A Role for Lysosomal PH Dysfunction in Alzheimer’s Disease and Strategies for its Restoration

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A Role for Lysosomal PH Dysfunction in Alzheimer’s Disease and Strategies for its Restoration

Abstract
Alzheimer’s disease (AD) is the most common form of dementia, leading to memory loss progressive cognitive decline over the course of what can be many years. Mutations in the catalytically active component of the γ-secretase complex, presenilin 1 (PS1), are the most common cause of familial Alzheimer’s disease (fAD), a less-prevalent but earlier-onset form of AD. PS1 mutation is associated with more severe lysosomal and autophagic pathologies than are found in sporadic AD; these pathologies may be a result of lysosomal pH dysregulation. The goal of this dissertation was to confirm a role for elevated lysosomal pH in cells from PS1-fAD patients, to investigate the repercussions of this lysosomal dysfunction, and to identify a therapeutic approach by which to restore both pH and lysosomal pathology to normal. Using human skin fibroblasts containing the PS1-fAD mutation A246E, the present work identified a small but significant increase in lysosomal pH in PS1-fAD mutant cells when compared to control fibroblasts. The pH data were supported by a reduction in mature cathepsin D (Cat D) and Cat D active site availability in PS1-fAD cells, as well as by substantial accumulation of autophagic substrates and up-regulation of components of the lysosomal and autophagic degradative systems, both at the mRNA and protein levels. Treatment with cAMP proved restorative, bringing lysosomal pH in PS1-fAD cells back to baseline while having minimal effect on control cells. cAMP increased Cat D active site availability, reduced autophagic backlog, and led both to mTOR phosphorylation and to down-regulation of genes involved in lysosomal function. Interestingly, cAMP-induced pH restoration, as well as Cat D increase and mTOR phosphorylation, was found to be PKA-dependent, suggesting a signaling pathway that may serve as a useful target for future treatment, and implicating PKA in the immediate, upstream response to cAMP treatment, and mTOR and gene expression modulation in the downstream response. This treatment proved effective in compromised lysosomes from primary neuronal cultures, as well, supporting the general utility of a cAMP-based approach. Together, these results identify lysosomal pH elevation as an important factor in AD pathology, and suggest several possible targets for future therapeutic investigation.

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A ROLE FOR LYOSOMAL PH DYSFUNCTION IN ALZHEIMER’S DISEASE
AND STRATEGIES FOR ITS RESTORATION

Erin E. Coffey

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Dedication

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ABSTRACT

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AND STRATEGIES FOR ITS RESTORATION

Erin E. Coffey
Claire H. Mitchell, Ph.D.

Alzheimer’s disease (AD) is the most common form of dementia, leading to memory loss progressive cognitive decline over the course of what can be many years. Mutations in the catalytically active component of the γ-secretase complex, presenilin 1 (PS1), are the most common cause of familial Alzheimer’s disease (fAD), a less-prevalent but earlier-onset form of AD. PS1 mutation is associated with more severe lysosomal and autophagic pathologies than are found in sporadic AD; these pathologies may be a result of lysosomal pH dysregulation. The goal of this dissertation was to confirm a role for elevated lysosomal pH in cells from PS1-fAD patients, to investigate the repercussions of this lysosomal dysfunction, and to identify a therapeutic approach by which to restore both pH and lysosomal pathology to normal. Using human skin fibroblasts containing the PS1-fAD mutation A246E, the present work identified a small but significant increase in lysosomal pH in PS1-fAD mutant cells when compared to control fibroblasts. The pH data were supported by a reduction in mature cathepsin D (Cat D) and Cat D active site availability in PS1-fAD cells, as well as by substantial accumulation of autophagic substrates and up-regulation of components of the lysosomal and autophagic degradative systems, both at the mRNA and protein levels. Treatment with cAMP proved restorative, bringing lysosomal pH in PS1-fAD cells back to baseline while having minimal effect on control cells. cAMP increased Cat D active site availability, reduced autophagic backlog, and led both to mTOR phosphorylation and to down-regulation of genes involved in lysosomal function. Interestingly, cAMP-induced pH restoration, as well as Cat D increase and mTOR phosphorylation, was found to be PKA-dependent, suggesting a signaling pathway that may serve as a useful target for future treatment, and implicating PKA in the immediate, upstream response to cAMP treatment, and
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CHAPTER 1:

Introduction
An introduction to the lysosome

The lysosome’s discovery came about, as many scientific discoveries do, as a fortuitous accident. In the early 1950s, the laboratory of Christian de Duve was devoted primarily to the study of insulin signaling in the liver, and in particular to the role that acid-precipitable glucose-6-phosphatase might play in modulating insulin’s effects. In attempting to distinguish the activity of this phosphatase from that of a related hepatic enzyme, acid phosphatase, the group found that, curiously, acid phosphatase activity seemed to disappear following fractionation, only to reappear following vigorous homogenization or cold storage. At the time, de Duve and colleagues reasoned, correctly, that these conditions that might have led to the release of acid phosphatase from an as-yet-unknown organelle. Only a few years later, they would go on to identify a host of degradative enzymes with similar acid optima, all of which appeared to be found in membrane-limited pockets throughout the cell. De Duve proposed the term “lysosome” to describe these pockets (De Duve et al., 1955). He suggested that this new organelle might have an important and multifaceted digestive function, including degradation and recycling of both extracellular and intracellular materials, protection from bacteria or other toxic agents, cellular differentiation, and perhaps clearance of dead or dying cells (De Duve et al., 1955, De Duve and Wattiaux, 1966). In many of these predictions, de Duve would be proven right.

Our understanding of lysosomal functions continues to evolve in the decades since the organelle’s discovery: we now know that it makes up one part of a dynamic and responsive intracellular network with many interacting players. It is involved in both endocytic and exocytic pathways, in cellular signaling, and in the degradation of organelles and bulk cellular material by way of autophagy, among other roles. This complex system comes complete with sophisticated, but still-poorly understood, feedback mechanisms by which the lysosome can communicate information about its internal state – including its catabolic efficiency and its contents – to cytoplasmic messengers that sit on the lysosomal surface (Zoncu et al., 2011, Cang et al., 2013). These messengers include mechanistic target of rapamycin (mTOR), a serine/threonine kinase
that negatively regulates autophagy, and transcription factor EB (TFEB), a member of the MITF family of transcription factors and the “master switch” for the co-expressed, lysosomal function-related genes that make up the Coordinated Lysosomal Expression And Regulation (CLEAR) network (Sardiello et al., 2009, Palmieri et al., 2011, Settembre et al., 2011, Rocznia-Ferguson et al., 2012, Settembre et al., 2013). TFEB phosphorylation blocks its nuclear translocation; interestingly, TFEB can be phosphorylated by mTOR (Sardiello et al., 2009, Martina et al., 2012, Rocznia-Ferguson et al., 2012, Settembre et al., 2012), or dephosphorylated by the phosphatase calcineurin (Medina et al., 2015), at several sites in response to changing cellular nutrient abundance and lysosomal efficacy. TFEB retention in the cytoplasm therefore leads to reduced expression of CLEAR genes, including genes for lysosomal degradative enzymes and genes that promote lysosomal biogenesis. This coordinated regulatory pathway ensures that the lysosomal degradative system responds quickly to the cell’s current energy needs.

The importance of these feedback mechanisms – which essentially generate an accurate, timely report on lysosomal function – cannot be overstated. The lysosomal lumen is normally maintained at an acidic pH range through the combined actions of the v-(H^+)/ATPase proton pump and a variety of ion channels and transporters (Mindell, 2012). Degradative enzymes that reside within the lumen tend to be highly pH-sensitive, both in their activity and, for some enzymes, in their maturation process. For example, the lysosome’s primary aspartyl protease, Cathepsin D (Cat D), exhibits pH-dependent maturation and pH-dependent activity (Barrett, 1970, Rosenfeld, 1982), with an activity maximum around 3.5 and a steep drop-off thereafter. By the time luminal pH reaches 5.0, Cat D will have lost over 80% of its activity. While Cat D has one of the lower-pH activity maxima of the lysosomal enzymes, many others are similarly pH sensitive, with pH optima over a range of acidic values (Barrett, 1973, Schwartz and Bird, 1977, Pentchev et al., 1978, Santa Cecilia et al., 1991, Ameis et al., 1994). Even the delivery of lysosomal enzymes to the lysosomal lumen is a pH-dependent process: without an acidic environment, the mannose-6-phosphate receptors that shepherd lysosomal enzymes from the Golgi to the lysosomes will not
release their cargo (Gonzalez-Noriega et al., 1980, Borden et al., 1990). On the one hand, this pH specificity helps reduce the risk of cellular damage should the lysosomal membrane rupture and its catabolic enzymes escape into the cytoplasm. On the other hand, such narrowly tuned functionality ensures that even very small increases in lysosomal pH can result in substantial loss of overall activity, and therefore of the lysosome’s ability to degrade its contents, including proteins, lipids, organelles and cellular structures, and protein aggregates. Lysosomal malfunction can also be a factor in both apoptotic and necrotic cell death (Zaidi et al., 2001, Boya et al., 2005, Golstein and Kroemer, 2007), for which the lysosome has gained the rather unfortunate moniker of “suicide bag” (Turk and Turk, 2009). These connections further underscore the lysosome’s importance in maintaining cellular health.

Unfortunately, lysosomal function grows less efficient with age. The so-called “aging pigment” lipofuscin – a dense, autofluorescent residue of incomplete lysosomal degradation – accumulates in lysosomes with age, and especially in post-mitotic cells, which cannot clear material by diluting it through cellular division (Terman and Brunk, 1998a, b, Brunk and Terman, 2002a, b, Gray and Woulfe, 2005). Lipofuscin accumulation and loss of lysosomal efficacy is associated with age-related macular degeneration, and lipofuscin is even believed to effect cellular damage in its own right (Brunk and Terman, 2002a). Several studies have also observed increased lysosomal permeability with age (Brunk and Brun, 1972, Nakamura et al., 1989), which would reduce the ability of the lysosome to maintain the pH and ion gradients necessary for its efficient function (Yao and Zhang, 1997, Deng et al., 2009). Lysosomal pH increase has been linked to increased lipid peroxidation (Guha et al., 2013), which has in turn been linked to increased lysosomal permeability (Johansson et al., 2010, Pourahmad et al., 2011) and to increased lysosomal susceptibility to osmotic stress (Zhang and Yao, 1997). As post-mitotic cells like neurons depend on a high rate of basal autophagy and lysosomal degradation to clear cellular debris (Boland et al., 2008), any one of these changes would make such cells more susceptible to additional damage, as well as substantially less capable of managing an increased degradative burden.
Lysosomal leakage may also prompt NLRP3 inflammasome activation in response to both chemical and pathological stimuli (Halle et al., 2008, Hornung et al., 2008), further strengthening the link between lysosomal health and overall cellular health. Interestingly, chemical treatment to increase the permeability of lysosomal membranes leads to aggregate formation in cultured neurons (Micsenyi et al., 2013), suggesting that lysosomal failure and aggregate formation may result in a “killer cycle” that further impairs lysosomal function. Aggregate formation, both extracellular and intracellular, also happens to be a pathological hallmark of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and others. The lysosome’s role in many of these diseases is only now beginning to be understood.

**The lysosome in neurodegeneration**

While the clinical neuropathology of lysosomal disorders was described in the 1880s, their cause would remain a mystery until the discovery of the lysosome in 1955 (De Duve et al., 1955), and the subsequent realization that catabolic enzymes with low pH optima might localize to that organelle. Prior to this discovery, these congenital “storage disorders” were classified only by the type of material that accumulated inappropriately in cells; most cases presented in early childhood and were accompanied by at least some central nervous system pathology, including cognitive and developmental impairment. Less than a decade after the lysosome’s discovery, however, patient tissue samples from glycogen storage disorder cases – i.e., Pompe’s disease – were found to be deficient in the activity of an α-glucosidase enzyme (Hers, 1963). This enzyme’s activity was also shown to have an acidic pH optimum; Hers and colleagues theorized (correctly) that α-glucosidase might be another lysosome-resident enzyme. This monumental finding proved to be the first instance in which a primary lysosomal deficit was directly linked to a systemic disease. Hers’s contemporaries soon realized that each known storage disorder might also be caused by genetic defect of a lysosomal enzyme and, conversely, that a primary lysosomal deficit might ultimately lead to storage pathology.
The lysosomal storage diseases, or LSDs, represent a diverse group of disorders, each prompted by a mutation in some sort of lysosome-resident protein. The loss of a single enzyme or scaffold protein can have devastating effects upon the lysosome. When the primary substrate of a defective enzyme builds up in quantity inside the lysosome, this accumulation can itself inhibit the activity of other enzymes that are not defective (Hers, 1963, Walkley and Vanier, 2009, Lamanna et al., 2011, Prinetti et al., 2011). In the case of mucopolysaccharidosis type IIIC, for example, a primary defect in the heparan sulfate catabolism enzyme acetyl–coenzyme A:α-glucosaminide N-acetyltransferase, encoded by the gene TMEM76 (Hrebicek et al., 2006), led not only to secondary storage of dermatan sulfate and gangliosides (Lamanna et al., 2011), but also to reduced activity of cathepsins B, H, and L (Kopitz et al., 1993). These findings indicate that even accumulations unrelated to a specific lysosomal enzyme’s activity may, given sufficient time, impact overall lysosomal degradative capacity. Therefore, depending on the disorder, a general build-up of material may lead to broader autophagy dysfunction (Curcio-Morelli et al., 2010), to depletion of lysosomal calcium stores (Lloyd-Evans et al., 2008), and even to neurodegeneration, though the most severely affected brain regions and cell populations may vary with the storage metabolite (Platt et al., 2012). In short, specific disruption of the lysosome can lead to a host of downstream, systemic defects, and effectively models the most extreme outcomes of lysosomal failure. Given that neurons rely upon high rates of basal autophagy and lysosomal degradation as a primary clearance mechanism (Boland et al., 2008), and that lysosomal dysfunction can lead to cell death (as described above), one might predict that mild, but persistent, lysosomal dysfunction could recapitulate many of the pathologies observed in LSDs, including cognitive impairment and a neurodegenerative phenotype.

The lysosome in Alzheimer’s disease

Since the discovery of the amyloid precursor protein nearly thirty years ago, much work in the Alzheimer’s field has focused on the consequences of specific and pathological protein accumulations: whether in extracellular “senile plaques,” or in intracellular “neurofibrillary tangles.”
Over the past two decades, however, accumulating evidence points toward a pivotal role played by dysfunctional lysosomes in Alzheimer’s disease. In many ways, the canonical Alzheimer pathologies resemble certain pathologies of both lysosomal storage disorders (Nixon, 2004, Lloyd-Evans et al., 2008, Nixon et al., 2008) and age-related retinal degenerative diseases (Isas et al., 2010, Ding et al., 2011, Kaarniranta et al., 2011, Ohno-Matsui, 2011, Sivak, 2013), which are also known to have a lysosomal component. Further strengthening this connection, patients with the LSD Niemann-Pick type C (NPC) have high cerebrospinal fluid levels of the same pathological proteins found in Alzheimer patients (Mattsson et al., 2011). The following section will provide a basic introduction to the disease’s clinical and pathological correlates, as well as an overview of the inherited genetic mutations that essentially guarantee that an individual will develop Alzheimer’s disease pathology.

A general introduction to Alzheimer's disease

Alzheimer's disease (AD) is the single most common form of dementia (Barker et al., 2002) and the sixth most common cause of death in the United States; it is currently estimated to affect about one-third of Americans aged 85 and older (Alzheimer's Association, 2015). Only a fraction of patients with AD develop the disease as a direct consequence of genetic mutation (“familial AD,” or fAD); the rest of AD cases (“sporadic AD”) are believed to arise from a combination of genetic and environmental risk factors (Bekris et al., 2010), of which the most prominent risk factor is increasing age (Lindsay et al., 2002). It is currently believed that by the time a patient presents with cognitive symptoms, the pathological processes of AD have been underway for decades (Jack et al., 2009, Villemagne et al., 2013). Clinically, the disease is characterized by progressive memory loss and increased cognitive deficit, including confusion, mood changes, impaired judgment, and even loss of basic skills and functions (Alzheimer's Association, 2015). After what can be many years of decline, AD results in widespread neuronal loss and cortical atrophy (Mattson, 2004). Advanced AD patients require long-term, round-the-clock care, posing a considerable emotional and financial burden on caregivers and patients alike. By 2050, the total
annual payments made for care of patients with AD and other dementias is projected to rise to $1 trillion or more (Alzheimer's Association, 2015). Unfortunately, current treatments do little more than temporarily slow the course of the disease (Raschetti et al., 2007; Raina et al., 2008). Even with treatment, AD patients ultimately succumb to their condition: in the final stages of disease, patients are typically immobile and more vulnerable to potentially fatal complications, including pneumonia, swallowing disorders, and malnutrition (Alzheimer's Association, 2015). Given the predicted increase in prevalence of the disease – both as the baby boomer generation ages, and as medical advances lead to overall longer life expectancy – Alzheimer’s disease represents not only a substantial public health issue for the 21st century, but an economic one, as well.

While AD often presents concurrently with other dementias, the most basic cellular pathology of the disease has been known for over a century. In 1906, German psychiatrist Dr. Alois Alzheimer was one of the earliest to describe the “characteristic serious disease of the cerebral cortex” (Maurer et al., 1997); he later went on to extensively detail the disease's neuropathology. Alzheimer's description of both plaques and tangles in the cortex of his patient would come to define the major pathological hallmarks of AD, although the primary components of each – aggregate fibrils of amyloid-β peptide in plaques, and hyperphosphorylated, microtubule-associated protein tau in tangles – would not be identified until decades later (Glenner and Wong, 1984a, b; Kosik et al., 1986; Nukina and Ihara, 1986). Pathology and consequent cell loss is first observed in the hippocampus, but later progresses beyond this region to affect much of the brain (Scalhille et al., 2002). By the final stages of disease, the most dramatically affected brain regions may have lost nearly 85% of their neurons and 66% of their volume (Bobinski et al., 1996). While neurofibrillary pathology appears to be more closely associated with regional atrophy and cognitive decline in humans (Bobinski et al., 1996), the amyloid-β production pathway is more immediately relevant to the present work, and will be the primary focus of the paragraphs to follow. For further information on tau, Hanger et al. recently authored a brief but comprehensive review of tau and neurodegenerative pathology (Hanger et al., 2014).
Amyloid-β peptide, which aggregates into oligomers and fibrils that form the primary constituents of extracellular neuritic plaques, is derived from the transmembrane amyloid precursor protein (APP). APP undergoes two sequential cleavage events that can generate a variety of peptides and peptide fragments. The first cleavage event is mediated by either α- or β-secretase – now known to be the enzymes ADAM10 (Jorissen et al., 2010, Kuhn et al., 2010) and BACE1 (Vassar et al., 1999), respectively. The second cleavage event is mediated by the catalytically active component of the γ-secretase enzyme complex: presenilin 1 (Selkoe and Wolfe, 2007). It is believed that one or both of these sequential APP cleavage events may take place within the endo-lysosomal system (Tagawa et al., 1992, Koo and Squazzo, 1994, Kawarabayashi et al., 1997, Pasternak et al., 2004, Yu et al., 2004). Cleavage of APP by β-secretase sets amyloid-β generation in motion. This form of the amyloid peptide is found as two primary species: amyloid-β_{40} and amyloid-β_{42} (Esler and Wolfe, 2001); the latter form appears to be more prone to aggregation into plaques (Roher et al., 1993). Early work implicated plaque formation in cellular toxicity; over the past two decades, however, the field has come to focus more upon the soluble oligomers of amyloid-β peptide (Terry et al., 1991, McLean et al., 1999, Mucke et al., 2000), as well as on its potential intracellular buildup and toxicity prior to plaque deposition (Zhang et al., 2002, Gouras et al., 2005, Oddo et al., 2006).

