vulnerability in AD and a precipitating factor in the accumulation of A β ₄₂ peptides in models of the disease (Nixon, Wegiel et al. 2005, Yu, Cuervo et al. 2005, Jiang, Mullaney et al. 2010).

We start by describing the structure and processing of APP and then move into describing specific antibodies that may be used to detect fragments along the two diverging processing routes. We then provide an immunoelectron microscopy protocol and details regarding the ultrastructural identification of endolysosomal components under the transmission electron microscope. Future studies in this area may reveal a wealth of information about the timing of aberrant accumulation of these APP-derived fragments, as well as the progression of abnormalities in the endosomal and lysosomal systems that sort and clear these peptides.

APP Structure and Processing

APP is a type I transmembrane protein synthesized in the rough endoplasmic reticulum (ER) and exists in 3 major isoforms ranging from 695 to 770 amino acids in length. While APP₇₇₀ has been chosen as the canonical sequence, the APP₆₉₅ isoform is the predominant form in neuronal tissue. The APP₆₉₅ isoform does not contain amino acids 290–364, also known as the Kunitz protease inhibitor (KPI) domain, distinguishing it from the APP₇₇₀ isoform. Isoforms APP₇₅₁ and APP₇₇₀ are widely expressed in non-neuronal tissues (D. Goodsell 2015). APP is composed of several domains that have been characterized using X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy (Rossjohn, Cappai et al. 1999, Wang and Ha 2004). Full length APP includes three domains that extend outside the cell, one transmembrane domain and one cytoplasmic intracellular domain. The N-terminal domain, copper-binding domain (Barnham, McKinsty et al. 2003), and cell adhesion domains comprise the extracellular portions of the protein and include amino acids 18–699 counting residues by APP₇₇₀. The transmembrane domain is a helical structure that is composed of amino acids 700–723, and the cytoplasmic domain is composed of amino acids 724–770 counting residues by APP₇₇₀. The various domains of APP and its complex structure enable a wide variety of functions including that of a cell surface receptor, processes involved in neurite growth, adhesion and axonogenesis, as well as cell mobility (Rossjohn, Cappai et al. 1999, Hoffmann, Twisselmann et al. 2000, Barnham, McKinsty et al. 2003, Muresan and Ladescu Muresan 2015). APP has also been implicated in the regulation of mitochondrial function, transcription, copper homeostasis and oxidative stress by modulating various protein-protein interactions (Barnham, McKinsty et al. 2003, Turner, O'Connor et al. 2003, Muresan and Ladescu Muresan 2015).

APP undergoes dynamic trafficking and processing resulting in the production of distinct fragments that are localized to different intracellular compartments and are thought to serve diverse functions (Figures 1 and 2). APP undergoes a number of post translational modifications that direct its trafficking throughout the cell. For example, phosphorylated APP species preferentially accumulate in filopodia, while modification by the peptidyl-prolyl cis-isomerase, Pin 1, or cytoplasmic glycosylation by O-glycNAcase control the

trafficking and targeting/anchoring to specific destinations of APP (Muresan and Ladescu Muresan 2015). The proteolytic processing of APP generally occurs via two divergent pathways that have discrete outcomes in terms of the fragments produced, and the functional impact on neurons. The non-amyloidogenic pathway (Figure 1) is the most common route of processing under normal physiological conditions. Cleavage of APP by α -secretases embedded in the plasma membrane results in the formation of sAPP α composed of amino acids 18–687 and a C83 carboxyl terminal fragment (CTF) composed of amino acids 688–770. APP cleavage via α -secretases precludes the formation of A β ₄₀ and A β ₄₂ peptides, the final products of the amyloidogenic pathway (LaFerla, Green et al. 2007). The amyloidogenic pathway (Figure 1) represents a smaller portion of APP processing, although in the brain this is a significant processing pathway (Choi, Berger et al. 2009). However, the products of amyloidogenic processing may have important physiological roles that are an important area of ongoing investigation. In this pathway, APP undergoes proteolytic cleavage by the aspartic protease β -secretase (BACE-1), which cleaves APP on the luminal side of the membrane, releasing a soluble APP β fragment (sAPP β) (Figures 1 and 2) composed of amino acids 18–671, and C99 CTF composed of amino acids 672–770 (Vassar, Bennett et al. 1999). BACE-1 cleavage results in the formation of a new N-terminus with the first aspartic amino acid 672 of A β (LaFerla, Green et al. 2007). Subsequent cleavage of this fragment, typically between 38–43 amino acids downstream from the BACE-1 cleavage site by the γ -secretase results in the release of the A β peptides (Figures 1 and 2).