*Presenilin: more than just an amyloid machine*

While sporadic AD constitutes the majority of AD cases, the etiology of the disease remains largely unknown. In contrast, the causes of familial AD are better understood and thus provide useful insights for the study of overall disease progression. In the majority of familial Alzheimer’s cases, the precipitating event is a mutation in the aspartyl protease presenilin 1 (PS1). PS1 (Fig. 1.1) is a membrane-bound enzyme with nine membrane-spanning domains (Spasic et al., 2006), and is the catalytically active subunit of the γ-secretase complex, of which the other components are nicastrin, aph-1, and pen-2 (De Strooper, 2003). To date, more than 150 mutations in PS1
have been linked to AD (De Strooper et al., 2012); many of these mutations are found in one of the transmembrane regions, rather than at the PS1 catalytic site (Dillen and Annaert, 2006, De Strooper et al., 2012). AD-associated mutations in presenilin are autosomal dominant and invariably lead to an accelerated, more severe onset of disease (Bateman et al., 2011).

Since its discovery, however, presenilin has been shown to play a role in more than just APP cleavage and amyloid production. Around the time that PS1 gene mutation was linked to Alzheimer’s disease (Sherrington et al., 1995), its Caenorhabditis elegans homolog was found to play a critical role in Notch cleavage and signaling (Levitan and Greenwald, 1995); human PS1 has since been found to cleave numerous type I membrane-spanning proteins, including Notch (Berezovska et al., 1999, Struhl and Adachi, 2000). The enzyme also interacts with the transmembrane domains of other proteins, including proteins that are not substrates of γ-secretase. PS1 has been shown to interact with the neuron-specific intercellular adhesion molecule telencephalin (Annaert et al., 2001); this protein accumulates in cells that lack PS1, and its trafficking appears to be misdirected (Esselens et al., 2004). PS1 may also play a role in the glycosylation and trafficking of various transmembrane proteins, including TrkB (Naruse et al., 1998) – the tyrosine kinase receptor for brain-derived neurotrophic factor (BDNF) – and fellow γ-secretase component nicastrin (Herreman et al., 2003), as well as APP itself (Kaether et al., 2002, Cai et al., 2003). In the absence of PS1, TrkB glycosylation was reduced and, if it reached the cell surface at all, was largely non-functional (Naruse et al., 1998). Given that the loss of TrkB signaling is linked to spatial memory deficits in AD model mice (Kemppainen et al., 2012), PS1 mutation may prove to play a more substantial role in this aspect of behavioral pathology. More recently, PS1 deficiency was shown to reduce expression of the neuroprotective cell surface tyrosine kinase receptor EphB2, presumably through a similar mechanism, and independently of PS1 γ-secretase activity (Barthet et al., 2013). PS1 may have been identified first for its APP processing ability, but subsequent studies in both humans and other animals indicate its cellular function is clearly far more diverse than originally suspected.
A role for the lysosome in Alzheimer pathology

A decades-long history of research connects Alzheimer’s disease with lysosomal and autophagic dysfunction. As early as 1967, Suzuki and Terry observed immunoreactivity of the lysosomal enzyme acid phosphatase in senile plaques (Suzuki & Terry 1967). Subsequent studies found that these plaques were also immunoreactive for cathepsins B and D (Cataldo et al., 1990), as well as other lysosomal hydrolases (Cataldo et al., 1991). The enzyme deposits were shown to be part of abundant extracellular granules of lipofuscin (Cataldo and Nixon, 1990) and lysosomal dense bodies (Cataldo et al., 1991), and were not seen in post-mortem tissue from patients with other neurodegenerative disorders. In other words, an abundance of early data implicated lysosomal leakage, and perhaps lysosomal exocytosis – a known mechanism by which cells attempt to clear debris (Medina et al., 2011) – in AD progression. Interestingly, extracellular hydrolases were found both in actively degenerating brain regions and in regions known to be affected in AD, but which were not currently degenerating, suggesting that lysosomal dysfunction might be an early sign of AD.

Other post-mortem data further implicated the lysosome in early disease. Abnormal lysosomal redistribution from the perikaryon to the axon hillock was observed in pyramidal neurons of the hippocampus and neocortex in AD brain; this redistribution occurred even in the absence of overt signs of degeneration, indicating that it may be an early event in disease (Nixon et al., 1992). These vulnerable regions had increased numbers of acid hydrolase-positive compartments (Cataldo et al., 1996) and were also characterized by increased expression of Cat D (Cataldo et al., 1995), neither of which was seen in non-AD brain. Neurons with more advanced degenerative pathology displayed similarly advanced lysosomal pathology, which persisted in the brain parenchyma beyond neuronal death (Cataldo et al., 1994). While some lipofuscin and lysosomal accumulation occurs in neurons in the course of normal aging, this accumulation paled by comparison to what was observed in AD patient tissue (Nixon et al., 1992, Cataldo et al., 1994).
Interestingly, while this lysosomal/autophagic pathology of AD is commonly observed in post-mortem AD brain tissue, PS1 mutations are known to exacerbate it (Cataldo et al., 2004).

The observed disruption of lysosome function in AD extends to the autophagic and endosomal systems, as well, indicating that the entire cellular degradative system is perturbed in AD. Immuno-electron microscopy has revealed extensive involvement of autophagy in AD, with autophagosomes at various maturation stages accumulating in abundance in both visually normal and dystrophic neurites (Nixon et al., 2005). Endosomal enlargement has been observed at early stages of AD; it was accentuated by inheritance of the known AD risk allele ApoE4 (Corder et al., 1993), and was specific to AD (Cataldo et al., 2000). Increased expression of several genes important for endosomal biogenesis and trafficking, including RAB4, RAB5, and RAB7, was observed in hippocampal CA1 neurons from AD patients; these increases correlated with severity of cognitive impairment (Ginsberg et al., 2010) and with up-regulated expression of Cat D in both mild and severe cognitive impairment.

Several non-human experimental models further validate the lysosome’s critical role in AD. Similarly to what has been seen in patient tissue, up-regulation of the lysosomal system was also an early event following neuronal injury of cultured rat hippocampal neurons (Adamec et al., 2000), while lysosomal disruption itself induced axon degeneration in cultured rat ganglia (Zheng et al., 2010). Selective inhibition of lysosomal proteases induced formation of enlarged neurites and tangle-like structures in the vulnerable regions of cultured rat entorhinohippocampal slices (Bi et al., 1999). Neuritic dystrophy following lysosomal proteolysis inhibition was also observed in cultured mouse primary neurons, and was accompanied by partially disrupted axonal transport and APP fragment accumulation; lysosomal restoration improved these AD-like phenotypes (Lee et al., 2011). Similar results were obtained in Drosophila melanogaster, in which Cat D inhibition was sufficient to promote accumulation of neurotoxic forms of tau that resemble the tau inclusions of AD (Khurana et al., 2010). Importantly, enhancement of lysosomal function was shown to be a
viable therapeutic strategy in several mouse models of AD (Butler et al., 2011, Yang et al., 2011, Yang et al., 2014), and has been suggested more generally as a strategy for addressing lysosomal dysfunction in neurodegeneration (Bahr, 2009).

The abundance of data connecting lysosomal dysfunction with AD led in 2010 to the first direct link between PS1 and lysosomal pH maintenance. In the absence of PS1, Lee et al. observed reduced glycosylation of the lysosomal proton pump’s V_o,a1 subunit; consequently, this subunit did not reliably arrive at the lysosome, leading to elevated lysosomal pH and consequent proteolytic and degradative deficits (Lee et al., 2010). The authors suggested that, as it does for other cellular constituents, PS1 might help to scaffold this subunit and to aid in its glycosylation. Loss or mutation of PS1 might therefore reduce PS1’s ability to interact with other proteins, including the proton pump, and thereby impair their efficient glycosylation and trafficking. While the pH deficit they described was only measured directly in PS1-ko mouse blastocysts, the authors also reported reduced degradative power in a variety of PS1-fAD human skin fibroblast genotypes. Importantly, treatment with the lysosomotropic weak base NH₄Cl (Ohkuma and Poole, 1978), which raises lysosomal pH, reduced starvation-induced proteolysis in control cells, but had no further impact on degradation in PS1-fAD cells. This finding implied that pH was already elevated in the PS1-fAD fibroblasts and that proteolysis could not be further impaired by addition of NH₄Cl.

A long history of research points toward a critical role for lysosomal and autophagic dysfunction in AD, though the magnitude of this dysfunction may vary with the severity and form of the disease. AD-like pathologies can be induced by lysosomal impairment, and treatments to improve lysosomal function also improved AD pathology in widely used AD models. The known loss of lysosomal efficacy in aging, in combination with the striking pathological similarities between the lysosomal storage disorders and Alzheimer’s disease (Nixon, 2004) lend further credence to the
theory that lysosomal failure in AD may lie somewhere in between normal aging and pathological youth on the spectrum of lysosomal dysfunction (Nixon et al., 2008).

**Confirming the role of lysosomal pH in Alzheimer’s disease: technical challenges**

Following this discovery of a proton pump trafficking defect in Alzheimer’s disease, several other groups attempted to replicate the findings with mixed success. Several subsequent studies found no effect of presenilin on lysosomal pH. Of these, one study found no change in mean vesicle pH in PS1 cells, failed to find a V₀a₁ trafficking defect, and saw no effects on Cat D maturation (Zhang et al., 2012). Presenilin was also shown to be required for efficient proteolysis in a manner independent of its γ-secretase activity, but again the lysosomal pH defect was not detected (Neely et al., 2011). Defects in lysosomal calcium storage, but not pH, were proposed to accompany presenilin loss or mutation (Coen et al., 2012), despite strong evidence that the ability of the lysosome to buffer and store calcium depends heavily on lysosomal pH (Christensen et al., 2002).

Over the same period, other investigators have found quite the opposite: that PS1 loss or mutation does, in fact, lead to lysosomal malfunction through pH deficiency. While the deficit has not been measured directly by these other groups, strong *in vivo* evidence has been gathered in support of AD-linked lysosomal pH failure – including in several transgenic mouse models of AD.

The 5 X FAD mouse is an accelerated amyloid deposition model that recapitulates many of the behavioral and pathological characteristics of human AD (Oakley et al., 2006). This mouse model is transgenic for five AD-associated mutations – including mutations in both APP and PS1 – and is now widely used in the Alzheimer’s field. In a recent study, V₀a₁ N-glycosylation was shown to be reduced in this mouse’s brain tissue, similar to what the Nixon laboratory had shown *in vitro* (Avrahami et al., 2013). The implicit acidification failure in this mouse was further supported by a reduction in mature Cat D (Avrahami et al., 2013). Reduced levels of mature Cat D and mature Cat B were also observed in areas near plaques in the PS1M146L/APP751s1 mouse, although in
this case no change was seen in V\textsubscript{o}a1 N-glycosylation (Torres et al., 2012) – likely due to incomplete loss of PS1 function (Wolfe et al., 2013). Finally, PS1-associated disruption of Wnt signaling was recapitulated in wild type cells through nothing more than elevation of lysosomal pH with chloroquine (Dobrowolski et al., 2012); given the dependence of Wnt signaling on a fully functional endocytic pathway (Blitzer and Nusse, 2006), these findings further implicate the endo-lysosomal axis in disease progression.

The apparent controversy regarding the role of lysosomal pH dysfunction in Alzheimer’s disease may be a primarily technical issue, as obtaining an accurate measure of lysosomal pH proves to be a complex procedure. The challenges and pitfalls of the various techniques for lysosomal assessment have been reviewed previously in extensive detail (Wolfe et al., 2013, Guha et al., 2014). A common and particularly critical point made by these reviews is that great care must be taken to optimize measurement protocols in each system; if quantitative measurement is desired, ratiometric dyes ought to be used in a moderately high-throughput context (e.g., a multiwell plate versus a microscope). Otherwise, any reported lysosomal pH measurements, especially those made with single-wavelength dyes, may be unreliable.

As an example, other laboratories commonly use the fluorescence of the single-wavelength LysoTracker Red DND-99 dye (Life Technologies Corp., Grand Island, NY, USA) as a quantitative measure of lysosomal pH, when in reality its readout ought to be treated as qualitative. Neely et al. (2011) found no apparent change in lysosomal pH using LysoTracker Red. Unfortunately, even when ratiometric dyes are used, they are often used incorrectly: Zhang et al. (2012) performed measurements using a microscope, rather than a microplate reader, and detected an average vesicular pH of 6.6; at more than two units above healthy lysosomal pH, this reading can hardly be considered informative for a lysosomal measurement. Similarly, while Coen et al. (2012) reported no difference in pH using LysoSensor Yellow/Blue DND-160 (Life Technologies), which is the same dye used in the present work, their group incubated cells with
the dye for 10 min or more; previous [unpublished] work from our laboratory indicates that incubations greater than 5 min quickly lead to a saturation of the signal, and a resulting inability to detect any pH differences that may exist. These caveats, in combination with possible issues with overexpression of exogenous of the \( V_0 a_1 \) subunit (Wolfe et al., 2013), may contribute to the mixed findings.

By contrast, our laboratory has devoted years to developing expertise in the use of a primary ratiometric lysosomal pH assay, and in supplementing these readouts with indirect measures of lysosomal pH and degradative efficacy (Liu et al., 2008, Baltazar et al., 2012, Guha et al., 2012, Liu et al., 2012, Guha et al., 2013). Therefore, we were particularly well positioned to provide firm answers to the lysosomal pH question, as well as to begin to address techniques by which to restore any elevated lysosomal pH that might be detected. With this technical skill, we produced one of the first studies to quantitatively and convincingly demonstrate lysosomal pH elevation in human skin fibroblasts from AD patients, and the first to correlate this specific dysfunction in human cells with Cat D deficiency and other measures of lysosomal and autophagic pathology. Unlike other laboratories, we focused our efforts on fibroblasts with the PS1-fAD mutation A246E, a mutation previously implicated in proteolytic impairment (Lee et al., 2010) and which is located in the enzyme’s sixth transmembrane region (Fig. 1.1). The use of PS1-fAD human skin fibroblasts in the present work was preferable to the use of PS1ko or PSdko cells, as reports from cells that wholly lack PS1 might not be representative of the mechanisms at work, or the degree of perturbation, in actual patient tissue. Our careful approach also ensured that we were the first to demonstrate that pH restoration may prove a therapeutically valid strategy in this model of AD; this finding represents an important step forward in understanding the lysosome’s role in disease.

In sum, the work described in this Introduction implicates lysosomal dysfunction in a complex array of neurodegenerative phenotypes, and suggests that treatments to restore lysosomal function may be effective in improving outcomes. While Alzheimer’s disease appears to dovetail
with poor lysosomal pH maintenance, this finding has not been widely reported, and is technically challenging to demonstrate. The following chapters will present studies that quantify a modest lysosomal pH elevation in human skin fibroblasts from AD patients (Chapter 2); identify certain of the downstream consequences of this pH problem (Chapter 2); and identify both a potential treatment to restore lysosomal pH (Chapters 2 and 3) and that treatment’s likely mechanisms of action (Chapter 3). Although considerable work remains to clarify the exact nature and chronology of lysosomal distress in Alzheimer’s disease, the work presented in this document represents a considerable advance in our understanding of lysosomal involvement in the disease process, and suggests important therapeutic strategies that may be explored in the future.
Figure 1.1. Structure of presenilin 1.
Presenilin 1 (PS1) is the catalytically active subunit of γ-secretase. PS1 was discovered to have nine transmembrane region topology, with the N terminus in the intracellular space and the C terminus in the extracellular or luminal space. The scissors represent a site of endoproteolytic cleavage that, while not explicitly required for enzyme activity, helps PS1 form stable complexes with the other components of γ-secretase. The active enzyme does, however, require association of the N-terminal fragment and the C-terminal fragment. Each fragment contributes one of the two aspartate residues that comprise the active site; the blue circles represent these residues. The red circle denotes the approximate location of the A246E mutation in transmembrane region VI. (Dillen and Annaert, 2006, Spasic et al., 2006)
CHAPTER 2:

Lysosomal alkalization and dysfunction in human fibroblasts with the Alzheimer’s disease-linked presenilin 1 A246E mutation can be reversed with cAMP

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Abstract

Mutation in presenilin 1 (PS1) is one of the leading causes of familial Alzheimer’s disease (fAD). PS1 mutation exacerbates the autophagic and lysosomal pathology in AD patients, leading to accumulation of partially degraded material in bloated lysosomes and autophagosomes—a pathology that bears some resemblance to other diseases characterized by elevated lysosomal pH, like age-related macular degeneration. In this study, we examined the effect of the PS1-fAD mutation A246E on lysosomal pH and lysosomal function, and asked whether restoration of lysosomal pH could reverse some of these changes. Lysosomal pH was elevated by 0.2–0.3 pH units in human fibroblasts with the PS1-fAD mutation. The lysosomal alkalization in PS1-fAD fibroblasts was supported by a reduction in the pH-dependent cleavage of cathepsin D and by a reduction in binding of boron-dipyrrromethene (BODIPY) FL- pepstatin A to the cathepsin D active site. PS1-fAD cells had increased LC3B-II/I ratios and p62 levels, consistent with impaired lysosomal degradation and analogous to changes induced by lysosomal alkalinization with chloroquine. PS1-fAD fibroblasts had increased expression of ATP6V1B2, ATG5, BECN1, and TFEB mRNA, and of ATP6V1B2, ATG5 and beclin at the protein level, consistent with chronic impairment of autophagic and lysosomal functions in the mutant cells. Critically, cyclic adenosine monophosphate (cAMP) treatment reacidified lysosomal pH in mutant PS1-fAD; cAMP also increased the availability of active cathepsin D and lowered the LC3B-II/I ratio. These results confirm a small elevation in the lysosomal pH of human PS1-fAD fibroblasts, demonstrate that this lysosomal alkalization is associated with chronic changes in autophagy and degradation, and suggest that treatment to reacidify the lysosomes with cAMP can reverse these changes.
Graphical abstract

The elevation of lysosomal pH associated with mutations in PS1-fAD prevents degradative enzymes from properly processing cellular waste material, leading to a backlog of autophagic makers LC3BII/I and p62, and a compensatory up-regulation of the genes ATP6V1B2, ATG5, BECN1, and TFEB. Treatment re-acidifies lysosomes and enhances autophagic throughput. Yellow dots indicate incompletely degraded and aggregate material.

Abbreviations

AD, Alzheimer’s disease; AMD, age-related macular degeneration; ANOVA, analysis of variation; BODIPY, boron-dipyrromethene; cAMP, cyclic adenosine monophosphate; EDTA, ethylenediaminetetraacetic acid; fAD, familial Alzheimer’s disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3, glycogen synthase kinase 3; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; MES, 2-(N-morpholino)ethanesulfonic acid; PKA, protein kinase A; PS1, presenilin 1; qPCR, quantitative polymerase chain reaction; RPE, retinal pigmented epithelium.
Introduction

Intracellular waste products, damaged organelles and other targets of bulk cellular degradation reach the lysosomes via the process known as macroautophagy (henceforward, simply “autophagy”). The efficient clearance of this material is of particular importance in post-mitotic cells such as cortical neurons (Boland et al., 2008). This degradation is highly dependent on lysosomal pH (pH$_L$): activity of lysosomal enzymes is optimal over a narrow range of acidic levels. Substantial shifts in pH$_L$, such as those induced by drugs like chloroquine or bafilomycin, can severely disrupt degradative enzyme activity and block the fusion of autophagosomes with lysosomes (Yamamoto et al., 1998, Klionsky et al., 2008). However, even an increase of only a few tenths of a unit is sufficient to depress the activity of key lysosomal proteases and lipases (Barrett, 1970, 1972, 1973, Schwartz and Bird, 1977, Ameis et al., 1994). These moderate elevations of pH$_L$ can perturb the clearance of cellular waste and lead to a backup of the autophagic pathway, resulting in a slow accumulation of waste with time.

Though the pH dependence of lysosomal enzyme activity has been recognized for decades, a role for impaired degradation has only been implicated in neurodegenerative diseases more recently (Pacheco et al., 2007, Cheung and Ip, 2009, Martinez-Vicente et al., 2010, Winslow et al., 2010, Elrick and Lieberman, 2013). Alzheimer’s disease (AD) is of importance in this regard, as fundamental defects in autophagy and autophagic degradation have been observed (Cataldo and Nixon, 1990, Cataldo et al., 1996, Cataldo et al., 2004, Nixon, 2005, Nixon et al., 2005, Nixon and Cataldo, 2006, Khurana et al., 2010, Lipinski et al., 2010). Although the canonical pathologies of AD include tau and amyloid-β deposition, the disease is also associated with the pathological build-up of partially degraded protein in bloated lysosomes and autophagosomes (Nixon et al., 2005). While this so-called “autophagic pathology” is observed in multiple forms of the disease, it is accentuated by mutations in the transmembrane protein presenilin 1 (PS1), the catalytically active component of the γ-secretase complex (Cataldo et al., 2004). As PS1 mutation is a common cause of early-onset, inherited, familial Alzheimer’s disease (fAD), these autophagic
defects may impact disease progression. The missense mutation A246E, one of the first PS1 mutations to be identified (Sherrington et al., 1995), is of particular relevance. While the protein with this point mutation is still capable cleaving amyloid precursor protein, it is associated both with elevated Aβ42/40 ratio (Scheuner et al., 1996, Qian et al., 1998) and with autophagic pathology (Lee et al., 2010). In addition, mice expressing the human A246E transgene show increased amyloid beta in the absence of plaques, as well as reduced performance (Lalonde et al., 2003). However, the mechanistic links between the mutation and these pathologies remain unclear.

The potential contribution of lysosomal alkalization to this impaired degradation is currently a matter of considerable interest. It has been suggested that the A246E mutation disrupts the trafficking of a v-(H+)ATPase subunit to lysosomes and that lysosomal pH is elevated in these mutant cells (Lee et al., 2010). However, others have been unable to detect a significant change in lysosomal pH using a variety of approaches (Neely et al., 2011, Coen et al., 2012, Zhang et al., 2012) or confirm a role for defective lysosomal pH in disease (Bezprozvanny, 2012). Given that accurate measurement of lysosomal pH is technically challenging, this discrepancy is understandable. However, we have spent the past decade developing a protocol that can accurately detect small changes in lysosomal pH. We have demonstrated the effects of elevated lysosomal pH in retinal pigmented epithelial (RPE) cells associated with age-related macular degeneration (AMD) and have screened to identify treatments that can reacidify damaged lysosomes and reverse the accumulation of waste material (Liu et al., 2008, Baltazar et al., 2012, Guha et al., 2012, Liu et al., 2012, Guha et al., 2013). AD has many parallels with AMD, including the slow accumulation of incompletely degraded material in the lysosome and in lysosome-associated organelles of aging post-mitotic cells (Isas et al., 2010, Ding et al., 2011, Kaarniranta et al., 2011, Ohno-Matsui, 2011, Sivak, 2013). We thus applied our technique for accurate detection of lysosomal pH to skin fibroblasts from humans with the PS1-fAD mutation and found an elevation in lysosomal pH, a decrease in pH-dependent processing of cathepsin D and
changes in molecular and protein markers.

**Experimental procedures**

**Culture of human skin fibroblast cells**

This study used two distinct sets of control (CTRL) and PS1-fAD (A246E) human skin fibroblasts from the NIA Aging Cell Culture Repository (Coriell, Camden, NJ, USA): cell numbers AG6840 and AG08170 were from two different PS1-fAD donors and termed “PS1-fAD” cells, while numbers AG07621 and AG07623 were the control cells from unaffected spouses of AD patients (termed “CTRL” cells). Cells were grown to confluence in 25 cm² primary culture flasks in minimum essential Eagle’s medium (Sigma–Aldrich, St. Louis, MO, USA) with 2 mM GlutaMAX™, 100 U/mL penicillin and 100 µg/ml streptomycin and 15% fetal bovine serum (all Life Technologies Corp., Grand Island, NY, USA). Cells were incubated at 37 °C in 5.5% CO₂ and sub-cultured by room temperature incubation in 0.53 mM EDTA in Hank’s balanced salt solution (HBSS) (−Mg²⁺/−Ca²⁺), followed by incubation with 0.05% trypsin/0.48 mM EDTA (Life Technologies) at 37 °C. Culture protocols followed in accordance with those provided by the cell supplier. Data presented from PS1-fAD fibroblasts represent composite data from both AG08640 and AG08170 lineages; data for CTRL fibroblasts represent composite data from both AG07621 and AG07623 lineages. No clear differences were detected between cells from different lineages but the same PS1 genotype.

**Measurement of pH<sub>L</sub> from fibroblast cells**

Lysosomal pH was measured as described using the dye LysoSensor Yellow/Blue DND-160 (Liu et al., 2008, Baltazar et al., 2012, Guha et al., 2012, Liu et al., 2012). The use of LysoSensor Yellow/Blue (Life Technologies) to measure lysosomal pH has the advantage, common to ratiometric dyes, that the readout is independent of concentration. Because the dye is membrane-permeable, readout is also representative of a broader range of lysosomes than those reached by endocytosis of a dextran-tagged probe. Extensive preliminary trials have optimized key
experimental parameters including incubation time, dye concentration, etc. to minimize variation and give the best signal-to-noise ratio with the lowest concentration of dye (Liu et al., 2008). For example, all readings used for a given experimental set were performed simultaneously in 96-well plates. Incubation times were monitored precisely, and all measurements were taken within 12–14 min of washing off the dye. Calibrations were performed simultaneously in adjacent wells to ensure the relevance of the measures.

In brief, CTRL and PS1-fAD fibroblasts from unaffected spouse donors were grown to at least 80% confluence in black 96-well plates. Cells were assayed after a minimum of 6 days in culture. The two cell types were plated in alternating rows to control for any signal variation across the plate. To begin the assay, culture medium was removed and cells were incubated for 3 min with 2 µM LysoSensor Yellow/Blue in isotonic solution (NaCl, 105 mM; KCl, 5 mM; HEPES-Acid, 6 mM; Na-HEPES, 4 mM; NaHCO₃, 5 mM; mannitol, 60 mM; glucose, 5 mM; MgCl₂, 0.5 mM; CaCl₂, 1.3 mM; pH, adjusted to 7.4; osmolality, 300 mOsm). Dye loading and incubation steps were carried out at room temperature and in the dark. After 3 min, cells were rinsed 3× with isotonic solution and incubated with additional isotonic solution, with a relevant drug, or with pH calibration buffers. All final drug applications (200 nM bafilomycin A1, 20 mM NH₄Cl, 30 µM tamoxifen, cyclic adenosine monophosphate (cAMP) cocktail (500 µM cpt-cAMP, 100 µM 3-isobutyl-1-methylxanthine (IBMX), 10 µM forskolin (forskolin from LC Laboratories, Woburn, MA, USA)) were made up in isotonic solution. After 10 min, fluorescence was measured with a Fluoroskan Ascent Microplate Fluorometer and recorded using the ASCENT software package (both Thermo Scientific, Waltham, MA, USA). Lysosomal pH was determined from the ratio of light excited at 340 nm over 380 nm (F₃₄₀nm/F₃₈₀nm, 527 nm emission). Mean light levels at both emission wavelengths were integrated over 60 ms and recorded for each well in sequence; this course was repeated every 30 s for 11 iterations. pH data are the mean levels from 3 to 7 measurements taken 12–14 min after removal of the dye. In certain recordings, absolute pH levels were obtained by calibrating the lysosomal pH against standards; calibration wells were
incubated with 15 µM monensin and 30 µM nigericin, each a proton–cation ionophore that permeabilizes the lysosomal membrane to Na⁺ and K⁺, respectively. These ionophores were added in a solution of 20 mM MES (2-(N-morpholino)ethanesulfonic acid), 110 mM KCl and 20 mM NaCl, at pH values of 4.0, 4.5, 5.0, 5.5 and 6.0. It should be noted that while the use of ionophores to calibrate fluorescent ratios into absolute values is relatively precise when working with a cytoplasmic readout such as with fura-2 (Gryniewicz et al., 1985), the complexities of permeating both the plasma membrane and vesicular membranes can lead to small variations in the absolute levels. However, our approach of measuring output simultaneously from cells grown on a single plate, and then calibrating and normalizing to the mean control level for each plate, ensures that the differences observed between cell types and between treatment conditions are repeatable and reliable.

**Confocal microscopy**

CTRL and PS1-fAD fibroblasts were grown to near-confluence on glass coverslips. For LysoSensor/LysoTracker staining, coverslips were first rinsed 3× in warm isotonic solution, then co-incubated in fresh growth medium with 2 µM LysoSensor Yellow/Blue and 50 nM LysoTracker DND-99 for just over 30 min at 37 °C. For boron-dipyrromethene (BODIPY) FL-pepstatin A staining, coverslips were rinsed and then co-incubated in fresh growth medium with 1 µg/ml BODIPY FL-pepstatin A (Life Technologies) for 1 h. Following incubation, coverslips were rinsed 3× times and then imaged on a warm stage using a Nikon A1R Laser Scanning Confocal Microscope and the University of Pennsylvania Live Cell Imaging Core. LysoSensor signal was excited at 407 nm using the microscope’s 405-nm Diode laser; emission was read at 450 nm. The LysoTracker signal was excited at 562 nm using the microscope’s diode-pumped solid-state laser (DPSS) laser; emission was read at 595 nm. The BODIPY FL-pepstatin A signal was excited at 488 nm using the microscope’s Argon laser; emission was read at 525 nm. Image capture and analysis were performed using Nikon Elements Advanced Research Software package 3.2. Image capture on live cells was completed within 15 min of completing dye incubation for
LysoSensor/LysoTracker staining, and within 30 min of completing dye incubation for BODIPY FL-pepsstatin A.

**Immunoblots**

CTRL and PS1-fAD fibroblasts were grown to confluence in adjacent wells of 6-well plates, and assayed after a minimum of 6 days in culture. On the day of protein collection, existing medium was replaced with one of three media solutions: fresh medium (components as described above: minimum essential Eagle’s medium, fetal bovine serum, GlutaMAX, penicillin/streptomycin), fresh medium plus 10 µM chloroquine (CTRL fibroblasts only), or fresh medium plus cAMP cocktail. Fibroblast cultures were incubated in these solutions for 6 h at 37 °C. Cells were rinsed 3× in Dulbecco’s phosphate-buffered saline (DPBS) (+Ca²⁺/+Mg²⁺) (Life Technologies). Total protein was extracted from fibroblasts using radio immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich or made in-house) plus Complete Mini Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN, USA) according to standard protocols. Protein concentration in cellular extracts was quantified on the BioPhotometer spectrophotometer (Eppendorf AG, Hamburg, Germany) using the Pierce® BCA Protein Assay Kit (ThermoFisher Scientific).

Protein samples were prepared for agarose gel electrophoresis according to standard protocols and run at 130 V on a 4–15% Mini-PROTEAN® TGX™ Gel with 50 µL well depth (Bio-Rad, Hercules, CA, USA). Gel-bound samples were then transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore Corp., Billerica, MA, USA) for immunoblotting. A solution of 5% non-fat milk (Bio-Rad) was used for membrane blocking. To detect p62, the mouse mAb p62/SQSTM1 (D-3) was used (#sc-28359, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To detect LC3B, the rabbit mAb LC3B (D11) XP® was used (#3868S, Cell Signaling Technology, Beverly, MA, USA). To detect vATPaseB2, the rabbit pAb Anti-ATP6V1B2 was used (#ab73404, Abcam, Cambridge, MA, USA). To detect Atg5, the rabbit mAb Atg5 (D1G9) was used (#8540, Cell Signaling Technology). To detect Beclin-1, the rabbit mAb Beclin-1 (D40C5) was used...
(#3495P, Cell Signaling Technology). To detect glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the rabbit mAb GAPDH (14C10) was used (#2118S, Cell Signaling Technology). The antibody used to detect cathepsin D was a generous gift from Kathleen Boesze-Battaglia. All primary Abs were used at a dilution of 1:1000. Secondary Abs were used at a dilution of 1:3000 and consisted of either sheep anti-mouse (GE Healthcare Life Sciences, Amersham, UK) or donkey anti-rabbit (GE Healthcare Life Sciences) IgG as horseradish peroxidase (HRP)-linked whole Ab for electrogenerated chemiluminescence (ECL). Visualization and band quantification were performed on an ImageQuant LAS 4000 biomolecular imaging system (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

**Quantitative polymerase chain reaction (qPCR)**

Total RNA was isolated from cultured human skin fibroblast cells using the TRIzol (Life Technologies) extraction protocol, with glycogen (Life Technologies) added to improve yield. RNA yield was determined by absorbance at 260 nm, and purity confirmed by measurement of 260 nm/280 nm ratio on Eppendorf Biophotometer Spectrophotometer. One microgram of total RNA was converted into cDNA using the High Capacity RNA-to-cDNA first strand synthesis kit (Applied Biosystems). Human primers were purchased from Life Technologies/Sigma–Aldrich and constructed according to specifications given in Table 2.1. qPCR was performed using the Power SYBR Green detector (Life Technologies) on a Life Technologies 7300 Real Time PCR System. Final primer concentration in each well was 1 µM sense and 1 µM antisense primer. The thermal cycling profile was as follows: Stage 1, 50 °C for 2 min (one cycle); Stage 2, 95 °C for 10 min (one cycle); Stage 3, 95 °C for 15 s followed by 60 °C for 1 min (40 cycles). Expression levels of genes of interest were normalized internally to each sample’s expression of the housekeeping gene ACTB (β-actin). ACTB expression did not differ between CTRL and PS1-fAD fibroblasts across all experimental trials. All runs included a final dissociation stage to confirm the amplification of only desired products. To control for genomic DNA contamination, PCR was also performed on samples from reverse transcriptase reactions in which the enzyme was omitted. No
products were observed from these samples, indicating that no genomic DNA contaminated our experimental samples.

**Data analysis**

All data are given as mean ± standard error of the mean. Significance was defined as p < 0.05 and was determined using a one-way analysis of variation (ANOVA) followed by an appropriate post hoc test using SigmaPlot statistics software (v11.0, Systat Software, Inc., San Jose, CA, USA) unless otherwise noted. When data were not normally distributed, ANOVAs were performed on ranks.

All chemicals and compounds used were from Sigma–Aldrich Corp., St. Louis, MO unless otherwise indicated.

**Results**

**pH$_L$ is elevated in PS1-fAD fibroblasts**

Initial experiments established the feasibility of performing reliable measurements of lysosomal pH from fibroblasts using our protocols. Fibroblasts derived from normal subjects (CTRL) were plated into black-walled 96-well plates and briefly incubated with different compounds known to increase pH$_L$ through varied mechanisms. These compounds included: 200 nM bafilomycin A1, a specific inhibitor of v-(H$^+$)ATPase (Bowman et al., 1988); 20 mM NH$_4$Cl, a lysosomotropic weak base (Ohkuma and Poole, 1978); 30 µM tamoxifen, a tertiary amine whose pH$_L$-elevating activity is distinct from its estrogen-like activity (Altan et al., 1999); and 10 µM chloroquine, a lysosomotropic agent that becomes "trapped" in the lysosome following protonation (de Duve et al., 1974, Chung, 1986). A representative pH measurement is shown to illustrate the strong effect of each compound upon pH$_L$ (Fig. 2.1, panels A–D). Bafilomycin A1 (Fig. 2.1.A), NH$_4$Cl (Fig. 2.1.B), tamoxifen (Fig. 2.1.C), and chloroquine (Fig. 2.1.D) led to a significant rise in lysosomal pH levels, as expected. While absolute level of baseline pH$_L$ varied somewhat, the baselines
recorded were comparable to lysosomal pH levels in other cell types, and were well within the usual acidic range of the lysosomal enzymes. Importantly, the differences found within the same experimental trial were constant. These findings verified our ability to effectively measure pH<sub>L</sub> in these fibroblasts, and showed that the pH<sub>L</sub> could be manipulated using standard procedures and compounds.

Subsequent experiments compared the baseline lysosomal pH levels between CTRL and PS1-fAD fibroblasts. The fibroblasts examined had the PS1-fAD missense mutation A246E, which is linked to both perturbed autophagy (Lee et al., 2010) and elevated Aβ<sub>42/40</sub> ratio (Scheuner et al., 1996, Qian et al., 1998). Fig. 2.1.E illustrates a typical experiment, with CTRL fibroblasts at a pH of from 4.38 ± 0.13 and PS1-fAD fibroblasts at 4.75 ± 0.17. This small but significant elevation in lysosomal pH was confirmed in numerous trials from both sets of donor pairs. In a total of 26 individual trials, a significant elevation in lysosomal pH was detected in the PS1-fAD cells, as indicated by an increased emission ratio (Fig. 2.1.F, the ratios were more reliable than calibration across double membranes – see Methods). This rise corresponds to an increase of approximately 0.2 pH units. No substantive difference was observed in autofluorescence between CTRL and PS1-fAD fibroblasts at wavelengths used for the pH<sub>L</sub> assay.

While fluorescence readings from the plate reader provided the most accurate comparison of lysosomal pH, it was important to examine the lysosomes microscopically. No major difference in overall LysoSensor staining patterns or fluorescence output from the two cell types was observed by confocal imaging (Fig. 2.1.G, H). Similarly, no substantial differences were observed between CTRL and PS1-fAD cells using LysoTracker dye (Fig. 2.1.I, J). With both dyes, the number and distribution of fluorescing organelles varied on a cell-to-cell basis. Bafilomycin treatment completely eliminated LysoTracker fluorescence for both cell types, confirming the readout.
Availability of active cathepsin D is reduced in PS1-fAD fibroblasts

Cathepsin D, the primary aspartyl protease of the lysosome, has a particularly acidic pH optimum (Barrett, 1970) as well as a pH-dependent maturation (Rosenfeld, 1982). It was therefore reasoned that the enzyme’s sharp tuning would make its activity and availability particularly sensitive to pH shifts over the range observed in the PS1-fAD fibroblasts. To this end, both CTRL and PS1-fAD fibroblasts were incubated with BODIPY FL-pepstatin A, which selectively binds to active cathepsin D (Chen et al., 2000a), and examined under a confocal microscope. PS1-fAD cells exhibited markedly reduced BODIPY FL-pepstatin A fluorescence when compared to CTRL fibroblasts (Fig. 2.2A). When mean fluorescence intensity per cell was calculated for both CTRL and PS1-fAD fibroblasts, BODIPY FL-pepstatin A fluorescence was significantly reduced by approximately 50%, indicating a substantial loss in the availability of active cathepsin D with PS1-fAD mutation (Fig. 2.2B).