Importantly, the fate of APP is dictated largely by its subcellular localization, thus highlighting the importance of trafficking in parallel with proteolytic cleavage (Figure 3). APP traffics through the plasma membrane alongside α -secretases, facilitating non-amyloidogenic processing. However, APP may also be internalized via endocytosis where it encounters the β - and γ -secretases that reside in acidic intracellular compartments particularly within the endolysosomal system, but may also include the trans Golgi network (TGN), the ER, and mitochondrial membranes (Mizuguchi, Ikeda et al. 1992, Xu, Greengard et al. 1995, Kinoshita, Shah et al. 2003), all of which may be sites for intracellular A β production (LaFerla, Green et al. 2007, Haass, Kaether et al. 2012). Typically, the internalization of APP favors amyloidogenic processing and the formation of A β , whereas its presence on the cell surface confers increased likelihood that it will encounter the α -secretase known to produce soluble APP α , and non-amyloidogenic C83 fragments (Hong, Huang et al. 2014). Once internalized, APP undergoes amyloidogenic processing in the early endosome followed by recycling in which A β peptides are released into the extracellular space, or alternatively, through subsequent endosomal-lysosomal compartments in which it may ultimately be degraded in the lysosome.

It has been observed that synaptic A β production may be significantly decreased by inhibiting clathrin-mediated endocytosis (Cirrito, Kang et al. 2008). Following neuronal depolarization and neurotransmitter release, synaptic vesicles must be formed and recycled in order to maintain an adequate number of vesicles for transmitter release during neuronal activation. Thus it is hypothesized that synaptic vesicle recycling during neuronal activation concurrently results in increased internalization of APP embedded in the plasma membrane, and therefore increases amyloidogenic processing by increasing the frequency of encountering β - and γ -secretases that reside in intracellular compartments.

The Endosomal-Lysosomal Pathway (Figure 3)

The endosomal-lysosomal pathway has become an active area of investigation in the context of neurodegenerative disorders characterized by an aberrant accumulation of proteins. Macromolecules and transmembrane proteins such as APP that are targeted for degradation may enter the endosomal-lysosomal pathway by undergoing endocytosis, autophagy or phagocytosis. As previously discussed, APP is commonly internalized from the plasma membrane via endocytosis and processed in the secretory and lysosomal pathways. APP synthesized in the ER and transported to the TGN can also traffic directly to the endosome by binding the adapter AP-4 (Burgos, Mardones et al. 2010). In addition, once in the endosome, APP may be transported back to the TGN via retromer proteins (Vieira, Rebelo et al. 2010), notably for APP by interacting with the sortilin related receptor (SORLA). Thus, transference of various forms of APP and its fragments occurs via the highly dynamic membrane enclosed vesicular structures that are compositionally and functionally distinct. These structures have been well characterized and include the early endosome, recycling endosome, late endosome and lysosome (Huotari and Helenius 2011).