Having observed a significant reduction in active site availability by BODIPY FL-pepstatin A, cathepsin D levels in both CTRL and PS1-fAD fibroblasts were next examined by Western blot. The antibody detected four primary bands: one each at 52 and 47 kDa, corresponding to immature/intermediate forms of cathepsin D (Gieselmann et al., 1983), and one each at 34 and 28 kDa, corresponding to processed, mature forms of cathepsin D (Fig. 2.2C) (Erickson et al., 1981). The ratio of 28-kDa/52-kDa cathepsin D was found to be significantly reduced in PS1-fAD fibroblasts, indicating impaired maturation of cathepsin D in mutant cells (Fig. 2.2D). Importantly, while no significant changes were observed in the relative levels of either the 52- or 47-kDa forms of cathepsin D (Fig. 2.2E, F), the reduction in levels of mature cathepsin D approached significance at 34 kDa (Fig. 2.2G) and was significant at 28 kDa when compared against CTRL fibroblasts (Fig. 2.2H). This indicated that the PS1-fAD cells have reduced maturation, and not initial production, of the protease.

Together, these data support the premise that even a small pH elevation has real consequences
for the activity of a major lysosomal enzyme, and suggest that PS1-fAD cells may have impaired degradation as a consequence of the loss of cathepsin D.

**Altered levels of key proteins support elevated pH**

The finding that cathepsin D availability and maturation is markedly reduced in PS1-fAD fibroblasts (Fig. 2.2) suggested that the autophagic degradative system may be perturbed as a result of pH elevation, and prompted an examination of autophagic markers. To provide a general index of autophagic activity, the levels of the proteins LC3B and p62 were determined in control and mutant cell types. LC3B is normally a cytosolic protein, the accumulation of which is often used to identify shifts in autophagic flux (Klionsky et al., 2012). Upon initiation of autophagy the cytosolic, LC3B-I form of the protein is cleaved and conjugated to phosphatidylethanolamine to form LC3B-II, which associates with autophagosomal membranes. Elevation of lysosomal pH can lead to a backlog of autophagy and can raise the ratio of LC3B-II to LC3B-I, as previously shown in LS174 cells exposed to bafilomycin A1 (Bellot et al., 2009). The ratio of LC3B-II/I was also elevated in PS1-fAD fibroblasts compared to CTRL (Fig. 2.3.A, B). This increase reflected a significant elevation in the relative level of LC3B-II in PS1-fAD fibroblasts (Fig. 2.3.A, C). Chloroquine also induced a rise in LC3B-II/I in CTRL fibroblasts (Fig. 2.3.D, E) which, as with the PS1-fAD fibroblasts, reflected a significant change in LC3B-II (Fig. 2.3.F). While qualitatively similar, the magnitude of this increase in LC3BII/I with chloroquine was considerably greater than that observed with PS1-fAD cells, consistent with the relative magnitude of lysosomal alkalinization under the two conditions.

The increased lysosomal pH levels in PS1-fAD cells are also supported by changes in the levels of the autophagy-associated protein p62, also known as sequestosome-1. p62 itself is primarily degraded by autophagy (Bjorkoy et al., 2005), and therefore its elevation can be indicative of impaired autophagic degradation. Levels of p62 were increased more than twofold in PS1-fAD cells as compared to control (Fig 2.3.G, H). Chloroquine also triggered an increase in p62 levels.
in control fibroblasts (Fig. 2.3.G, I). Together, elevation of the LC3B-II/I ratio and of p62 provides support for an elevated lysosomal pH in PS1-fAD cells on a protein level, and implies functional consequences consistent with this rise, although the use of these two measures does not rule out other mechanisms by which autophagic degradation might be inhibited in PS1-fAD fibroblasts, such as through the mTOR signaling pathway.

**PS1-fAD mutation results in altered expression of lysosome- and autophagy-associated genes**

Additional support for a perturbed degradative system in PS1-fAD cells was sought at a molecular level by examining the expression of genes associated with lysosomal pH and autophagy. Lysosomal pH is regulated by a complex series of feedback systems, with pH elevation leading to alterations in mRNA message for certain key genes (Settembre et al., 2012). qPCR demonstrated that relative expression of the B2 subunit of the v-(H+)ATPase proton pump rose 89% in PS1-fAD fibroblasts (Fig. 2.4.A). For ATG5, whose gene product is associated with autophagosome elongation, relative expression rose 87% in PS1-fAD fibroblasts (Fig. 2.4.B). Similarly, expression of BCN1, whose product beclin is involved in the genesis of autophagosomes, was also increased by 92% in PS1-fAD fibroblasts (Fig. 2.4.C). Finally, there was a significant increase in the expression of TFEB, the transcription factor responsible for lysosomal biogenesis, which has also recently been identified as a link between lysosomal and autophagic processes (Settembre et al., 2011, Rocznik-Ferguson et al., 2012); relative expression rose 59% in PS1-fAD fibroblasts (Fig. 2.4.D). All expression data were first normalized internally to the ubiquitously- and highly-expressed β-actin (ACTB) message prior to comparison, as no difference in ACTB expression was detected between CTRL and PS1-fAD fibroblasts.

**Protein level shifts in PS1-fAD fibroblasts of lysosome- and autophagy-associated proteins mirror gene expression changes**

While disruption of the lysosome- and autophagy-associated genes identified in Fig. 2.4 provided
support for perturbed autophagy in PS1-fAD fibroblasts, validation at the protein level was desired to confirm the functional effect of the increased mRNA levels. To this end, the levels of vATPaseB2, Atg5, and beclin-1 were evaluated by Western blot. TFEB levels were not examined at this time, since the primary mechanism of TFEB’s action is through a nuclear translocation event (Settembre et al., 2012), and not purely through increased expression.

The protein level of vATPaseB2 in CTRL fibroblasts was found to be unaffected by 6-h incubation with CHQ, but was significantly increased in PS1-fAD fibroblasts when compared against CTRL cells (Fig. 2.5.A–C). Similar results were observed for the protein level of Atg5 (Fig. 2.5.D–F) and for the level of beclin-1 (Fig. 2.5.G–I): in these cases, as well, CHQ incubation proved insufficient to increase protein levels, but PS1-fAD mutation reliably produced this increase. Together, these data provide further support for a perturbation of the degradative system in PS1-fAD cells, while also highlighting a possible difference between the effect of acute and chronic lysosomal pH elevation upon that system.

Intracellular cAMP elevation re-acidifies lysosomes and reduces LC3B accumulation

Since pH measurement, protein level, and gene expression data all support the conclusion that the lysosomal pH is defective in the PS1-fAD fibroblasts, attempts were made to restore pH using the intracellular signaling molecule cAMP. Previous work from our laboratory has demonstrated that intracellular elevation of cAMP can partially restore pH, that has been elevated by either pathological or by pharmacological means (Liu et al., 2008, Guha et al., 2012). Importantly, cAMP re-acidified RPE lysosomes in cells from old mice whose lysosomes had been damaged for an extended time, suggesting the approach might also restore acidity to lysosomes in the PS1-fAD fibroblasts. cAMP also has been shown to promote acidification in a variety of contexts and across a range of cell types (Alzamora et al., 2010, Paunescu et al., 2010).

Increased intracellular cAMP levels reduced pH in PS1-fAD fibroblasts (Fig. 2.6.A). A cAMP-
elevating cocktail, composed of cell-permeable cAMP, IBMX, and forskolin, consistently restored pH\textsubscript{L} in the mutant fibroblasts. The cAMP mix led to a small but insignificant drop in the lysosomal pH of control cells. Interestingly, preliminary data indicate that cAMP induced a larger acidification of lysosomes from control fibroblasts whose lysosomes had been alkalized either with NH\textsubscript{4}Cl or with tamoxifen. The re-acidifying effect of cAMP was also larger in epithelial cells with alkalized lysosomes (Liu et al., 2012), consistent with the findings in fibroblasts.

To validate the functional implications of lysosomal acidification of PS1-fAD fibroblasts by cAMP, the consequences of cAMP-induced pH\textsubscript{L} restoration on both cathepsin D active site availability and on autophagy were examined. PS1-fAD cells treated with cAMP cocktail had greater BODIPY FL-pepstatin A fluorescence when compared against untreated PS1-fAD fibroblasts (Fig. 2.6.B). When mean fluorescence intensity per cell was calculated for both treated and untreated PS1-fAD fibroblasts, BODIPY FL-pepstatin A fluorescence was significantly increased by about 10% in treated fibroblasts, indicating that 6-h cAMP treatment and pH restoration brings about at least partial recovery of active cathepsin D (Fig. 2.6.C). Still, even partial restoration of enzyme activity proved sufficient to improve clearance through the autophagosomal degradation pathway; incubation with the cAMP cocktail reduced the LC3B-II/I ratio by 60% (Fig. 2.6.D, E), and significantly decreased LC3B-II levels while increasing LC3B-I (Fig. 2.6.D, F). As cAMP interferes with p62 independently through the ubiquitin–proteasome degradative pathway (Myeku et al., 2012), an examination of p62 levels was not pursued here.

**Discussion**

In this study, human skin fibroblasts containing the PS1-fAD mutation A246E were found to exhibit elevated lysosomal pH (Fig. 2.1), reduced availability of active cathepsin D and reduced cleavage to the mature form of the enzyme (Fig. 2.2), and also impaired degradation of autophagic substrates (Fig. 2.3) as compared to levels from control fibroblasts. Substantive increases in the expression of genes associated with lysosomal and autophagic degradative
Machineries were also detected in PS1-fAD fibroblasts (Fig. 2.4), with increases mirrored by protein levels (Fig. 2.5). Finally, lysosomal acidification was restored by elevation of intracellular cAMP, leading to a partial restoration of cathepsin D active site availability, as well as reduction in the LC3B-II/I ratio (Fig. 2.6). Together, these changes indicate a small but significant steady-state dysfunction in lysosomal pH and degradation as a consequence of the A246E PS1-fAD mutation, and offer some clues as to how to address this problem therapeutically.

**Elevation of pH**

The concurrence of effects on pH, cathepsin D, protein and gene levels strongly support the conclusion that lysosomes in PS1-fAD fibroblasts are alkalized as compared to controls. Our protocol to measure lysosomal pH enables the accurate detection of small changes in this pH. While the elevation of lysosomal pH by 0.2 units may appear small, this change occurs across the sharpest part of the pH/activity curve for many lysosomal enzymes, where even a minimal rise in pH is sufficient to depress enzymatic activity and slow down degradation of cellular materials (Barrett, 1970). A pH elevation of only 0.2 units was recently shown to impair proteolysis in macrophage phagosomes (Jiang et al., 2012), confirming that modest alkalizations are capable of inducing pathological changes. The decreased cleavage of cathepsin D into the mature form and the reduction in binding of BODIPY FL-pepstatin A to the active site of this major lysosomal protease are consistent with functional consequences of lysosomal alkalization. This deficit is likely small enough that most cellular processes can progress unhindered under normal circumstances. However, the gradual accumulation of improperly processed material may decrease efficient cellular function over time. This slow accumulation is predicted to be more severe in active post-mitotic cells like neurons, which rely upon high baseline levels of autophagic degradation as a primary clearance mechanism (Boland et al., 2008). Given the differences between acute modulations of autophagy and the chronic changes in PS1-fAD fibroblasts, however, the situation is likely to be complex, and differences in the magnitude of the LC3 response in PS1 cells compared to control and those treated with CHQ are not unreasonable. Of
interest in this regard is a study demonstrating that chronic treatment with chloroquine or NH\textsubscript{4}Cl, but not the autophagy induction inhibitor 3-MA, leads to increased p62 and LC3B-II/I in rat cortical neurons (Myeku and Figueiredo-Pereira, 2011). Whether that result reflects the short-term actions of 3-MA (Wu et al., 2010), or merely different feedback systems activated by lysosomal alkalization, is not clear.

While modest alkalization may have functional consequences over time, the small magnitude of the change may explain why this difference has been so difficult to detect. Measuring lysosomal pH is a difficult task under ideal conditions, and single-wavelength assays such as LysoTracker Red may not be sensitive enough to detect the small changes demonstrated here (Neely et al., 2011, Coen et al., 2012). Similarly, while the microscopy-based assay for measuring pH\textsubscript{L} used by Zhang et al. (2012) may enable specific localization of acidic organelles, its readout of average vesicular pH apparently yields a value far higher, both in WT- and PS1k/PSdko-ES, than would be found in functional lysosomes, and therefore the utility of such an approach to address the question of lysosomal pH is rather unclear. Our assay has been designed to minimize variation and detect small differences in vesicular pH; the use of a membrane-permeable pH probe may also enable access to different compartments than dextran-conjugated probes, the readout of which is necessarily restricted to that lysosomal sub-population undergoing fusion with endosomes, and thus may not be indicative of compromised lysosomal subpopulations. In particular, the concordance of our direct pH measurement data with the more indirect BODIPY FL-pepstatin A data, as well as with the immunoblots showing impaired maturation of cathepsin D, provides strong support for the accuracy of our approach.

This study does not identify the ultimate source of pH\textsubscript{L} dysregulation in these fibroblast culture types. It may indeed result from improper trafficking of v-(H\textsuperscript{+})ATPase due to a defect in the v0a1 subunit, as the Nixon laboratory has suggested (Lee et al., 2010), or perhaps from some other underlying defect or defects in the autophagic or endo-lysosomal axes. Our qPCR results
indicating an increase in message for the proton pump suggest a compensatory mechanism is being attempted. It is important to note that our qPCR probes were designed to amplify ATP6V1B2, the B2 subunit of the proton pump, which is the more ubiquitously expressed variant of the B subunit of v-(H+)ATPase (Puopolo et al., 1992, van Hille et al., 1994). No trafficking issue with this subunit has been identified thus far in AD.

The gene expression and protein data are generally consistent with feedback attempts to compensate for perturbed lysosomal pH. The fact that a relatively short-term incubation with CHQ is insufficient to induce the protein increases observed in PS1-fAD suggests, too, that these fibroblasts may initially attempt to manage pH increases by some other means than increased autophagic initiation. It is possible, however, that sustained, life-long increase in pH, in comparison to a temporary, pharmacological increase, may differentially affect the degradative system along the entirety of its axis. Interestingly, BCN1 expression is also increased in fibroblasts from patients with certain storage diseases (Pacheco et al., 2007), further substantiating the similarities observed between primary lysosomal disorders and AD (Nixon, 2004, Nixon et al., 2008), and suggesting that up-regulation of autophagy in response to accumulated material, while perhaps a conserved strategy, may not be effective in all cases. The small-but-significant up-regulation of TFEB in PS1-fAD fibroblasts is particularly intriguing, given that Zhang et al. (2012) reported no difference from wild type in the expression of TFEB while investigating gene expression profiles of PS1ko and PSdko-ES cells, as well as PScdko mice. The group also saw no change in the expression of ATG5 and BCN1 in any of these PSko models. These discrepancies suggest that cells containing mutated, but otherwise intact, PS1 exhibit a differential expression profile from cells that lack PS1 and/or PS2 all together; some caution should therefore be observed in conflating and comparing results obtained through the use of a variety of models and techniques. Together, the elevated expression and protein levels of these genes critical for autophagy and lysosomal acidification provide additional evidence for perturbed lysosomal pH and, moreover, may be indicative of a degradative system attempting to
compensate for a chronic defect at the lysosomal pH level.

Regardless, the major difference between control and PS1-fAD fibroblasts is the A246E mutation in PS1, and as multiple groups have implicated autophagic disruption in AD, the evidence points toward a basic deficit in cells with the AD-associated PS1 mutation that predisposes the cells toward a phenotype of accelerated aging, including lysosomal failure and protein buildup.

Re-acidification by cAMP

Importantly, our data show that re-acidifying lysosomes via elevated intracellular cAMP can help to restore the lysosomal pH and autophagic turnover associated with the PS1-fAD (A246E) mutation. The mechanism by which cAMP may do this in these fibroblasts is currently unknown, though similar acidifying processes have been linked to protein kinase A (PKA) activity in other cells (Liu et al., 2008, Alzamora et al., 2010, Paunescu et al., 2010) and may involve the activation of a Cl− channel (Liu et al., 2012). Another intriguing mechanistic possibility for cAMP’s effect on pH could involve PKA-mediated inhibition of glycogen synthase kinase 3 (GSK3) (Fang et al., 2000). While the activity of both the α and β isoforms of GSK3 has been associated with AD pathology (Cho and Johnson, 2003, Phiel et al., 2003), a number of recent studies have also identified links between GSK3 and lysosomal dysfunction, whether pharmacologically- or pathologically-induced (Dobrowolski et al., 2012, Parr et al., 2012, Avrahami et al., 2013). GSK3 inhibition reportedly restores lysosomal acidification defects in both the 5XFAD mouse model and in cells lacking PS1/2 (Avrahami et al., 2013) and also improves amyloid pathology (Parr et al., 2012, Avrahami et al., 2013). Interestingly, low PKA activity has been reported in brain tissue from AD patients (Liang et al., 2007), consistent with a reduction in regulated lysosomal acidification. Augmenting this system by increasing intracellular cAMP levels and re-acidifying lysosomes may help to enhance clearance in older neurons and prevent additional consequences of lysosomal defect, such as perturbed calcium signaling (Christensen et al., 2002). The ability of cAMP to reverse the rise in LC3B-II/I ratio seen in the PS1-fAD mutant cells also supports the
central role of lysosomal pH in controlling this ratio. In particular, the fact that cAMP simultaneously increases LC3B-I while decreasing LC3B-II points to its considerable power to affect autophagy and autophagic clearance, perhaps through some more fundamental effect on the system than pH alone. Intriguingly, the effect of short-term cAMP treatment apparently reduces LC3B-II to a level below the control baseline; we suspect that this may be due to differences between chronic and acute effects on autophagic states. By contrast, the reduction of baseline pH\textsubscript{L} observed in control fibroblasts was minimal and not significant. Preliminary results suggest, however, that cAMP lowers lysosomal pH in control fibroblasts when that pH is artificially elevated, which is consistent with the idea that the cAMP cocktail proves most effective when pH\textsubscript{L} is perturbed from the baseline.

Overall, this study suggests that small deficits in autophagic degradation, linked to improper maintenance of lysosomal pH, may contribute to pathologic build-up of protein in PS1-fAD fibroblasts, and that lysosomal re-acidification may offer a strategy by which this accumulation can be ameliorated. In other words: regardless of whether lysosomal failure is the precipitating event in disease, or simply a consequence of PS1-mutation-associated phenotypes, re-acidification could equalize the cellular playing field.

**Acknowledgements**

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## Tables

Table 2.1. qPCR primers.

<table>
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<tr>
<th>Target</th>
<th>NCBI code</th>
<th>Primer pair</th>
<th>Tm (°C)</th>
<th>Product length (bp)</th>
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</thead>
</table>
| ACTB   | NM_001101.3 | Sense: AGAAAAATCTGGCACCACACC  
Antisense: GGGGTGTGAAGGTCTCAAA | 59.97  
59.94 | 142 |
| ATP6V1B2 | NM_001693.3 | Sense: GAGGGGCAGATCTATGTGGA  
Antisense: GCATGATCCTTCCTGGTCAT | 60.00  
59.89 | 128 |
| ATG5   | NM_004849.2 | Sense: GCAAGCCAGACAGGAAAAAG  
Antisense: GACCTTCAGTGGTCCGGTAA | 59.99  
59.97 | 137 |
| BECN1  | NM_003766.3 | Sense: AGGTTGAGAAAGCGAGACA  
Antisense: GCTTTGTCCACTGCTCCTC | 59.99  
60.00 | 139 |
| TFEB   | NM_001167827.2 | Sense: GTCCGAGACCTATGGGAACA  
Antisense: CGTCCAGAAGCATATGGTG | 58.52  
57.28 | 218 |
Figures

Figure 2.1. PS1-fAD mutation yields elevated pH\textsubscript{L} in human skin fibroblasts. A–D, Fifteen-minute treatment with pH\textsubscript{L}-elevating compounds significantly increases lysosomal pH in fibroblasts from controls. A, Bafilomycin A1 (Baf A1) 200 nM. B, NH\textsubscript{4}Cl 20 mM. C, Tamoxifen (TMX) 30 µM. D, Chloroquine (CHQ) 10 µM; for each n = 6–14 wells. E, Lysosomal pH levels in CTRL vs. PS1-fAD fibroblasts, n = 10. F, Summary of baseline lysosomal pH levels across 26 separate experiments, expressed as uncalibrated ratios of fluorescence excited at 340 vs. 380 nm – ∼pH\textsubscript{L} (lysosomal pH). Each experiment from 7 to 10 wells of each genotype, total 222 CNTL and 224 PS1-fAD. All values normalized to the mean control for each experimental set. G, H, Confocal images of 2 µM LysoSensor staining at ex/em 405/450 nm for CTRL, G, and PS1-fAD fibroblasts, H. I, J, Confocal images of 50 nM LysoTracker staining at ex/em 405/450 nm for CTRL, I, and PS1-fAD fibroblasts, J. Scale bars = 10 µm. Throughout, “∗” signifies p < 0.05 and CTRL = untreated control fibroblasts.
Figure 2.2. Cathepsin D active site availability and maturation reduced in PS1-fAD fibroblasts.
A, BODIPY FL-pepstatin A fluorescence is markedly reduced in PS1-fAD fibroblasts when compared to CTRL. Scale bar = 100 µM. B, Mean intensity per cell analysis of BODIPY FL-pepstatin A fluorescence reveals a significant loss in fluorescence; n = 42 CTRL and 40 PS1-fAD cells. C, A sample Western blot showing reduction of mature cathepsin D in PS1-fAD fibroblasts. D, The ratio of 28-kDa/52-kDa cathepsin D is reduced in PS1-fAD fibroblasts. E, The level of 52-kDa or, in F, 47-kDa cathepsin D, is the same in PS1-fAD and control fibroblasts. The reduction in the mature 34-kDa band approaches significance in G at p = 0.07, while the mature 28-kDa band is significantly reduced in PS1-fAD cells, H. For D–H, n = 3. Throughout, "∗" signifies p < 0.05 and CTRL = untreated control fibroblasts.
Figure 2.3. Elevated pH$_i$ impairs autophagic degradation through the lysosomes.

A, A sample Western blot showing elevation of LC3B-II in PS1-fAD fibroblasts. B, LC3B-II/I is elevated in PS1-fAD fibroblasts. C, LC3B-I is unchanged in PS1-fAD fibroblasts, while LC3B-II is significantly elevated. For B, C, n = 15 CTRL, 16 PS1-fAD, 9 blots total. D, A sample Western blot showing elevation of LC3B-II in CTRL cells treated with 10 µM chloroquine (CHQ) for 6 h. E, LC3B-II/I is elevated in CTRL fibroblasts treated with CHQ. F, LC3B-I is unchanged in CHQ-treated fibroblasts, while LC3B-II is significantly elevated. For E, F, n = 5 CTRL, 5 CTRL + CHQ, 5 blots total. G, A sample Western blot showing elevation of p62 in PS1-fAD fibroblasts and in CTRL cells treated with 10 µM CHQ for 6 h. H, p62 is elevated in PS1-fAD fibroblasts, (n = 13 CTRL, 14 PS1-fAD, 8 blots total). I, p62 is elevated following 6 h chloroquine incubation, n = 5 per condition, 5 blots total. Throughout, "∗" signifies p < 0.05 and CTRL = untreated control fibroblasts. Protein level of each protein was normalized to GAPDH as an internal control.
Figure 2.4. PS1-fAD fibroblasts exhibit altered gene expression profile. Results from quantitative PCR experiments indicating the increased expression of genes in fibroblasts from the PS1-fAD cells as compared to unaffected controls. A, ATP6V1B2 expression elevated in PS1-fAD fibroblasts. B, ATG5 expression elevated in PS1-fAD fibroblasts. C, BECN1 expression elevated in PS1-fAD fibroblasts. D, TFEB expression elevated in PS1-fAD fibroblasts. n = 4 independent trials for each. ** signifies p < 0.05 for entire figure. Expression level of each gene was first normalized to ACTB expression as an internal control.
Figure 2.5. PS1-fAD fibroblasts have increased levels of lysosome- and autophagy-associated proteins.

A, A sample Western blot showing elevation of vATPaseB2 (ATP6V1B2) in PS1-fAD fibroblasts, but not in CTRL cells treated with 10 µM CHQ for 6 h. B, vATPaseB2 is not elevated following 6-h CHQ incubation; n = 4 CTRL, 4 CHQ, 4 blots total. C, vATPaseB2 is elevated in PS1-fAD fibroblasts; n = 4 CTRL, 4 PS1-fAD, 4 blots total. D, A sample Western blot showing elevation of Atg5 in PS1-fAD fibroblasts, but not in CTRL cells treated with 10 µM CHQ for 6 h. E, Atg5 is not elevated following 6-h CHQ incubation; n = 5 CTRL, 5 CHQ, 5 blots total. F, Atg5 is elevated in PS1-fAD fibroblasts; n = 7 CTRL, 8 PS1-fAD, 6 blots total. G, A sample Western blot showing elevation of beclin-1 in PS1-fAD fibroblasts, but not in CTRL cells treated with 10 µM CHQ for 6 h. H, Beclin-1 is not elevated following 6-h CHQ incubation; n = 4 CTRL, 4 CHQ, 4 blots total. I, Beclin-1 is elevated in PS1-fAD fibroblasts; n = 8 CTRL, 8 PS1-fAD, 6 blots total. Throughout, "*" signifies p < 0.05 and CTRL = untreated control fibroblasts. Protein level of each protein was normalized to GAPDH as an internal control.
Figure 2.6. cAMP restores pH$_L$ and improves clearance in PS1-fAD fibroblasts.

A. Treatment of PS1-fAD cells with cAMP reduced lysosomal pH levels back to control levels. Treatment of control cells with the cocktail had a minimal effect. LysoSensor ratios normalized to mean control of each day’s experiment. N = 14 plates with 7–10 wells each. cAMP cocktail = 500 µM cpt-cAMP + 100 µM IBMX + 10 µM forkoslin. *p < 0.05 vs control, †p < 0.05 vs PS1-fAD alone. B, BODIPY FL-pepstatin A fluorescence is increased in PS1-fAD fibroblasts treated with cAMP cocktail. Scale bar = 100 µM. C, Mean intensity per cell analysis of BODIPY FL-pepstatin A fluorescence reveals a significant increase in fluorescence; n = 60 PS1-fAD and 81 PS1-fAD cells. D, Western blot showing incubation with cAMP cocktail decreases LC3B-II/I in PS1-fAD fibroblasts. E, Summary of six Western blots showing a significant decrease in LC3B-II/I in PS1-fAD fibroblasts treated with the cAMP cocktail. F, LC3B-I is significantly increased in cAMP-treated fibroblasts, while LC3B-II is significantly reduced. For E, F, n = 11 PS1-fAD, 8 PS1-fAD + cAMP, 6 blots total. *p < 0.05 for the entire figure.
CHAPTER 3:

cAMP acts through PKA and mTOR to restore lysosomal pH in presenilin 1 A246E Alzheimer fibroblasts

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Abstract

Mutations in the transmembrane protein presenilin 1 (PS1) lead to the early-onset, inherited, familial form of Alzheimer’s disease (fAD). Some PS1 mutations are known to exacerbate the autophagic and lysosomal pathology of AD, including the A246E mutation. This mutation results in increased lysosomal pH, and induces a compensatory response from the lysosomal and autophagic machinery. Defective lysosomes can be acidified by treatment with a cAMP-elevating cocktail. In this study, we confirmed the beneficial effects of cAMP on lysosomal pH in A246E mutant fibroblasts, and explored the mechanisms underlying this improvement. Re-acidification was supported by increased binding of boron-dipyrromethene (BODIPY) FL-pepstatin A to the cathepsin D active site. cAMP’s beneficial effects on both lysosomal pH and cathepsin D active site availability were found to be dependent on protein kinase A (PKA) activity. cAMP treatment increased levels of phosphorylated mTOR; both pH restoration and mTOR phosphorylation were dependent on PKA. cAMP treatment resulted in dramatically reduced expression of transcription factor EB after 6 h, and reduced ATP6V1B2 expression after 12 h. cAMP’s ability to restore lysosomal pH in compromised cells was replicated in primary rat cortical cultures with pharmacologically-elevated lysosomal pH, thus demonstrating a broader application for cAMP treatment. Together, these results confirm cAMP’s ability to restore lysosomal pH, implicate PKA in pH restoration, and indicate that the downstream response to pH restoration is a complex, PKA-mediated process that engages the mTOR-TFEB signaling pathway.
Introduction

Ever since the discovery of the lysosome in 1955, defects in essential lysosomal catabolic enzymes were hypothesized, and later found, to play a critical role in several storage diseases. More recently, however, we have come to understand that lysosomal dysfunction is not limited to these severe, though relatively rare, inherited disorders. Instead, even mild disruptions of the lysosomal environment have proven to have far wider reach, with lysosomal underperformance implicated in macular degeneration and in a host of age-related neurodegenerative disorders, including Alzheimer’s disease (Cataldo and Nixon, 1990, Cataldo et al., 1996, Cataldo et al., 2004, Nixon, 2005, Nixon et al., 2005, Nixon and Cataldo, 2006, Khurana et al., 2010, Lee et al., 2010, Lipinski et al., 2010, Avrahami et al., 2013, Coffey et al., 2014).

The neuropathology of Alzheimer’s disease (AD) is traditionally associated with the build-up of extracellular senile plaques of the peptide fragment amyloid-β, and of intracellular neurofibrillary tangles associated with hyperphosphorylated tau protein (Glenner and Wong, 1984a, b, Kosik et al., 1986, Nukina and Ihara, 1986). For some years, however, another pathology has been linked to disease: the build-up of incompletely degraded material in swollen lysosomes and autophagosomes (Nixon et al., 2005). Alzheimer’s-associated mutations of the transmembrane protein presenilin 1 (PS1), a protein that is also responsible for the final cleavage step in amyloid-β production, appear to exacerbate this pathology (Cataldo et al., 2004). Of note, a major lysosomal protease, Cathepsin D (Cat D) has been identified as a component of senile plaques (Cataldo and Nixon, 1990), further implicating the lysosomal/autophagic degradative pathway in the disease.

The role of PS1 mutation in accelerating autophagic pathology in AD has been a subject of much debate over the past few years. In 2010, PS1 loss, and even its AD-associated mutation, was linked to increased lysosomal pH – possibly through a failure to glycosylate and target the V0a1 subunit of the lysosomal proton pump, v-(H⁺)ATPase (Lee et al., 2010). While several other
laboratories were unable to confirm this pH elevation (Neely et al., 2011, Coen et al., 2012, Zhang et al., 2012), this may be due to the considerable technical challenge of in making accurate measurements of lysosomal pH (Wolfe et al., 2013, Guha et al., 2014). We, and others, confirmed the link between increased lysosomal pH and PS1 mutation (Avrahami et al., 2013, Coffey et al., 2014). Our laboratory also demonstrated that elevated cAMP is able to restore pH and lysosomal function (Coffey et al., 2014), though cAMP’s mechanisms of action and signaling targets were still uncertain. The observed disruption of autophagy in PS1-fAD fibroblasts, however, suggests several possible targets of cAMP-induced lysosomal re-acidification.

It has been known for some time that the mechanistic target of rapamycin complex 1 (mTORC1) negatively regulates mammalian autophagy, but its role in, and response to, lysosomal health and stability was discovered much more recently. mTORC1 was shown to interact with the lysosomal “master switch” transcription factor EB, or TFEB, which regulates both autophagy and lysosomal biogenesis (Sardiello et al., 2009, Palmieri et al., 2011, Settembre et al., 2011). Interestingly, Settembre and others have shown that TFEB not only positively regulates its own transcription (Settembre et al., 2013), but that it can be phosphorylated by active mTOR on the lysosomal membrane in a rapamycin-independent manner (Martina et al., 2012, Rocznia-Ferguson et al., 2012, Settembre et al., 2012). We have previously found TFEB expression to be itself up-regulated in PS1-fAD fibroblasts, along with several other TFEB targets in the Coordinated Lysosomal Expression and Regulation (CLEAR) network (Coffey et al., 2014), presumably as a compensatory response to autophagy failure. These findings, in combination with recent work implicating mTORC1 in both TFEB activity and in the lysosomal response to nutrients (Martina et al., 2012, Rocznia-Ferguson et al., 2012, Settembre et al., 2012), suggested that both TFEB and mTOR might mediate cAMP’s effects.

Our previous work validated that human skin fibroblasts containing the familial PS1 mutation A246E are characterized by increased lysosomal pH, and that this increase results in myriad
other problems: in degradative deficiency, lysosomal enzyme loss, and compensatory up-regulation of the machinery (Coffey et al., 2014). We also found that elevation of intracellular cAMP improves both lysosomal pH and reduces autophagic backlog in PS1-fAD fibroblasts. In this study, we asked how cAMP restores lysosomal pH. We first asked whether protein kinase A, the activity of which is regulated by cAMP, might mediate cAMP’s action in PS1-fAD fibroblasts. We also investigated whether mTOR and TFEB, as known mediators of lysosomal and autophagic responses, might also respond to cAMP treatment, thereby providing a potential downstream mechanism for cAMP’s action. Here, we demonstrate the general applicability of the cAMP therapeutic approach, find that cAMP treatment restores lysosomal pH and Cat D availability, increases mTOR phosphorylation in PS1-fAD cells, and decreases mRNA expression of both TFEB and ATP6V1B2; these effects appear to depend upon PKA activity.

Materials and Methods

Culture of human fibroblast skin cells
Culture of human skin fibroblast cells was carried out as described (Coffey et al., 2014). Briefly, human skin fibroblast cultures were purchased from the National Institute of Aging’s Cell Culture Repository at Coriell Institute of Medical Research (Camden, NJ); catalog numbers AG08170 (PS1-fAD fibroblasts with the A246E mutation) and AG07621 or AG07623 (unaffected spousal fibroblasts) were used in this study. Cells were cultured in Eagle’s Minimum Essential Medium supplemented with 15% fetal bovine serum, 1% penicillin/streptomycin (Life Technologies Corp., Grand Island, NY, USA), and 1X GlutaMAX (Life Technologies). Cultures were maintained at 37°C in 5.5% CO₂. Cells were cultured a minimum of 6 d before assays were performed, as this was previously found to affect the development of lysosomal pathology (Coffey et al., 2014).

Culture of rat primary cortical cells
Primary cortical cultures from neonatal Sprague Dawley rats were obtained through the University
of Pennsylvania Perelman School of Medicine’s “Neurons R Us” service, which is run by Dr. Marc
Dichter. On the day of isolation, cultures were plated into dishes pre-coated with 0.01% poly-L-
lysine (Peptide Institute, Osaka, Japan); cultures were then grown in neurobasal medium + B27
supplement (both Life Technologies). Cells were cultured a minimum of 11 d before assays were
performed. Cultures were determined by antibody staining to be primarily neuronal (data not
shown); astrocytes were stained for GFAP, and neurons for Pgp9.5 (see Microscopy section of
Materials and Methods for details). In some experiments, cells were treated with the anti-mitotic
compound β-D-cytosine arabinofuranoside (Ara-C) to halt astrocytic growth and increase the
proportion of neurons; results for these experiments were similar with and without Ara-C.

Measurement of lysosomal pH

Lysosomal pH (pH_L) was measured as previously described (Coffey et al., 2014). Briefly, fibroblast
cells or neuronal cultures were plated into black-walled, clear-bottom 96-well plates and
grown for a period of time appropriate to each cell type (see above). On the day of the
experiment, cells were pre-treated in complete growth medium with appropriate drugs; then
medium was removed and cells were incubated for 3 min in 2 µM LysoSensor Yellow/Blue (Life
Technologies). All steps including and following this incubation were performed under foil. Cells
were rinsed 3x in room temperature isotonic solution (NaCl, 105 mM; KCl, 5 mM; HEPES-Acid,
6 mM; Na-HEPES, 4 mM; NaHCO₃, 5 mM; mannitol, 60 mM; glucose, 5 mM; MgCl₂, 0.5 mM;
CaCl₂, 1.3 mM; pH, adjusted to 7.4). Cells were then incubated for 10 min in either isotonic
solution or in isotonic solution containing the relevant drug(s). Final drug concentrations were:
30 µM tamoxifen, cyclic adenosine monophosphate (cAMP) cocktail (500 µM cpt-cAMP, 100 µM
3-isobutyl-1-methylxanthine (IBMX), 10 µM forskolin (forskolin from LC Laboratories, Woburn,
MA, USA)) and 1 µM myristoylated protein kinase inhibitor 14-22 amide, henceforward “PKI”
(Tocris Bioscience, Bristol, UK). The pH assay with γ-secretase inhibitor IX (#565784, EMD
Millipore Corp., Billerica, MA, USA) referenced in the Results section involved a 24-48h
preincubation with the inhibitor; differing incubation times did not impact experiment outcomes.
For pH experiment shown in Fig 3.2.A, pH was measured using LysoSensor Yellow/Blue dextran (Life Technologies). Cells were incubated with 25 µg/ml fluorophore overnight in medium at 37ºC. The following day, cells were chased for 6 h in fresh medium, with 1 µM PKI added in medium for the final hour as required. The assay then proceeded as above, although the 3 min LysoSensor incubation step was omitted.

**Microscopy**

Live-cell confocal microscopy was carried out as previously described (Coffey et al., 2014). Briefly, for boron-dipyrromethene (BODIPY) FL-pepstatin A (Life Technologies) staining, cells were pre-treated for 6 h with fresh medium or with medium plus cAMP cocktail. In the last hour of incubation, BODIPY FL-pepstatin A was added to each treatment condition to a concentration of 1 µg/ml. Samples were rinsed and imaged in Dulbecco’s Phosphate-Buffered Saline (DPBS) (+Ca²⁺/+Mg²⁺) (Life Technologies) using the University of Pennsylvania Live Cell Imaging Core’s Nikon A1R Laser Scanning Confocal Microscope (excitation 488 nm/emission 525 nm). Fluorescence imaging was performed using the Argon laser; images were captured at 60X.