The maturation model is based on the concept that endosomes go through defined stages as they mature, and that the compositional changes that occur during this process result in functionally distinct roles for endosomes in each stage of maturation. The early endosome is associated with the small GTPase rab5 and has a slightly acidic intraluminal pH, allowing for receptor cargo to readily dissociate and be sorted. This allows receptors to recycle back to the cell surface while ligands proceed to the late endosome and lysosome for degradation. As the early endosome accumulates an increasing number of intraluminal vesicles (ILVs) and increases intraluminal acidity, it also moves toward the microtubule organizing center and associates with different rab proteins. These alterations indicate a transition from the early endosome stage to the late endosome stage. The function of the late endosome is to generate ILVs and to serve as a sorting compartment for macromolecules destined for lysosomal degradation. Critical to this process, are the endosomal sorting complexes required for transport (ESCRT) machinery. ESCRT is comprised of complexes 0-III that bind ubiquitinated cargo and generate intraluminal vesicles that are delivered to lysosomes and degraded (Edgar, Willen et al. 2015). Late endosomes are associated with the small GTPase rab 7, and are the stage in which multivesicular bodies (MVB) may form (Hu, Dammer et al. 2015). MVBs may fuse with the cell surface for the release of ILVs as exosomes, fuse with autophagosomes (Figure 5B) to generate amphisomes, or continue down the endosomal-lysosomal pathway. Facilitated by ESCRT machinery, MVBs or late endosomes may fuse with lysosomes, resulting in a highly acidic intraluminal environment in which hydrolases degrade cargo contents. Thus, the endolysosomal pathway is a complex and branching pathway with multiple points of entry and exit. Understanding the transport of APP through this complex system under normal physiological conditions and conditions that drive amyloidogenic processing in the rodent and the human is a crucial knowledge gap that must be investigated to leverage the intrinsic ability of neurons to remove and degrade A β ₄₂ and β -CTFs, and perhaps develop disease modifying therapeutics at increasing the efficacy of these systems in doing so.

Investigation of ESCRT machinery in the context of APP processing, and A β ₄₂ production have suggested that APP is trafficked in a subpopulation of MVBs that are destined to the lysosome for degradation, and further, suggests a role for early ESCRT components in limiting A β ₄₂ accumulation by promoting lysosomal targeting (Edgar, Willen et al. 2015). Closely paralleled with these observations, other investigators have found that autophagosomes are key organelles that connect with the MVB/lysosomal pathway for efficient turnover of CTFs. These investigators demonstrated that the fusion of autophagosomes with endolysosomal compartments cause an increase in C99 levels that accumulated primarily in structures resembling ILVs of MVBs, while activation of autophagosome formation enhanced lysosomal clearance of C99 CTFs. Thus supporting a model in which autophagosomes cooperate in the turnover of C99 and APP, forming an amphisome-like compartment by fusing with MVBs before fusion with lysosomes. Another pathway for removal of CTFs from the MVB is within exosomes secreted from the cell, which are enriched in β -CTFs in particular (Levy 2017). Further, conditions that compromise the fusion of autophagosomes to endosomal compartments accumulate C99 CTFs (Gonzalez, Munoz et al. 2017). Interestingly, another recent study identified Tetraspanin 6 (TSPAN6), a protein enriched in MVBs and ILVs, as a pivotal point in the pathway of ILV towards exosomes or lysosomal degradation. Heightened expression of TSPAN6 resulted in increased endosomal size, increased number of ILVs and increased secretion of exosomes containing APP CTFs (Guix, Sannerud et al. 2017). Electron microscopy and ultrastructural analysis indicated a higher number of endosomal compartments with non-degraded material in the lumen as well as a larger number of ILVs (Guix, Sannerud et al. 2017).

Protocol

Electron Microscopy (Figure 4)

Selection of Antibodies and Experimental Design—Schmidt et al. (Schmidt, Jiang et al. 2005, Schmidt, Mazzella et al. 2012) have previously described tissue homogenate treatments to extract membrane-bound and soluble fragments of APP prior to Western Blot (WB), immunoprecipitation (IP) and enzyme-linked immunosorbent assay (ELISA), techniques that have been applied to mouse brain to characterized each APP fragment derived from the endogenously expressed APP (Morales-Corraliza, Mazzella et al. 2009). Here, we describe the antibodies routinely used in our laboratories that may be used on total homogenate or separate soluble and membrane fractions to reveal various fragments of APP. We also describe antibodies that may be used on tissue sections for immunohistochemistry. The C1/6.1 antibody binds the C-terminus of APP, thus detects all full-length APP and related CTFs. Following BACE-1 cleavage, antibody JRF/N25 can detect the C99 CTF in models expressing human APP, as well as subsequent fragments A β ₄₀ and A β ₄₂. Antibody m3.2 has similar reactivity against murine APP derived fragments. Subsequent cleavage of the C99 CTF, between 38–41 amino acids by the γ -secretase results in the release of the A β ₄₀ and A β ₄₂ peptides, respectively. To readily distinguish fragments A β ₄₀ and A β ₄₂, the N-terminal specific antibodies JRF/cA β ₄₀/10 and JRF/cA β ₄₂/26 may be used, respectively [Figure 1, (Morales-Corraliza, Mazzella et al. 2009, Schmidt, Mazzella et al. 2012, D. Goodsell 2015)].