Fixed-cell fluorescent microscopy was carried out using a Nixon Eclipse E600 optical microscope. Images were captured at 40X magnification. For fibroblasts, cultures were grown to desired confluence on glass coverslips, then rinsed briefly in DPBS and fixed in 4% paraformaldehyde. Cells were rinsed with DPBS and permeabilized at room temperature in quenching buffer (20 mM glycine, 75 mM NH₄Cl) + 0.1% Triton X-100 for 8 min, followed by quenching buffer + 0.1% Triton X-100 + 0.05% SDS for 2 min; coverslips were then rinsed with DPBS. Coverslips were blocked for 2 h in blocking solution (1.4 cold fish skin gelatin from Sigma, 500 µl 10% w/v saponin stock in H₂O, and 5% normal goat serum, with DPBS to 200 ml). All coverslips were rinsed with DPBS and incubated with primary antibody in blocking solution overnight at 4ºC. The following day, coverslips were rinsed first in DPBS and then in blocking solution, and then incubated with
secondary antibody in blocking solution for 1 h. Cells were washed in first DPBS, then blocking solution, then DPBS; fibroblast morphology was then stained by incubating with the beta-actin dye Alexa Fluor 568 phalloidin (#A12380, Life Technologies) at 5 U/ml for 20 min, followed by a quick DPBS/blocking solution/DPBS rinse; nuclei were then stained by incubating with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) from Life Technologies (#D1306) at 0.5 µg/ml in DPBS for 1 min. Coverslips were rinsed in DPBS, mounted onto glass slides in Slow Fade Gold Antifade Mountant (#S36936, Life Technologies), and then sealed for imaging. To detect lysosomes, the mouse primary antibody for LAMP2 (#ab25631, Abcam, Cambridge, MA) was used at a dilution of 1:100; the secondary antibody Goat anti-mouse IgG (H+L) Alexa Fluor® 488 (#A11001, Life Technologies) was used at a dilution of 1:500.

For neurons, cultures were grown on glass coverslips, then rinsed briefly in DPBS and fixed in 4% paraformaldehyde. Coverslips were then rinsed in DPBS and permeabilized in 0.1% Triton X-100 for 20 min at room temperature. Cells were blocked with 10% goat serum, and incubated with primary antibodies overnight at 4°C. The following day, coverslips were rinsed in diluted blocking buffer at room temperature, incubated with secondary antibodies for 2 h in the dark, rinsed again, and mounted onto glass slides using SlowFade Gold + DAPI (#S36938, Life Technologies), and then sealed for imaging. To detect neurons, the rabbit primary antibody for Pgp9.5 (#AB5975, EMD Millipore Corp.) was used at a dilution of 1:1000; to detect astrocytes, the murine primary antibody for GFAP (#MAB360, EMD Millipore Corp.) was used at a dilution of 1:400. Secondary antibodies used were a goat anti-rabbit antibody at 1:1000, and a goat anti-mouse antibody at 1:2000 (Life Technologies #A11006 and #A10035, respectively). Neuronal images were captured at 20X.

**Quantitative measurement of BODIPY FL-pepstatin A fluorescence**

For quantitative assessment of BODIPY FL-pepstatin A fluorescence in neuronal cultures, cells were cultured in clear-bottom, black-walled 96-well plates, using the medium components
described above, for a minimum of 11 d. To begin assay, cells were pre-incubated for 1 h with 10 ng/ml BODIPY FL-pepstatin A in complete medium. Medium, along with fluorophore, was removed, cells were rinsed 3x with isotonic solution, and then 100 µl of the appropriate treatment condition was added to each well. Cells were assayed after 10 min incubation with drugs, and fluorescence measured with a Fluoroskan Ascent Microplate Fluorometer and recorded using ASCENT software (Thermo Scientific, Waltham, MA, USA). Fluorescence was measured at 485 nm excitation/527 nm emission. For measuring BODIPY FL-pepstatin A fluorescence in PS1-fAD fibroblasts, cells were cultured in 96-well plates as above; cells were grown for at least 6 d and to confluence. To begin assay, cells were pre-treated with one of 3 conditions: control medium; cAMP cocktail for 6 h; or PKI for 1h, then PKI + cAMP cocktail for 6h. In the final hour of pre-treatment, 1 ug/ml BODIPY FL-pepstatin A (Life Technologies) was added to each treatment condition. Medium, along with treatment conditions and fluorophore, was removed, cells were rinsed 3x with isotonic solution, and then 100 ul isotonic solution was added to each well. After a brief interval (about 5 min), fluorescence was measured as above. Also as above, fluorescence was measured at 485 nm excitation/527 nm emission.

**Immunoblots**

Immunoblotting was performed according to standard protocols used previously (Coffey et al., 2014). Briefly, fibroblasts were grown to confluence in 6-well plates and treated with drug solutions as required by each experiment. Protein was extracted in radio immunoprecipitation assay (RIPA) buffer and protein concentration was quantified using the Pierce® BCA Protein Assay Kit (ThermoFisher Scientific). Samples were prepared for electrophoresis and run on a pre-cast 4-15% Mini-PROTEAN® TGX™ Gel (Bio-Rad, Hercules, CA, USA). Sample transfer to a PVDF membrane (EMD Millipore Corp.) was performed according to standard protocols, and membranes were then blocked for 1 h in 5% non-fat milk solution (powdered milk from LabScientific, Highlands, NJ). Primary antibody incubations were carried out overnight at 4°C, with all primary dilutions at 1:1000. The secondary, an anti-rabbit, horseradish peroxidase (HRP)-
conjugated whole antibody (GE Healthcare Life Sciences, Amersham, UK) was diluted at 1:3000 and incubated with the membrane the following day for 1 h. Bands were developed using electrogrenated chemiluminescence (ECL) and the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences). Development and analysis of membrane images was performed using the ImageQuant LAS 4000 system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Each membrane included an n of 2-3 samples per condition; for analysis, band volume of the protein of interest was first normalized to the loading control for each lane, and then normalized across the membrane to the mean band volume of the protein of interest in the untreated control condition.

Primary antibodies: for phosphorylated mammalian target of rapamycin (p-mTOR), rabbit polyclonal antibody against the S2448 phosphorylation site was used (#2971, Cell Signaling Technology, Beverly, MA, USA). For mTOR, rabbit polyclonal antibody was used (#2972, Cell Signaling Technology). For the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rabbit monoclonal antibody GAPDH (14C10) was used (#2118, Cell Signaling Technology).

**Quantitative polymerase chain reaction (qPCR)**

Total RNA was isolated from cultured human skin fibroblast cells using the TRIzol (Life Technologies) extraction protocol, then quantified and converted to cDNA as previously described (2014). qPCR was also performed as previously described, and expression levels of each gene were normalized to the expression of housekeeping gene ACTB (β-actin). Human primers were purchased from Life Technologies/Sigma–Aldrich according to sequences listed in Table 1. Primer sequences for ACTB, TFEB, and ATP6V1B2 were derived from Primer3.

**Data analysis**

Data were compiled and analyzed using SigmaPlot statistics software (v11.0, Systat Software,
Inc., San Jose, CA, USA) unless otherwise noted. A p-value of p < 0.05 was considered significant; data were analyzed by ANOVA and then the appropriate post-hoc test. Bar graph values represent means plus or minus standard error. When the distribution of data was non-normal, further analysis was performed on ranks.

All materials were purchased from Sigma–Aldrich Corp. in St. Louis, MO, unless otherwise noted.

Results

cAMP restores lysosomal pH and Cat D active site availability in human fibroblast cultures

Lysosomal pH was elevated by about 0.2 pH units in PS1-fAD fibroblasts when compared to non-Alzheimer’s fibroblasts (Coffey et al., 2014); this effect was not dependent upon PS1 activity, as treatment of CTRL cells with γ-secretase inhibitor IX had no effect upon pH, and treatment of PS1-fAD cells with the inhibitor had no further effect upon pH [data not shown]. 10 min treatment with a cAMP-elevating cocktail (consisting of 500 µM cpt-cAMP, 100 µM IBMX, and 10 µM forskolin), however, partially restored lysosomal pH in CTRL cells treated with tamoxifen [data not shown] and also lowered lysosomal pH in PS1-fAD cells (Fig. 3.1.A). These data indicated that cAMP-induced lysosomal pH restoration was rapid and most likely did not depend upon additional transcription or translation. Next, CTRL, PS1-fAD, and PS1-fAD fibroblasts were treated with 6 h with cAMP and stained for LAMP2 to detect lysosomes, and for phalloidin to detect basic cellular morphology. Though cell size and morphology did differ somewhat between CTRL and PS1-fAD fibroblasts, no substantial differences in lysosome number or distribution were observed following cAMP treatment; representative images are shown in Fig. 3.1.B. These findings support the observed cAMP effect in PS1-fAD cells as a product of pH reduction, and not as a product of changes in lysosome number.
Given the potential impact of restoring defective lysosomal pH in cells with PS1 mutations, we sought to test whether cAMP elevation restores lysosomal proteolytic function, using cathepsin D activity as a readout. The fluorophore BODIPY-FL-pepstatin A is fluorophore is conjugated to the highly specific Cat D inhibitor pepstatin A, and therefore offers a measure of Cat D active site availability (Chen et al., 2000a). The technique allows for an indirect confirmation of lysosomal pH, as both Cat D function (Barrett, 1970) and maturation (Rosenfeld, 1982) require an acidic lysosomal milieu. 6 h treatment with cAMP cocktail visibly increased fluorescence of BODIPY FL-pepstatin A (Fig. 3.1.C), supporting the ability of cAMP to re-acidify compromised lysosomes and implying enhanced activity of Cat D.

**PKI blocks cAMP-induced pH restoration**

Prior work from a number of laboratories, including our own, has linked the activity of protein kinase A (PKA) to lysosomal acidification (Liu et al., 2008, Alzamora et al., 2010, Paunescu et al., 2010). We have previously shown that cAMP-induced lysosomal re-acidification in cultured human retinal pigmented epithelial cells can be blocked when cells are also treated with a cell-permeable, myristoylated protein kinase inhibitor (PKI)14-22 amide (Liu et al., 2008). Henceforward, this inhibitor will be referred to as “PKI.” The ability of PKI to block cAMP-induced pH restoration was therefore examined in PS1-fAD fibroblasts. The inhibitor was applied at 1 µM for all experiments as this concentration of the pseudosubstrate fragment reduces PKA activity in both rat islet cell extracts and in intact islets (Harris et al., 1997). Cells were pre-incubated with PKI for approximately 1 h prior to addition of the cAMP cocktail, ensuring minimal access to endogenous PKA substrate prior to the start of each experiment.

PKI completely and significantly blocked cAMP-induced lysosomal pH restoration in PS1-fAD fibroblasts (Fig. 3.2.A), indicating the short-term effects of cAMP upon lysosomal pH were dependent upon PKA activity. When PS1-fAD fibroblasts were treated with both cAMP and PKI for 6 h, and when this treatment was preceded by 1 h pre-incubation with PKI, the cAMP-induced
increase in Cat D active site availability was also completely blocked (Fig. 3.2.B). These data further implicated PKA in cAMP-induced pH restoration, and in its functional consequences for Cat D.

cAMP increases mTOR phosphorylation in PS1-fAD fibroblasts

mTOR complex 1 is a known regulator of autophagy. When the mTOR kinase component of this complex is activated, it initiates a signaling cascade that promotes cellular growth and blocks autophagy initiation (Ma and Blenis, 2009, Duvel et al., 2010, Kim et al., 2011, Laplante and Sabatini, 2012). Under conditions of starvation or following exposure to upstream signals or chemicals like rapamycin, mTOR itself becomes inhibited, and autophagy proceeds as needed until cellular nutrient balance is restored (Chang et al., 2009). Phosphorylation at residue S2448 has been shown to correlate with mTORC1 activity (Rosner et al., 2010); therefore, given our earlier observation that autophagy was perturbed in PS1-fAD fibroblasts (Coffey et al., 2014), the protein level of phosphorylated S2448 (henceforward, “p-mTOR”) was examined in both CTRL and PS1-fAD fibroblasts. No difference was observed in the protein level of either p-mTOR or mTOR when comparing CTRL and PS1-fAD fibroblasts (Fig. 3.3.A, B).

mTORC1 is highly responsive to changing nutrient conditions within the cell, and recently was found to modulate its activity based on the level of free amino acids within the lysosome; this changing activity depended on the lysosomal proton pump (Zoncu et al., 2011). As cAMP lowers lysosomal pH and increases Cat D active site availability, it was postulated that the resulting increase in degradation would lead to changes in mTOR phosphorylation. Therefore, it was examined whether incubation with cAMP led to increased mTOR phosphorylation in PS1-fAD fibroblasts. After 6 h treatment with cAMP cocktail, a significant increase in the level of p-mTOR was observed (Fig. 3.3.C, D). While a lesser increase in mTOR was also observed with cAMP, regression analysis showed that these increases in p-mTOR and mTOR were not correlated (data not shown). To probe whether p-mTOR was a cause or an effect of cAMP-mediated pH
restoration, PS1-fAD fibroblasts were treated with cAMP for 15 min. At this timescale, no change in either p-mTOR or mTOR was observed (Fig. 3.3.E, F). If mTOR phosphorylation were directly mediated by cAMP, one would expect to see the phosphorylation event happening concurrently with pH restoration. Instead, this timing suggests that lysosomal pH is restored first, and that mTOR phosphorylation and activation occurs later, once lysosomal degradative activity is back to normal.

**TFEB expression is reduced with cAMP**

Having previously observed increased gene expression of transcription factor EB (*TFEB*) in PS1-fAD fibroblasts with elevated lysosomal pH (Coffey et al., 2014), it was hypothesized that cAMP-induced pH restoration would result in a reduction in *TFEB* expression, as detected by quantitative real-time polymerase chain reaction (qRT-PCR). After 6 h incubation with the cAMP cocktail, the level of *TFEB* transcript in PS1-fAD fibroblasts was reduced by over 80%, though no decrease in expression of TFEB target genes *ATP6V1B2* was observed at this time (Fig. 3.4.A). After 12 h incubation with cAMP cocktail, however, the expression level of *ATP6V1B2* transcript was also significantly reduced, while expression of *TFEB* had returned to its approximate baseline level (Fig. 3.4.B).

**mTOR phosphorylation with cAMP Is blocked by PKI**

Finally, it was investigated whether, by blocking lysosomal pH elevation via PKI pre-incubation, the effects of pH restoration on mTOR activation would also be blocked. PS1-fAD fibroblasts were pre-incubated for 1 h with PKI, and then treated with either fresh medium, cAMP cocktail, or PKI + cAMP cocktail for 6 h. As in Fig. 3.3, a cAMP-dependent increase in p-mTOR was observed; preliminary data, while not yet significant, indicate that this increase may be blocked by PKI (Fig. 3.5.A, B). No changes in mTOR were observed, corroborating the hypothesis that the short-term, downstream events of cAMP treatment are dependent primarily on mTOR phosphorylation, and not on any baseline change in mTOR protein expression.
**cAMP restores lysosomal pH and Cat D active site availability in rat primary cortical cultures**

While the use of human fibroblasts permits study of actual patient tissue, it was also important to demonstrate that the cAMP approach worked in neurons. Because cAMP treatment is less effective when lysosomal pH is ideal than when pH is elevated (Coffey et al., 2014), lysosomal pH first needed to be elevated above healthy baseline. The tertiary amine tamoxifen quickly elevates lysosomal pH and does so independently of its estrogen-like activity (Altan et al., 1999); therefore, tamoxifen was an ideal drug to use in this experiment. Importantly, it was found that aberrant lysosomal pH, induced by tamoxifen, was restored by cAMP cocktail in rat primary cortical cultures. A representative image of these cultures is shown in Fig. 3.6.A, and shows the primarily neuronal composition of the cultures. In cortical cultures treated for 10 min with tamoxifen, treatment with cAMP cocktail lowered lysosomal pH (Fig. 3.6.B). When these same cultures were assayed using BODIPY FL-pepstatin A, the decreased Cat D active site availability caused by 10 min tamoxifen exposure could also be reversed by simultaneous treatment with cAMP (Fig. 3.6.C). Even on this short time scale, cAMP was able to restore lysosomal pH in both fibroblast and neuronal cell types.

**Discussion**

In this study, elevating cAMP restored both lysosomal pH and Cat D active site availability in PS1-fAD (A246E) human skin fibroblasts (Fig. 3.1). Lysosomal pH restoration was found to be dependent on PKA; without PKA activity, neither pH nor Cat D active site availability was restored (Fig. 3.2). Persistent pH restoration via cAMP was shown to increase p-mTOR (Fig. 3.3) and to down-regulate TFEB expression after 6 h, and ATP6V1B2 expression after 12 h (Fig. 3.4). The mTOR response appears be PKA-dependent and not related to baseline mTOR levels (Fig. 3.5). Finally, cAMP elevation restored lysosomal pH in rat primary cortical cultures treated with tamoxifen (Fig. 3.6). Together, the data support the more general applicability of cAMP elevation
as a pH-lowering strategy; more specifically, they implicate PKA in the immediate, upstream response to cAMP, and mTOR and TFEB in the eventual output of treatment.

**PKA: an upstream actor in lysosomal re-acidification**

The present findings directly linked cAMP’s beneficial actions in PS1-fAD fibroblasts to protein kinase A activity. cAMP treatment resulted in a PKA-dependent pH restoration; when PKA activity was blocked with a PKI inhibitor, the downstream effects of cAMP treatment, including increased Cat D active site availability and mTOR phosphorylation, were also blocked. While ECLTogether, these data demonstrate the necessity of active PKA for effective cAMP treatment, and suggest that pH restoration may be required for cAMP’s other positive outcomes. Of note, brain tissue from AD patients has been reported to exhibit low PKA activity (Liang et al., 2007); based on the current work, this PKA hypoactivity might considerably disrupt the ability of patient lysosomes to maintain appropriate pH or to restore pH once elevated, thereby hastening the onset of observed autophagic and lysosomal phenotypes in AD brain (Nixon et al., 2005).

The finding that cAMP-induced lysosomal acidification in PS1-fAD fibroblasts works through PKA adds to a growing body of literature linking PKA to acidification mechanisms. PKA mediates lysosomal acidification in retinal pigmented epithelial cells (Liu et al., 2008), while in kidney, PKA activity regulates the localization of v-(H\(^+\))ATPase on the plasma membrane (Alzamora et al., 2010, Paunescu et al., 2010). A likely possible mechanism is suggested by studies in yeast, in which PKA plays a role in the reversible dissociation of the proton pump’s V\(_0\) and V\(_1\) domains – a quickly-flipped “on/off” switch for lysosomal acidification (Forgac, 2007, Qi et al., 2007, Bond and Forgac, 2008). Finally, it is possible that anion conductances, which constitute an important secondary mechanism for endo- and lysosomal pH regulation after the proton pump (Faundez and Hartzell, 2004), may be activated by cAMP. Chloride channel activation by cAMP has been demonstrated in prior work from our laboratory (Liu et al., 2012); given that chloride conductances are commonly regulated by phosphorylation (Cheng et al., 1991, Miyazaki et al., 2012), chloride
channel or transporter may represent another potential target of PKA activity. Investigation of these various possibilities may be illuminating and further our understanding of the specific lysosomal deficits and restorative mechanisms in AD.