Tissue Preparation—Male and female rodents used for immunohistochemistry (IHC) were deeply anesthetized using isoflurane (Vedco, St. Joseph, MO) and subsequently transcardially perfused through the ascending aorta with the following fixatives: 10 ml of 1000 units/ml heparinized saline and 50 ml of 3.75% acrolein in 2% formaldehyde pH 7.4. Acrolein fixation yields optimal tissue preservation for ultrastructural analysis, and has been shown to preserve A β ₄₂ peptides within the intracellular compartments of the cell, in contrast to paraformaldehyde-only perfused tissues (Takahashi, Milner et al. 2002). The brains were removed, cut into 4–5 mm coronal blocks, stored in 2% paraformaldehyde fixative for 30 minutes and then sectioned (30–40 μ m) on a vibrating microtome (Vibratome; Pelco EasiSlicer, Ted Pella, Redding, CA). Brains were cut in 40 μ m coronal sections, and sections from regions of interest (LC) were selected for IHC processing using the rat brain atlas of Paxinos and Watson (Paxinos, 1986).

Immunohistochemistry—Acrolein-perfused tissues were incubated in 1% sodium borohydride in 0.1 M PB to remove reactive aldehydes, and then blocked in 0.5% BSA in 0.1 M Tris-buffered saline (TBS). Tissue sections were incubated for 48 hours at 4°C in primary antibody in 0.1% BSA in 0.1 M TBS.

Secondary antibodies used for electron microscopy include biotinylated (1:400) and 1 nm gold particle conjugated IgG (1:50) (Electron Microscopy Science, Hatfield, PA). Immunoperoxidase labeling may be performed in addition to immunogold labeling for dual electron microscopy. The examples provided in figures 2 and 4 show immunoperoxidase labeling of tyrosine hydroxylase (TH) to identify cell bodies of the locus coeruleus, though investigators could use a variety of other antigens to identify other cell populations, or intracellular markers of interest. Electron-dense labeling is detected via silver intensification of immunogold particles using a silver enhancement kit (Aurion R-GENT SE-EM kit, Electron Microscopy Science). The examples provided in figures 2 and 4 show immunogold conjugated secondary antibodies that are bound to MOAB-2 primary antibody. Tissues were prepared for visualization under the electron microscope with osmification, serial dehydration, flat-embedding, and tissue sectioning at 74 nm on an ultramicrotome (Commons, Beck et al. 2001). Sections were collected on copper mesh grids and examined using an electron microscope (Morgani, Fei Company, Hillsboro, OR). Digital images were viewed and captured using the AMT advantage HR HR-B CCD camera system (Advance Microscopy Techniques, Danvers, MA). Electron micrograph images were then prepared using Adobe Photoshop to adjust the brightness and contrast.

Ultrastructural Analysis

Controls and Criteria—Adequate preservation of ultrastructural morphology is one of the criteria imposed when selecting tissue sections to be used for ultrastructural analysis. A minimum of 3 sections per region of each animal were used for analysis. At least 10 grids containing 4–7 thin sections each were collected from plastic-embedded sections of the regions of interest from each animal. Quantitative evaluation of immunoreactive elements was applied only to the outer 1–3 μ m of the epon–tissue interface where penetration of antibodies is optimal. To prevent the inclusion of spurious labeling in quantification, only profiles with a minimum of 2 gold particles were considered immunoreactive and used for

quantification. For dual labeling, only micrographs containing both peroxidase and gold–silver markers were used for the tissue analysis to ensure that the absence of one marker did not result from uneven penetration of markers (Leranth and Pickel, 1989).

For each animal, comparable levels of the region of interest were selected for ultra-thin sectioning. Dendritic and axon-terminal profiles were sampled from at least 5 copper grids of ultrathin tissue sections near the tissue-plastic interface. All profiles were scanned and selected for analysis based on the following criteria. Dendrites with a maximal cross-sectional diameter between 0.7 μm and 5 μm , and a mix of dendrites of sizes from across this range were included in the analysis. Large profiles were excluded to avoid the bias towards positive labeling of larger structures. Extremely small, large, longitudinal and irregularly shaped profiles were excluded from the analysis due to possibly higher perimeter/surface ratios and risk of biasing the silver grain counts towards the membrane. Any profiles containing large, irregularly shaped silver grains of more than 0.25 μm were excluded from the analysis. Cellular profiles that fail to meet any of the described criteria were excluded from analysis.

Trafficking and Identification of Organelles—Cellular elements were isolated and classified based on Fine Structure of the Nervous System (Peters 1991). Somata were identified by the presence of a nucleus, Golgi apparatus, and smooth endoplasmic reticulum. Proximal dendrites contain endoplasmic reticulum, were typically apposed to axon terminals, and were larger than 0.7 μm in diameter. Synapses were verified by the presence of a junctional complex, a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or associated postsynaptic thickening. A synaptic specialization was only designated to the profiles that form clear morphological characteristics of either Type I or Type II (Gray 1959). Asymmetric synapses were identified by thick postsynaptic densities (Gray's Type I; Gray 1959), while symmetric synapses had thin densities both pre- and post-synaptically (Gray's Type II; Gray 1959). An undefined synapse was defined as an axon terminal plasma membrane juxtaposed to a dendrite or soma devoid of recognizable membrane specializations and no intervening glial processes. Axon terminals were distinguished from unmyelinated axons based on synaptic vesicle presence and a diameter of greater than 0.1 μm .

The recognition of endolysosomal compartments at various stages of processing were recognized morphologically using electron microscopy (Figures 2, 5). Early endosomal compartments may be identified by the presence of a central vacuole of ~100–500 nm diameter, that is often referred to as the sorting endosome. This structure is primarily electron lucent, or clear, and is frequently visualized with a cytoplasmic clathrin coat. Extending from the main sorting endosome, are smaller tubules structures that are part of the recycling endosomal system (Klumperman and Raposo 2014) (Figure 3, green). Occasionally, endosomes in this state contain a few ILVs, which range in size from 40 to 100 nm (Murk, Humbel et al. 2003, Klumperman and Raposo 2014). Late endosomal compartments are comprised of a vacuole 250–1000 nm in diameter, and budding compartments that connect to the TGN (Figure 3, blue). Various EM studies have used the increasing number of ILVs within these compartments to pinpoint the switch from the early endosome to the late endosome that is thought to occur in the range of five to eight ILVs

(Mobius, van Donselaar et al. 2003, Murk, Humbel et al. 2003, Mari, Bujny et al. 2008). Thus, late endosomes are more readily distinguishable when there are greater numbers of ILVs, at which point they are frequently referred to as MVBs (Klumperman and Raposo 2014) or dense core vesicles (dcv) (see Figure 5D).

Lysosomes (Figure 5 A, E) are the terminal degradative compartment receiving cargo from the endosomal and autophagy pathways. They are spherical organelles with diameters between 200 nm and $>1\ \mu\text{m}$, and typically have an electron dense lumen, reflecting high protein concentrations (Bainton 1981, Klumperman and Raposo 2014). When lysosomes are supplied with cargo from the autophagy pathway, autolysosomes are formed (Figure 5B). Autolysosomes are generally known to be larger and more irregularly shaped than lysosomes, with a highly variable content. Autophagosomes are known to have a double limiting membrane and the cytoplasm inside has the same appearance as the cytoplasm outside the autophagosome. As noted in a cautionary piece of literature on the morphological analysis of autophagy-related structures, this is frequently a source of inaccuracy, as empty vacuoles and sometimes enlarged mitochondria may be identified as endolysosomal and autophagy compartments based on the presence of their limiting membrane alone (Eskelinen 2008). For an extensive ultrastructural analysis of how these components are altered under conditions of degeneration, and how disruption of the endolysosomal system can influence the efficiency of autophagy we refer the reader to (Nixon, Wegiel et al. 2005).