When considering any of these potential mechanisms, it is important to recall that cAMP was observed to have minimal effect on lysosomal pH in CTRL fibroblasts (Coffey et al., 2014). This observation has several implications for further work. First and most importantly, it suggests that cAMP treatment will not disrupt normal lysosomal pH maintenance in healthy cells, making cAMP-based treatment options more appealing as a future therapeutic avenue. Second, it is possible that these proposed mechanisms for PKA-mediated lysosomal pH restoration contribute most to pH homeostasis when pH is above baseline; therefore, it may be important from a technical perspective to explore these mechanisms in PS1-fAD cells or in other cell types characterized by elevated lysosomal pH.

mTOR and TFEB signaling mediate the downstream, sustained response to cAMP

The mechanistic target of rapamycin (mTOR) is a large serine/threonine kinase that is found as part of two distinct complexes in cells: mTORC1 and mTORC2. While both complexes are known to play a role in regulating cellular metabolism, the known targets of mTORC2 are still limited to a small number of processes, such as cytoskeletal organization and cellular proliferation. mTORC1, on the other hand, is a versatile and ubiquitous cellular player. This complex plays a critical role in the regulation of cell growth and in the energy balance of catabolic and anabolic processes within the cell (reviewed in Laplante and Sabatini, 2012). It is responsive to a wide variety of inputs – both intra- and extracellular – including glucose level, growth factors, stress, and others (reviewed in Rabinowitz and White, 2010). Recently, both mTORC1 docking on the lysosome and mTORC1 activity have been directly correlated with lysosomal abundance of amino acids (Zoncu et al., 2011); this relationship implies that nutrient abundance drives mTORC1 to the lysosome, where it is activated so that further autophagy can be halted.
Given the pH sensitivity of the lysosome’s various catabolic enzymes, especially Cat D (Barrett, 1970, Rosenfeld, 1982), and the responsiveness of mTOR to changes in lysosomal contents, we asked whether pH restoration would lead to an increase in active mTOR, as monitored indirectly by mTOR phosphorylation. 6 h cAMP treatment did indeed lead to increased mTOR phosphorylation at S2448, a site whose phosphorylation is positively correlated with mTOR activity (Rosner et al., 2010), while no baseline changes in mTOR protein levels was observed with cAMP. Interestingly, although 15 min cAMP treatment is sufficient to restore lysosomal pH, no change in phosphorylation was observed after 15 min cAMP treatment. These data suggest that cAMP and PKA do not act directly upon mTOR, but rather that mTOR phosphorylation increases at a later point, presumably as an effect of or in response to pH restoration. The inhibitory effect of PKI upon both the immediate- and long-term outcomes of cAMP treatment, including mTOR phosphorylation and Cat D active site restoration, further supports this proposed sequence of events.

Active mTORC1 has also been shown to interact with and to phosphorylate transcription factor EB (TFEB), the “master switch” of lysosomal biogenesis and function (Sardiello et al., 2009, Palmieri et al., 2011), on the lysosomal surface. Phosphorylation of TFEB prevents its nuclear translocation (Settembre et al., 2011, Rocznia-Ferguson et al., 2012) and results in down-regulated mRNA expression of genes linked to lysosomal biogenesis (Martina et al., 2012, Rocznia-Ferguson et al., 2012, Settembre et al., 2012), including but not limited to its own expression (Settembre et al., 2013). The present findings support this connection between mTOR and TFEB, as phosphorylated and, by extension, activated mTOR is accompanied by a substantial decrease in TFEB expression. This down-regulation may reflect the action of other transcription factors upon TFEB expression; it may also be indicative of TFEB auto-regulation. While TFEB auto-regulation has previously been demonstrated in both the nematode Caenorhabditis elegans and in a murine system (Settembre et al., 2013), this finding would
represent, to our knowledge, one of the earliest observations of possible TFEB auto-regulation in a human cell type, and may suggest a conserved mechanism for controlling its expression.

Of note, 6 h cAMP treatment was insufficient to cause down-regulation of the TFEB target gene ATP6V1B2, consistent with other reports demonstrating a lag time of up to 4 h between a change in nutrient conditions and a change in TFEB localization (Martina et al., 2012) and transcription (Settembre et al., 2013). By 12 h treatment, however, ATP6V1B2 expression was down-regulated, while TFEB mRNA expression had returned to baseline. It is postulated that incubation with cAMP-elevating drugs of greater half-life than the ones used here would yield even more persistent down-regulation of relevant genes. These data also support the hypothesis that pH restoration has an immediate, PKA-mediated effect, followed by a delayed, downstream response, in which both mTOR and TFEB-mediated changes in gene expression are involved. A simplified model of how these pathways may interact with potential PKA-mediated acidification mechanisms is given in Figure 3.7.

While no baseline difference in mTOR phosphorylation was observed between CTRL and PS1-fAD fibroblasts, this may be a consequence of using dividing cell types in this study. Post-mitotic cells like neurons rely on high rates of basal autophagy to clear cellular debris (Boland et al., 2008), whereas dividing cells can partially dilute lysosomal debris by distributing their lysosomes among daughter cells (Bergeland et al., 2001). As a consequence, autophagic pathology is likely to be more severe in a post-mitotic cell type than in a dividing cell type. It is therefore possible that a difference in baseline mTOR phosphorylation would be detectable in PS1-fAD neurons where it was undetectable in fibroblasts. Another possibility is that an mTOR-independent regulatory pathway mediates the baseline disruption of autophagy observed in PS1-fAD fibroblasts (Coffey et al., 2014). Pharmacological elevation of intracellular calcium, for example, is known to reduce autophagic flux independently of mTOR activity (Williams et al., 2008, Ganley et al., 2011). Given that elevated lysosomal pH reduces the lysosome’s ability to buffer calcium...
(Christensen et al., 2002) and that the lysosomes are the second-largest intracellular calcium store after the endoplasmic reticulum (Alvarez et al., 1999, Lloyd-Evans and Platt, 2011), the baseline differences in autophagy between CTRL and PS1-fAD fibroblasts may be due to changes in this or other mTOR-independent pathways (reviewed in Sarkar, 2013).

Of interest, the data reported in the present study agree with those reported in a recent study by Avrahami et al. involving the 5 X FAD mouse model of Alzheimer’s disease. Considerable autophagic and lysosomal pathology was observed in the cortex of the 5 X FAD mouse, including decreased maturation of Cat D, as observed in PS1-fAD fibroblasts, and reduced mTOR activity (Avrahami et al., 2013). Upon treatment to improve lysosomal function, increased mTOR activity was observed; these data agree with our findings that restoring lysosomal pH with cAMP, and thus improving degradation, leads to increased mTOR phosphorylation. Importantly, the parallels between the Avrahami study and the present study suggest that mTOR pathway engagement may prove crucial to successful therapy. While the exact pathological relationship of mTOR to AD progression is still a subject of some debate, reduced mTOR activation has been observed after treatment with amyloid-β_42, but not amyloid-β_40 (Lafay-Chebassier et al., 2005); of these two forms, amyloid-β_42 is considered the more toxic. Reduced mTOR activation has also been linked to issues with long-term synaptic plasticity and memory storage (Stoica et al., 2011), another pathological hallmark of AD (reviewed in Pozueta et al., 2013).

Although directly targeting autophagy through mTOR has been proposed previously as a blanket strategy for neurodegenerative diseases characterized by aberrant accumulation, such an approach will be inevitably thwarted if the disease in question involves a fundamental dysfunction of the lysosome (Harris and Rubinsztein, 2012). A better therapeutic approach may therefore be to fix the lysosome first and, in so doing, address autophagy indirectly. The cAMP-based approach to lower lysosomal pH, which has been demonstrated to be effective even in aged lysosomes from pathological mice (Liu et al., 2008), offers a possible solution.
cAMP signaling as a common mechanism for lysosomal re-acidification

Our laboratory has now demonstrated that cAMP effectively restores lysosomal pH in a number of different cell types, including: human retinal pigmented epithelial (RPE) cells (Liu et al., 2008), murine RPE cells from mice with pathologically damaged lysosomes (Liu et al., 2008), human skin fibroblasts from Alzheimer patients (Coffey et al., 2014), and rat primary cortical cultures. We have also demonstrated in epithelial cells, fibroblasts, and neurons that Cat D active site availability, an important functional outcome of pH restoration, is improved by cAMP treatment. The neuronal work indicates that even a small, short-term pH elevation significantly affects Cat D and that, even at this time scale, simultaneous treatment with cAMP restores both pH and the availability of the enzyme’s active site. At the shorter time scale of the neuronal experiments, Cat D active site loss more likely reflects reduced activity of existing Cat D than reduced maturation of precursor Cat D, as pulse-chase experiments in rat hepatocytes do not begin to show mature Cat D until at least 5 h after labeling (Rosenfeld, 1982). These experiments therefore indicate that BODIPY FL-pepstatin A can detect both maturation- and activity-dependent changes in Cat D active site availability. It will be interesting to examine whether prolonged lysosomal pH elevation in neurons yields similar, or even more dramatic, results to what is observed in fibroblasts, and to test whether longer-term cAMP treatment is a similarly effective tool to restore both pH and its downstream outcomes.

Overall, this study suggests that pH restoration, as well as Cat D active site availability and mTOR phosphorylation, is ultimately dependent upon PKA activity, and that strategies to re-acidify the lysosomes may be intimately tied to the functionality of this enzyme. The variety of cell types, species, and timecourses in which cAMP effectively lowers lysosomal pH suggests that cAMP may play a universal role in lysosomal pH maintenance, and therefore that cAMP-dependent acidification mechanisms should be considered a viable therapeutic target for Alzheimer’s disease and for other diseases characterized by failing lysosomes.
Acknowledgements

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Table 3.1. qPCR primers.

<table>
<thead>
<tr>
<th>Target</th>
<th>NCBI code</th>
<th>Primer pair</th>
<th>Tm (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
</table>
| ACTB    | NM_001 101.3 | Sense: AGAAAATCTGGCACCACACC  
Anti-Sense: GGGGTGTTGAAGGTCTCAAA | 59.97  
59.94 | 142 |
| ATP6V1B2 | NM_001 693.3 | Sense: GAGGGGCAGATCTATGTGGA  
Anti-Sense: GCATGATCCTTCCTGGTCAT | 60.00  
59.89 | 128 |
| TFEB    | NM_001 167827.2 | Sense: GTCCGAGACCTATGGGAACA  
Anti-Sense: CGTCCAGACGCAATAATGGTGA | 58.52  
57.28 | 218 |
Figures

Figure 3.1. cAMP restores elevated lysosomal pH.
A, 10 min treatment with cAMP cocktail significantly decreases lysosomal pH in PS1-fAD fibroblasts from controls; normalized, representative example shown. cAMP cocktail = 500 µM cpt-cAMP + 100 µM IBMX + 10 µM forskolin. p = 0.009; n = 7-8 wells per condition. B, Representative fluorescence micrograph of CTRL fibroblasts, PS1-fAD fibroblasts, and PS1-fAD fibroblasts + cAMP, stained for actin (red), lysosomal marker LAMP2 (green) and nuclear stain DAPI (blue). Scale bar = 50 µm. C, 6 h treatment with cAMP cocktail increases availability of Cat D active site, as visualized by the binding of BODIPY FL-pepstatin A; representative images shown. Scale bar = 50 µm.

Figure is located on following page.
Figure 3.2. PKI blocks restorative effect of cAMP treatment on lysosomal pH.

A, 1 h of 1 µM PKI pre-incubation blocks pH restorative effect of 10 min treatment with cAMP cocktail; n = 25-28 wells per condition from 2 experiments; p = 0.041, PS1-fAD v. PS1-fAD + cAMP; p = 0.027, PS1-fAD + cAMP v. PS1-fAD + cAMP + PKI. LysoSensor ratios normalized to mean control of each experiment.

B, 1 h of 1 µM PKI pre-incubation blocks restorative effect of 6 h cAMP cocktail treatment on Cat D active site availability, measured with BODIPY FL-pepstatin A; n = 22-25 wells per condition from 2 experiments; p = 0.005 by ANOVA; p = 0.006, PS1-fAD v PS1-fAD + cAMP; p = 0.017, PS1-fAD + cAMP v. PS1-fAD + cAMP + PKI.
Figure 3.3. PS1-fAD fibroblasts have increased p-mTOR with cAMP.

A and B, No difference was observed in either p-mTOR or mTOR between CTRL and PS1-fAD fibroblasts at baseline protein levels; n = 3 samples per genotype. Molecular weight = 289 kDa for both p-mTOR and mTOR, 37 kDa for GAPDH. C and D, 6 h cAMP treatment significantly increases p-mTOR in PS1-fAD fibroblasts (n = 20 samples per condition, p = 0.003), while mTOR does not increase significantly (n = 17 samples per condition). E and F, No significant difference was observed in either p-mTOR or mTOR when PS1-fAD cells were treated for 15 min with cAMP cocktail; n = 3 samples per condition.
Figure 3.4. cAMP treatment reduces mRNA expression of TFEB and ATP6V1B2. 
A, PS1-fAD fibroblasts treated for 6 h with cAMP exhibit significantly reduced expression of TFEB compared to untreated fibroblasts, as detected by qRT-PCR; ATP6V1B2 expression was unchanged. n = 6-7 samples per condition, p < 0.001 for TFEB, n.s. for ATP6V1B2. B, PS1-fAD fibroblasts treated for 12 h with cAMP exhibit no significant change in TFEB expression compared to untreated fibroblasts; ATP6V1B2 expression was significantly reduced after 12 h treatment. n = 6-7 samples per condition, n.s. for TFEB, p < 0.001 for ATP6V1B2.
Figure 3.5. PKI blocks cAMP-induced increase in p-mTOR.
A and B, PKI blocks the increase in p-mTOR observed with cAMP; n = 13-14 samples per condition, p = 0.026 for PS1-fAD vs. PS1-fAD + cAMP, n.s. for PS1-fAD vs. PS1-fAD +cAMP + PKI and n.s. for PS1-fAD + cAMP vs. PS1-fAD +cAMP + PKI. Molecular weight = 289 kDa for both p-mTOR and mTOR, 37 kDa for GAPDH. No significant change observed in mTOR across all conditions.
Figure 3.6. cAMP restores lysosomal pH and Cat D availability in rat primary cortical cultures.

A, Representative picture of rat primary cortical cultures, stained for neuronal marker Pgp9.5 (green), astrocyte marker GFAP (red) and DAPI (blue), with cultures estimated to be about 90% neuronal. Scale bar = 100 µm. B, 10 min treatment with cAMP cocktail significantly decreases lysosomal pH in rat primary mixed cortical cultures also treated with tamoxifen (TMX) 30 µM. n = 39-42 wells per condition from 3 experiments; p < 0.001, CTRL v. TMX; p = 0.013, TMX v. TMX + cAMP. LysoSensor ratios normalized to mean control of each day’s experiment. C, 6 h cAMP treatment significantly increases availability of Cat D active site in rat primary mixed cortical cultures treated with TMX 30 µM, as measured with BODIPY FL-pepstatin A. n = 10 wells per condition; significant to p = 0.007 by ANOVA; in post-hoc pairwise comparisons, p = 0.017, t-test, CTRL v. TMX; p = 0.006, Mann-Whitney Rank-Sum test, TMX v. TMX + cAMP; CTRL v. TMX + cAMP, not significant.
Figure 3.7. A mechanistic model for cAMP-induced pH restoration.

A, PS1-fAD cells are characterized by elevated lysosomal pH and reduced levels of Cat D active site availability; these pathologies correlate with lesser mTOR phosphorylation and increased transcription of TFEB and ATP6V1B2 (from this work and work published in Coffey et al., 2014).

B, cAMP re-acidifies lysosomes and restores Cat D active site availability; these events are PKA-dependent and re-acidification is proposed to occur through one of two potential mechanisms: through increased insertion of v-(H\(^+\))ATPase into the lysosomal membrane, or through increased activation of lysosomal chloride channels. Lysosomal re-acidification leads to increased mTOR phosphorylation and reduced transcription of TFEB and ATP6V1B2 at 6 and 12 h cAMP treatment, respectively. These transcriptional changes may be due to retention of phosphorylated TFEB in the cytoplasm.

Figure is located on following page.
CHAPTER 4:

Discussion and Future Directions
Adult-onset neurodegenerative disease represents a substantial public health problem for the industrialized world. Of this multitude of diseases, Alzheimer's disease (AD) has the greatest prevalence and is among the most devastating, progressively and inevitably stripping patients of their cognitive ability over the course of decades, and ultimately resulting in death (Alzheimer's Association, 2015). Only a handful of approved treatments are available to patients, and none of these does more than temporarily slow the course of disease (Raschetti et al., 2007, Raina et al., 2008). This paucity of therapeutic options exists in part because the precipitating event (or events) in Alzheimer’s disease is still largely unknown; by the time a patient develops cognitive symptoms, the pathological processes underlying disease have been underway for decades (Jack et al., 2009, Villemagne et al., 2013). Better understanding of these disease mechanisms will enable the development of better, and earlier, treatment options for AD patients. The goal of this dissertation work, therefore, was to investigate the role played by elevated lysosomal pH in a cell culture model of Alzheimer’s disease, and to determine whether the pathologies observed in this model could be reversed through pH restoration.

The work described in the preceding chapters supports a critical role for lysosomal pH maintenance in the pathology of disease. Here, we have presented evidence that lysosomal pH is significantly elevated in human skin fibroblasts from familial Alzheimer (fAD) patients with the presenilin 1 (PS1) mutation A246E (Chapter 2). The work demonstrated that this pH elevation, though small, has a profound impact on the level of mature, active Cathepsin D (Cat D) present in the lysosomal lumen, reducing the availability of a critical catabolic enzyme. This degradative failure leads to a build-up of autophagosomes, as indicated by a rise in the levels of LC3B-II and p62 in PS1-fAD fibroblasts. Several genes critical to autophagy and lysosomal degradation are also up-regulated as a compensatory response to the PS1-fAD degradative backlog, with a consequent increase in the expression of their respective proteins. It was also shown that treatment with a cAMP-elevating cocktail not only lowers lysosomal pH, but increases the availability of Cat D and improves degradation through the autophagic-lysosomal pathway
(Chapter 2). Extended treatment with this cocktail ultimately leads to an increase in mechanistic target of rapamycin (mTOR) phosphorylation and in decreased expression of both transcription factor EB (TFEB) and one of its targets, ATP6V1B2 – the gene for the B2 subunit of the v-(H⁺)ATPase proton pump (Chapter 3). Both the immediate effects of cAMP on lysosomal pH and its downstream effects on Cat D active site restoration and mTOR phosphorylation appear to be mediated by protein kinase A (PKA) activity (Chapter 3). Together, these data strongly implicate pH dysfunction in certain AD-associated phenotypes, and suggest that treatment to improve lysosomal pH may be a therapeutic approach worth further investigation in an AD context.

Expanding upon the mechanisms of lysosomal pH dysfunction and restoration

While the findings presented in this dissertation represent an important step forward in our understanding of the lysosome’s importance in Alzheimer’s disease, much work remains to be done. At the end of Chapter 3, several possible mechanisms were proposed by which cAMP-induced PKA activation may directly mediate lysosomal re-acidification in PS1-fAD cells, including through v-(H⁺)ATPase membrane insertion, v-(H⁺)ATPase domain assembly, and chloride channel or transporter activation – or possibly by some combination of the above. Of these mechanisms, the most likely possibility, and one for which strong precedent exists, is that cAMP induces increased association of the v-(H⁺)ATPase v₁ and v₀ functional domains at the lysosomal membrane. cAMP/PKA-induced activation of lysosomal chloride conductance has also been widely observed in lysosomal systems and seems the next most likely mechanistic possibility, while insertion of v-(H⁺)ATPase from an existing pool, though intriguing and not without parallels in other cell types, remains a primarily speculative mechanism. Several technical approaches exist that could help clarify which of these possible mechanisms may be at work in the fibroblast model.