Quantification—Quantification and analysis may be conducted as previously reported (Commons, Beck et al. 2001, Oropeza, Mackie et al. 2007). To further define the subcellular distribution of the APP fragments of interest and their trafficking under various treatment conditions, distribution ratios may be calculated for each subcellular compartment of interest for each animal in naïve and experimental treatment conditions, as we have previously reported (Oropeza, Mackie et al. 2007). To calculate the distribution ratio, investigators counted the number of immunogold particles within each subcellular compartment of interest. A ratio of immunogold particles identified within the compartment of interest to total immunogold particles per profile was computed. An average of ratios for each animal was taken. A one-way analysis of variance (ANOVA) was used to determine if there were within-group differences in the distribution ratio among animals receiving identical experimental conditions. If no differences were detected between animals within the same experimental group, data from these animals were pooled and an average distribution ratio from the animals under each experimental condition was calculated. ANOVA was used for within group comparisons to determine the presence of statistically significant shifts in the distribution of immunoreactivity. Tukey's post-hoc tests were used for between group comparisons.

Discussion and Recommendations

Previous studies have utilized electron microscopy to identify the subcellular location of A β_{42} peptides in AD transgenic mice as well as in human brains, and have demonstrated that accumulation, which increases with age, occurs in MVB prior to plaque formation. Additionally, the cell bodies in which these MVB were formed had abnormal morphology

and dystrophic neurites (Takahashi, Milner et al. 2002). In another electron microscopy study, this group went on to describe a subtle change in the distribution of oligomerized A β in vitro and in vivo. Monomeric A β ₄₂ aggregated on the outer membranes of endosomal vesicles and MVBs, while A β ₄₂ oligomers were distributed to the inner membranes of morphologically abnormal endosomal organelles and to microtubules (Takahashi, Almeida et al. 2004).

Combining the use of antibodies specific to various fragments of APP and electron microscopy may yield important information about subtle changes in the distribution of various fragments throughout the cell, as well as indicate signs of neuronal injury or altered morphology of the organelles to and from which the fragments are being trafficked. This may be particularly important for studies aiming to assess early markers of disease such as early-endosome abnormalities, altered cholesterol metabolism or other signs of neuronal injury and dysregulation of lysosomes such as the presence of lipofuscin, which is identified morphologically at the ultrastructural level using electron microscopy. Of note, we describe here a protocol that utilizes a combination of paraformaldehyde and acrolein as tissue fixatives, which has been shown to preserve intraneuronal A β species, in contrast to tissues perfused with paraformaldehyde alone which can show APP and its cleavage products primarily on the plasma membrane.

We have previously used various antibodies to successfully localize endogenous murine A β by EM: MOAB2 specifically recognizes intraneuronal 42 amino acid long A β , while D54D2 recognizes endogenous A β ₄₂, A β ₄₀, A β ₃₉, A β ₃₈, and A β ₃₇ that may result from variable γ -secretase cleavage. Each antibody was determined to be specific using APP KO mice and rat liver slices, where APP expression and A β are low (Ross, Reyes et al. 2017). Inclusion of negative controls either lacking APP expression or, for instance β -cleaved APP products such as β -CTFs and A β in a BACE-1 KO model, are valuable tools when assessing the specificity of an antibody immuno-EM signal. Additionally, antibodies such as those described in Table 1, can be used to detect APP itself as well as other APP fragments. For example, C1/6.1 binding will illustrate the distribution of APP and the CTFs within a cell. The distribution of additional APP fragments can be inferred by the localization of two antibody-binding patterns overlaid in a single section. For example, luminal JRF/N25 labeling in an endosome may be either β -CTFs or A β based upon the fragment-specificity of this antibody. Close juxtaposition of this signal with cytoplasmic C1/6.1 is consistent with β -CTF, which contains the C-terminal APP domain recognized by C1/6.1 but missing in the A β peptides. While these types of analyses are demanding, and require differentially directly-conjugated primary antibodies when both antibodies are mouse monoclonal antibodies, these are the approaches that are necessary to precisely define the subcellular localization of APP fragments. As noted above for A β identification, the addition of KO tissue where a specific APP cleavage event does not occur can be valuable. In the above scenario using JRF/N25 and C1/6.1 to identify β -CTFs, concurrent immunolabeling of BACE-1 KO would show the distribution of APP and α -CTFs detected by C1/6.1 in cells lacking any specific JRF/N25 binding and lacking β -CTFs.

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Figure 1: APP Fragments and Specific Antibody Recognition Sites.

The proteolytic processing of APP generally occurs via two divergent pathways. The non-amyloidogenic pathway is the most common route of processing in most cells, and results from cleavage of APP by α -secretases embedded in the plasma membrane. This cleavage results in the formation of sAPP α composed of amino acids 18–687 and a carboxyl terminal C83 fragment (α -CTF) composed of amino acids 688–770. The amyloidogenic pathway accounts for a smaller portion of APP processing. In this pathway, APP undergoes proteolytic cleavage by the aspartic protease β -secretase (BACE-1), which cuts APP on the luminal side of the membrane, releasing a soluble APP β fragment (sAPP β) composed of amino acids 18–671, and carboxyl terminal C99 fragment (β -CTF) composed of amino acids 672–770 (Vassar, Bennett et al. 1999). BACE-1 cleavage results in the formation of a new N-terminus with the first aspartic amino acid 672 of A β (LaFerla, Green et al. 2007), which is a neo-epitope detected by some antibodies. The C1/61 antibody binds the C-terminus of APP, thus detecting all full-length APP and related CTFs. Following BACE-1 cleavage, antibody JRF/N25 detects the C99 β -CTF. Subsequent cleavage of this β -CTF, at 38–43 amino acids downstream of this β -cleavage site by the γ -secretase, results in the release of the A β ₄₀, A β ₄₂, and to a lesser extent other A β peptides. To readily distinguish fragments A β ₄₀ and A β ₄₂, the N-terminal specific antibodies JRF/cA β 40/10 and JRF/cA β 42/26 may be used, respectively. The structure of the APP molecule is attributed to David S. Goodsell and the RCSB Protein Data Bank (PDB), and was modified to depict fragments following proteolytic cleavage (D. Goodsell 2015).

Figure 2. Distinguished labeling patterns of 22C11, APP-β, and MOAB-2 antibodies in the naïve rat.

The epitopes labeled by three different antibodies within the APP protein readily distinguish three distinct fragments with unique distributions. The 22C11 (green) antibody labels an epitope that reveals full length APP, while the APP-β (red) antibody labels sAPP-β, the fragment resulting from BACE-1 cleavage of full-length APP, and MOAB-2 (blue) an antibody that labels the intracellular fragment that results from γ-secretase cleavage of the sAPP-β fragment. can be readily distinguished using low-resolution immunofluorescence techniques. Individually labeled puncta in close proximity (panel a,a') may represent full length, membrane-bound APP and highlights the distinct region of the APP peptide in which the antibody-specific epitope is present; for example, 22C11 labeling (green) is the N-terminal extracellular region of APP, while MOAB-2 (blue) embedded within the membrane, and APP-β (red) that labels the C-terminus is facing the cytoplasmic side of the plasma membrane. There are several occurrences of co-localization, which primarily occur between the 22C11 and APP-β antibodies (yellow puncta), that indicate β-CTF labeling. Importantly, there are few occasions in which MOAB-2 labeling is co-localized with APP-β (magenta), which may be readily distinguished from γ-secretase cleavage products identified by MOAB-2 labeling alone. There were no occurrences of MOAB-2 immunoreactivity with 22C11 labeling. MOAB-2 is also more frequently visualized as individual puncta, and further away from 22C11 and APP-β immunoreactivity, alluding to the putative intracellular

localization of A β ₄₂ peptides. However, it should be noted that electron microscopy is necessary to define the subcellular localization of these fragments with any certainty.

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Figure 3: APP Processing and the Endolysosomal System.

The fate of APP is dictated largely by its subcellular localization, thus highlighting the importance of trafficking in parallel with proteolytic cleavage. Transmembrane proteins such as APP that are targeted for degradation enter the endosomal-lysosomal pathway by undergoing endocytosis, autophagy or phagocytosis. APP is internalized from the plasma membrane via endocytosis and further processed in endocytic, recycling and lysosomal compartments. In addition, once in the endosome, APP may be transported back to the TGN (**G, blue**) via retromer proteins (Vieira, Rebelo et al. 2010), following recognition by the sortilin related receptor (SORLA). Thus, transference of various forms of APP and its fragments occurs via the highly dynamic membrane enclosed vesicular structures that are compositionally and functionally distinct. These structures have been well characterized and include the early endosome, recycling endosome, late endosome (**End, green**) and lysosome (**Lys, red**) (Huotari and Helenius 2011). Arrow heads point to immunogold labeled A β_{42} .