In dendritic cells of the immune system, increased assembly of the lysosomal proton pump’s v₁ and v₀ domains may occur in response to various maturation signals (Trombetta et al., 2003). A
similar phenomenon has been reported in yeast in response to glucose and is both rapid (Parra and Kane, 1998) and promoted by PKA (Bond and Forgac, 2008). This assembly event may take as little as 5 min (Parra and Kane, 1998), which would be consistent with the 10 min re-acidification observed with cAMP (Chapters 2 and 3). These findings suggest that the rapid, PKA-dependent lysosomal re-acidification reported in Chapter 3 may occur via a similar reversible association mechanism. To determine whether or not cAMP treatment promotes increased assembly of the $v_1$ and $v_0$ domains, immunohistochemistry could be used to detect increased colocalization of one of the peripheral $v_1$ subunits, such as the A or B subunit, with one of the $v_0$ integral membrane subunits, such as the c subunit (Forgac, 2007). A protocol for $v_1$-targeted immunoprecipitation of the $v_0$ domain has also been described (Bond and Forgac, 2008) and would serve as an excellent additional measure of association. Lysosomal localization could then be confirmed by staining for lysosomal markers such as LAMP1 or LAMP2. While this last experiment was in fact attempted and the data suggested increased association of the peripheral v-(H$^+$)ATPase subunit B2 with the lysosomal markers LAMP1 and LAMP2, the v-(H$^+$)ATPase antibody used proved insufficiently specific to yield conclusive results [data not shown]. It is also possible that these initial results indicate that cAMP leads increased insertion of existing v-(H$^+$)ATPase into the lysosomal membrane. A cAMP/PKA-linked proton pump cycling mechanism has a physiological precedent in kidney cells (Alzamora et al., 2010, Paunescu et al., 2010) and would also enable rapid lysosomal acidification without requiring new transcription or translation; however, this mechanism has not previously been demonstrated to be at work in lysosomes, and as such is primarily speculative.

As an alternative and to provide confirmation of these initial findings, v-(H$^+$)ATPase domain association and/or association with the lysosomal membrane could be assessed by isolating lysosomes from cAMP-treated and untreated PS1-fAD cells, and then by probing for v-(H$^+$)ATPase subunit content via Western blot or ELISA. Various differential centrifugation protocols would permit crude isolation of the lysosomal fraction, but may be complicated by the
fact that lysosomes containing incompletely degraded and aggregate material may have different densities from their healthy counterparts, and therefore might not fractionate appropriately without specialized isolation techniques (Diettrich et al., 1998). More recently, however, the Lloyd-Evans laboratory successfully used superparamagnetic ferrofluids for high-yield, ultrapure lysosomal isolation in multiple cell types, including those with a storage disorder phenotype (Walker and Lloyd-Evans, 2015). This technique may prove useful in future work.

Another mechanistic possibility worth exploring is the potential activation of lysosomal chloride currents by cAMP/PKA, as phosphorylation events commonly regulate chloride channel and transporter activity (Cheng et al., 1991, Miyazaki et al., 2012). A previous study demonstrated that the cAMP re-acidification treatment used in this work also activates lysosomal chloride channel conductance (Liu et al., 2012). The cystic fibrosis transmembrane conductance regulator (CFTR), for example, can be phosphorylated and activated by PKA at four separate serine residues; mutation of these residues, however, completely blocked CFTR activity (Cheng et al., 1991). PKA-dependent acidification improved the ability of microglia, which have an abnormally high baseline lysosomal pH, to degrade fibrillar amyloid-β (Majumdar et al., 2007); efficient degradation was later shown to require the delivery of the chloride transporter ClC-7 to the lysosomal membrane (Majumdar et al., 2011). Phosphorylation status of CFTR and other lysosomal chloride channels or transporters could therefore be investigated via Western blot to indirectly assess PKA-induced chloride channel activity. Anionic currents could also be measured directly via the lysosomal patch-clamping technique that, though unquestionably challenging, has been previously described (Cang et al., 2013). Together, these approaches will increase understanding of PKA’s specific role in lysosomal pH maintenance as suggested by Chapter 3; these findings may also prove informative to the general study of lysosomal homeostatic regulation, as well as to the more specific study of lysosomal dysfunction and recovery in AD.
The importance of studying a cell-based, living model of Alzheimer’s disease cannot be overstated, as many of the techniques used in this dissertation work – including direct pH measurement and fluorescent visualization of Cat D active site availability – would be impossible to perform in post-mortem tissue. Even for murine AD models characterized by apparent lysosomal pH dysfunction, such as the 5 X FAD mouse (Avrahami et al., 2013), direct measurement of lysosomal pH from isolated regions of interest would prove extremely challenging. The fibroblast model used in this dissertation, on the other hand, enabled side-by-side, live-cell comparison of actual patient tissue with unaffected tissue (Chapter 2), and permitted evaluation of immediate, short-term, and long-term responses to drug treatment (Chapters 2 and 3), including Western blotting and expression analysis via qPCR. The absence of other physiological systems and cell types in this culture model also reduced possible confounds resulting from the interaction of different cell types. In future studies, the fibroblast approach could therefore be used to detect lysosomal pH deficiency in patient fibroblasts containing other PS1-fAD mutations than the A246E mutation examined here. The work presented in this dissertation, in combination with the known association between PS1-fAD mutation and severity of lysosomal pathology (Cataldo et al., 2004), suggests that such investigations may find pH elevation to be a common phenomenon in familial AD with PS1 mutation. Given that average age of onset is known for many fAD-causing PS1 mutations, it will be especially important to determine how early the pH deficit arises in fAD progression. This knowledge may then inform future treatment, or perhaps even contribute to the development of preventive strategies based on lysosomal pH restoration.

In Chapter 3, it was demonstrated that lysosomal pH can be pharmacologically elevated in rat primary neuronal cultures, that the pH elevation corresponds with loss of Cat D active site availability, and that cAMP treatment corrects these failures. Most importantly, these data indicate that lysosomal pH changes are measurable in living neurons, and that at least some of the consequences of these pH changes recapitulated what was observed in the fibroblast culture
model. It would therefore be of great interest to determine whether the pH elevation detected in PS1-fAD mutant fibroblasts can also be detected in PS1-fAD patient neurons. Given the practical and ethical concerns associated with the use of living adult human neural tissue, however, a particularly appealing future direction for this work would be to study lysosomal dysfunction in induced pluripotent stem (iPS) cells derived from both the CTRL and the PS1-fAD fibroblasts used in this study. iPS cells, which can be generated from human skin fibroblasts and other cell types, have been used successfully to model both familial (Yagi et al., 2011, Israel et al., 2012, Kondo et al., 2013) and sporadic AD (Israel et al., 2012, Hossini et al., 2015), so a strong precedent exists for using this approach.

**General implications of lysosomal pH dysfunction in Alzheimer's disease**

While the autophagic and lysosomal pathologies of Alzheimer's disease may be worsened by PS1 mutation (Cataldo et al., 2004), they are nevertheless present in post-mortem brain tissue from sporadic AD patients (Nixon et al., 2005). The simplest explanation for these remarkably similar pathologies is that lysosomal pH is elevated in both familial and sporadic AD. A concerning corollary of possible vesicular pH dysfunction is that poor acidification would interfere with neurotransmitter loading into synaptic vesicles in Alzheimer neurons, thereby further contributing to observed synaptic dysfunctions in AD (Roseth et al., 1995, Zhou et al., 2000, Pozueta et al., 2013). In fAD, pH rises as a result of a PS1 mutation that directly impacts lysosomal acidification (Lee et al., 2010, Coffey et al., 2014), as reported in Chapter 2; in sporadic AD, pH may be elevated through an indirect mechanism. For example, although individuals accumulate amyloid-β (Funato et al., 1998, Morishima-Kawashima et al., 2000) as a normal consequence of aging, amyloid-β is known to perturb membrane fluidity and permeability on its own (Soreghan et al., 1994, Kanfer et al., 1999, Lin et al., 2001, Wood and Igbavboa, 2003, Quist et al., 2005, Yang et al., 2010). At sufficiently high concentration, it can even induce complete lysosomal membrane permeabilization (Yang et al., 1998, Ditaranto et al., 2001). Over time, the sum of such aging-related insults to the lysosome may interfere with its ability to maintain pH,
leading to AD lysosomal pathology. It will be informative to determine whether pH increases correlate with lysosomal permeabilization in both the PS1-fAD fibroblasts used in this work, and also in tissue samples from patients diagnosed with probable sporadic AD. Lysosomal pH and its effect on (im)permeability could then be assessed with the endocytosed, membrane-impermeable dye Lucifer yellow (Page et al., 1994, Burdick et al., 1997), yielding important insights about the resting stability of Alzheimer lysosomes. When used in conjunction with BODIPY FL-pepstatin A for Cat D active site assessment (Chen et al., 2000a), these experiments could even enable determination of whether long-term in vitro treatment with physiologically relevant cerebrospinal fluid levels of amyloid-β peptide – i.e. in the 1-10 nM range (Pearson and Peers, 2006) – substantially affects lysosomal membrane stability and lysosomal function.

Another possible mechanistic link between the lysosomal pathology of fAD presented in this dissertation and the lysosomal pathology of sporadic AD is suggested by a report that PKA activity is significantly down-regulated in Alzheimer brain (Liang et al., 2007). In Chapter 3 of the present work, it was reported that PKA activity is necessary for both cAMP-induced pH restoration and its longer-term consequences of Cat D active site restoration and mTOR phosphorylation. Should PKA play a general role in lysosomal pH maintenance, progressive loss of PKA in AD could contribute to lysosomal acidification defects and subsequent reduction in Cat D activity. Of note in this respect, Cat D is known to degrade amyloid-β (McDermott and Gibson, 1996), linking lysosomal defects with canonical Alzheimer pathologies; Cat D is also thought to preferentially degrade the more toxic, aggregation-prone amyloid-β_{42} species over the shorter amyloid-β_{40} (Saido and Leissring, 2012). Once impaired by even mild pH elevation (Barrett, 1970), reduced Cat D activity could contribute to a lysosomal dysfunction snowball effect, and even to the development of further AD pathology. PKA down-regulation would also compromise the aging lysosome’s ability to restore pH once elevated. Therefore, depending on the level of pre-existing lysosomal damage an individual sustains over the natural course of aging, further lysosomal distress from any source might serve as a precipitating event in AD.
**Strategies for pH restoration in Alzheimer’s disease**

Whether or not elevated lysosomal pH proves to be a universal mechanism in AD progression, the ability to effectively treat even a fraction of AD patients would be a considerable step forward. Chapters 2 and 3 of this dissertation presented evidence that lysosomal pH restoration via cAMP treatment proved effective in ameliorating lysosomal pathology, both in PS1-fAD fibroblasts and in pharmacologically compromised lysosomes from rat primary neuronal cultures. In the following section, the therapeutic implications of these findings will be examined in greater detail, and several strategies for lysosomal pH restoration will be explored. From a practical perspective, the next logical step is to focus on endogenous targets whose activation or inhibition leads to cAMP elevation. The efficacy of the cAMP cocktail used in the present work suggests two initial targets: adenylyl cyclase and phosphodiesterase, each of which was targeted by a component of the cocktail ( forskolin and 3-isobutyl-1-methylxanthine (IBMX), respectively). While clearly effective, neither forskolin nor IMBX is selective in its activity. Therefore, it behooves us to identify drugs that act in a more targeted fashion to increase intracellular cAMP in the brain.

*Transmembrane adenylyl cyclases as a potential therapeutic target*

Adenylyl cyclases in mammalian tissue come in many isoforms, but belong to two major families: the membrane-associated transmembrane adenylyl cyclases (tmACs) and the soluble adenylyl cyclases (sACs). While both families produce cAMP once activated, their cellular localization and triggers for activation are markedly different. tmACs are linked to Gαs-coupled G protein-coupled receptors (GPCRs) (May et al., 1985) and primarily act at the plasma membrane; this association between tmACs and GPCRs facilitates the intracellular response to extracellular cues. sACs, on the other hand, are distributed throughout the cytoplasm and also associated with specific organelles (Zippin et al., 2003, Bundey and Insel, 2004), creating microdomains of cAMP signaling (Bitterman et al., 2013, Rahman et al., 2013, Wertheimer et al., 2013). Such local control of cAMP generation would limit the reach of the ubiquitous cAMP/PKA and mTOR...
signaling pathways and make sACs an appealing target for therapeutic purposes; however, the sACs are activated only by increased local levels of calcium and bicarbonate (Chen et al., 2000b, Jaiswal and Conti, 2003, Litvin et al., 2003), and are insensitive to modulation by G proteins or forskolin (Buck et al., 1999). This unresponsiveness to forskolin makes the sACs unlikely to be targeted by the cocktail used in Chapters 2 and 3 of this work. Therefore, until a specific pharmacological activator of the sACs is developed, future pharmacological efforts involving ACs should instead be focused on tmACs and their associated GPCRs.

While forskolin is membrane-permeable and is well-known to activate tmACs in isolation, it may also be able to act upon Gαs-coupled GPCRs and enhance adenylyl cyclase activation in that way (Jasper et al., 1990). It is therefore possible that both direct and indirect adenylyl cyclase activation contribute to increased intracellular cAMP and its beneficial effects for the PS1-fAD cells, as reported in the present work. Consequently, focusing on Gαs-coupled GPCRs may serve as a useful strategy for elevating cAMP in AD. Interestingly, a deficit in GPCR-associated tmAC activity was observed in AD brain as early as the 1990s (Ross et al., 1993, Schnecko et al., 1994, Bonkale et al., 1996, Yamamoto et al., 1997), further supporting the importance of targeting cAMP production as a therapeutic approach. A potentially interesting target in this respect is pituitary adenylate cyclase 1 receptor (PAC1R), for which the endogenous ligand is the pituitary adenylate cyclase activating polypeptide (PACAP). PACAP levels in brain have been shown to inversely correlate with the severity of cognitive decline in AD (Han et al., 2015), while treatment to increase PACAP levels, and thereby increase cAMP production, improved pathology (Rat et al., 2011, Han et al., 2014). The exact mechanism by which PACAP-induced cAMP production achieved these beneficial outcomes, however, remains somewhat unclear. Given that human skin is known to express PAC1R (Steinhoff et al., 1999), it would be interesting to test PACAP on the PS1-fAD cells used in this work and to determine whether sustained PACAP treatment to increase intracellular cAMP also restores lysosomal pH. Importantly, PACAP was found to readily cross the blood-brain barrier (Nonaka et al., 2002), and was also efficacious when delivered
intranasally (Rat et al., 2011); therefore, targeting tmACs using PACAP may prove a viable therapeutic strategy. Prior to a clinical trial, however, global and local effects of this neuropeptide should be carefully assessed in rodents to ensure that the peptide does not lead to unexpected or unacceptable adverse outcomes in “off-target” brain regions.

*Phosphodiesterase inhibitor therapy*

When considering endogenous cAMP-elevating enzymes, the phosphodiesterases (PDEs) may also prove to be an ideal target. The phosphodiesterase family includes 11 subtypes of enzyme, as well as a host of different splice variants for each subtype (Bender and Beavo, 2006). While these subtypes may have a variable distribution based upon tissue and cell type, all perform the same function: breaking apart the ester bond that cyclizes the mononucleotides AMP and GMP (Bender and Beavo, 2006). Depending on subtype, a PDE may be specific for either cAMP or cGMP, or have some affinity for both substrates. We have already demonstrated the efficacy of this approach *in vitro*: a critical component of the cAMP cocktail used in Chapters 2 and 3 is 3-isobutyl-1-methylxanthine (IBMX), a general PDE inhibitor. Many PDE inhibitors are orally bioavailable (Garcia-Osta et al., 2012), as they have been used successfully to treat a range of unrelated disorders, including erectile dysfunction (Fusco et al., 2010). Many PDE inhibitors are also predicted to cross the blood-brain barrier with high probability based on structure – a critical first step in testing PDE inhibitors in a therapeutic context for neurodegenerative disease. However, given the sheer number of PDE isozymes in the brain, as well as their variable distributions and expression levels (Lakics et al., 2010), careful screening would be necessary in order to ensure that the appropriate PDE(s) is/are targeted. For example, PDE-3 is ubiquitously expressed at low levels throughout most brain regions, while PDE-4 isoforms are among the more highly expressed in hippocampus (Lakics et al., 2010). Even a single cell may express multiple PDE subtypes in order to better regulate local cyclic nucleotide signaling (Bender and Beavo, 2006), raising the possibility that at least one of these PDE subtypes might have a lysosomal localization and thus make an ideal target. The complexity of this signaling network
underscores the importance of choosing specific targets and delivery systems (e.g., intranasal delivery to more directly target the brain). Such refinements will ensure maximum treatment efficacy while simultaneously limiting the reach of the therapeutic agent(s) to desired systems and cell types.

While certain of these compounds have been tested previously and successfully against Alzheimer’s disease in murine models, their exact mechanisms of action remain unclear. Most hypothesized benefits of PDE inhibitors in AD are related to the demonstrated, general role of cyclic nucleotides in neuroplasticity (Jancic et al., 2009) and neuroprotection (Paintlia et al., 2009), and not necessarily to disease-specific mechanisms (Comery et al., 2005). For example, treatment with the PDE-4 inhibitor rolipram improved performance of not only AD model mice in a contextual conditioning task, but also of control mice (Comery et al., 2005). These improvements may have been due to increased cAMP-response element binding protein (CREB) phosphorylation with rolipram, leading to increased expression of genes critical for learning and memory, and consequent enhancement of long-term memory formation (Cheng et al., 2010). By contrast, we have observed minimal effects of cAMP treatment on fibroblasts with normal lysosomal pH (Chapter 2). It will be particularly interesting to test inhibitors with some prior success against AD pathology in our PS1-fAD fibroblast model, and to then determine which of these various compounds most effectively lowers lysosomal pH while also having minimal effect on CTRL cells. Promising results have been reported thus far for PDE-3 (Hiramatsu et al., 2010, Park et al., 2011, Lee et al., 2014), PDE-4 (Gong et al., 2004, Sierksma et al., 2014), and PDE-5 inhibitors (Puzzo et al., 2009, Cuadrado-Tejedor et al., 2011, Garcia-Barroso et al., 2013), so these PDE subtypes may prove useful as initial targets; to the best of our knowledge, however, no PDE inhibitor has yet completed a Phase III clinical trial. Given that most PDE-4 inhibitors tested so far have severe emetic side effects (Bender and Beavo, 2006), it will also be useful to determine whether lower concentrations of these PDE inhibitors are required to restore lysosomal pH than are required to effect neuroplastic change. Less concentrated dosing might reduce some
of the PDE inhibitors’ observed side effects, making them a more reasonable option for early treatment of AD.

Conclusions

Alzheimer’s disease is a complex and heterogeneous disorder; the work in this dissertation represents an important step forward in understanding certain of the subcellular mechanisms that may play into disease development and progression. Evidence was presented that PS1-fAD mutation leads to lysosomal pH dysfunction, as well as to deficits in lysosomal Cat D maturation and active site availability. This chronic pH dysfunction correlates with a perturbation in the autophagic degradative system at both at the mRNA and protein levels. Treatment with cAMP results in a PKA-dependent restoration of many of these pathologies, including lowered lysosomal pH, increased Cat D availability, and reduced autophagic backlog. cAMP treatment ultimately promotes sustained responses through mTOR phosphorylation and through transcriptional changes in TFEB and one of its targets, ATP6V1B2. The work presented here proposes an important place for lysosomal dysfunction in AD chronology, and suggests several treatment possibilities that may prove fruitful in the future. Critically, this dissertation work further emphasizes the importance of investigating malfunctions in basic cellular systems like the lysosome that may contribute to neurodegenerative pathology and its progression. This merger between translational efforts and basic science has the potential to offer novel therapeutic approaches and creative new ways of thinking about neurodegenerative disease. While many years undoubtedly remain before the entire picture of AD becomes clear, it will be exciting to see how critical consideration of the once-lowly lysosome informs the solution.
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