Figure 4: Electron microscopy.

Following immunohistochemical procedures, tissues are prepared for visualization under the electron microscope with osmification, serial dehydration, flat-embedding, and tissue sectioning at 74 nm on an ultramicrotome (Commons, Beck et al. 2001). Sections are collected on copper mesh grids and examined using an electron microscope (Morgani, Fei Company, Hillsboro, OR). Digital images are viewed and captured using the AMT advantage HR HR-B CCD camera system (Advance Microscopy Techniques, Danvers, MA). Electron micrograph images are then prepared using Adobe Photoshop to adjust the brightness and contrast.

Figure 5. A β ₄₂ subcellular localization.

A. Immunoelectron micrographs of TH-immunoreactive dendrites (**TH-d**), one of which is dually labeled with immunogold A β ₄₂ (arrow heads). More specifically, A β ₄₂ is localized to a lysosomal (**Lys**) compartment within the dendrite, identified at the ultrastructural level. **B.** Example of an autolysosome that contains heterogeneous mixture of electron dense materials, including immunogold labeled A β ₄₂. **C.** Immunogold labeled A β ₄₂ is localized to axon terminals (**at**) presynaptic to TH immunolabeled dendrite. Here, immunogold labeled A β ₄₂ is associated with mitochondrial membranes (**m**). **D.** Immunoelectron micrograph of

immunogold labeled A β ₄₂ localized to an axon terminal filled with dense core vesicles (**dcv**), a subcellular compartment derived from multivesicular bodies that frequently contain neuropeptides co-packaged with fast acting neurotransmitters that may be released from synaptic sites. **E.** TH-immunolabeled cell body that contains several lysosomes with immunogold labeled A β ₄₂; immunogold labeled A β ₄₂ is also present on the cell surface, potentially indicating secretion into the extracellular space.

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Table 1:

Antibodies for IHC, IP, WB

Antibody	Source	Epitope	Fragments Detected	Applications	References
22C11	Millipore (MAB348)	Amino acids 66–81 of N-terminal APP	All three isoforms of APP: immature ~110kDa, sAPP ~120kDa, and mature ~130kDa	ELISA, IHC, IP and WB	(Hoffmann, Twisselmann et al. 2000)
C1/6.1	Laboratory of Dr. Paul Mathews New York University & Nathan Klein Institute	C-Terminus residues 676 – 695 of APP695	APP holoprotein but not sAPP	IP, ICC, IHC, WB	(Mathews, Jiang et al. 2002) (Jiang, Mullaney et al. 2010)
Alternative Commercially available: APP-β	Thermo Fisher (51–2700)	22 amino acid residues of C-terminus	sAPP- β	WB, IF,IHC,ELISA	
M3.2	Laboratory of Dr. Paul Mathews New York University & Nathan Klein Institute	residues 1–15 of the A β peptide	APP holoprotein, sAPP α , β -CTF and A β	ELISA, IHC, IP, WB	(Choi, Berger et al. 2009) (Morales-Corraliza, Mazzella et al. 2009)
Alternative Commercially available: D54D2	Cell Signaling	N-terminus of A β	A β ₄₂ , A β ₄₀ , A β ₃₉ , A β ₃₈ , and A β ₃₇ .	IHC 1:100	(Ross, Reyes et al. 2017)
JRF/cAβ 42/26	Laboratory of Dr. Paul Mathews New York University & Nathan Klein Institute	recognizes the C-terminus of A β ₄₂ ; does not detect A β ₄₀ or full-length APP	residues 33–42 of the A β 1–42 peptide	A β 1–42	(Vandermeeren, Geraerts et al. 2001, Mathews, Jiang et al. 2002, Schmidt, Nixon et al. 2005) (Janus, Pearson et al. 2000)
Alternative Commercially available: MOAB2	Kerafast	Amino acids 1–4 of A β ₄₂	A β ₄₂	IHC; 1:1000	(Youmans, Tai et al. 2012, Ross, Reyes et al. 2017)