Thermal Response of Amyloidogenic Elements in Cultured N2a Cells: Potential Relevance to Alzheimer's Disease Pathology

by

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ABSTRACT

Alzheimer's disease (AD) weighs a large burden on health care systems, families and caregiving networks around the world. It is the most common dementia afflicting elderly individuals, and causes many debilitating symptoms, including memory loss, mood swings and confusion which progress in severity as the individual declines throughout the disease course. Amyloid-β (Aβ) plaques and tau neurofibrillary tangles (NFTs) are key features of AD, as these insoluble aggregates deposit themselves and spread throughout the brains of AD patients, causing loss of neuronal structure and function and eventual death. Familial AD (FAD) is the less prevalent but more defined form, as genetic mutations drive disease processes. Alternatively, sporadic disease (SAD) is multifactorial in nature, with a complex interplay between a variety of risk factors guiding pathogenesis. A newly emerging risk factor has been posited to contribute to AD: environmental temperature. So far, hypothermia has been studied in the context of tau, while there is not much known about how a reduced temperature can influence the amyloidogenic processes that lead to Aβ production.

Thus, in the present study, we utilized a cell culture system to investigate the effects of different ambient temperatures on amyloid precursor protein (APP) metabolism. Murine neuroblastoma cells harboured either endogenous mouse APP (N2a) or had the human Swedish APP transgene expressed (N2a-APP). We exposed these cells to hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) for either 6, 12, or 24 hours and evaluated cellular processes associated with APP metabolism by a variety of methods. The data obtained throughout this project implicate environmental temperature as capable of influencing APP metabolism, as low temperature caused accumulation of APP α/β-C-terminal fragments as well as a decrease in secreted $A\beta_{1\text{-}40/42}$. Some

of these changes may be due to a decrease in the efficiency of the cellular clearance mechanisms associated with the removal of these APP products. We also note alterations in the endolysosomal autophagic system, with an increase in LC3-II, as well as a qualitative increase in the colocalization of Aβ with endolysosomal autophagic constituent proteins LC3 and LAMP1 at hypothermic temperatures. These results obtained in our *in vitro* hypothermia model describe a temperature-dependent effect on APP processing that may be relevant to AD pathogenesis.

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ABBREVIATIONS

AP2	Adaptor protein 2
Aβ	Amyloid beta
AD	Alzheimer's disease
ADAM10	A disintigrin and metalloprotease 10
ANOVA	Analysis of variance
APH1	Anterior pharynx defective 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BAG2	B-cell lymphoma-associated athanogene 2
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
BAT	Brown adipose tissue
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cirp	Cold-inducible RNA binding protein
CSP	Cold shock protein
CNS	Central nervous system
α/β-CTF	Alpha/beta C-terminal fragment
Chx.	Cycloheximide

DWI	Diffusion weighted imaging
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
DS	Down syndrome
ECL	Enhanced chemiluminescent
ELISA	Enzyme-linked immunosorbent assay
EL	Endolysosomal
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
GSK3α/β	Glycogen synthase kinase 3α/β
HSP	Heat shock protein
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
iPSCs	Induced pluripotent stem cells
LAMP1	Lysosomal-associated membrane protein 1
LC3	Microtubule associated protein 1A/1B light chain 3
LDH	Lactate dehydrogenase
MAPT	Microtubule-associated protein tau

MCAO	Middle cerebral artery occlusion
MCI	Mild cognitive impairment
MTT	3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide
NFT	Neurofibrillary tangle
N2a	Neuro-2A
N2a-APP	Neuro-2A-APP
PBS	Phosphate buffered saline
PICALM	Phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PEN2	Presenilin enhancer 2
PP2A	Protein phosphatase 2A
PS1/2	Presenilin ½
Rbm3	RNA binding motif 3
RNA	Ribonucleic acid
RIPA	Radioimmunoprecipitation assay
SAD	Sporadic Alzheimer’s disease
SDS	Sodium dodecyl sulfate

RT	Reverse transcription
qPCR	Quantitative PCR
sAPPα/β	Soluble amyloid precursor protein α/β
TFEB	Transcription factor EB
TGN	Trans-Golgi network

1. INTRODUCTION

1.1 An overview of Alzheimer's disease

Alzheimer's disease (AD) is the leading cause of dementia afflicting the elderly. This deadly neurodegenerative disease has become a worldwide epidemic, with an estimated 41.5 million people suffering from AD, and the financial burden associated with their care approximately USD$ 815 billion according to data collected in 2015 (Martin Prince et al., 2015). The worldwide prevalence is projected to double every 20 years, with a disproportionate increase of cases in developing countries that have rapid population growth and a gradually increasing life expectancy (Martin Prince et al., 2015). The socioeconomic burden of AD extends beyond the illness suffered by the patient to encompass costs associated with the necessary familial and professional caregiving networks, as well as losing productive members of society to this disease. Individuals suffering from AD experience a wide array of symptoms, from memory loss and erratic mood swings to depression (Masters et al., 2015). There is a clear and urgent need to understand and manage AD, as it is quickly becoming an overwhelming burden on society.

Despite decades of research, there are currently no effective treatments to slow the progression or manage AD, and since the gold standard of diagnosis is *post-mortem*, the processes behind early stages of the disease are elusive as well. There is consensus, however, regarding the pathogenic accumulation of two insoluble proteins within the brains of AD patients. The first of these proteins are amyloid-β (Aβ) peptides, which are derived *via* sequential proteolysis of the amyloid precursor protein (APP) while the second is the tau protein, which becomes hyperphosphorylated and accumulates in vulnerable neurons (Masters et al., 2015).

APP is a type I integral transmembrane protein with one membrane-spanning domain, a large extracellular N-terminal domain and a short cytoplasmic C-terminal domain. APP is expressed ubiquitously throughout the body, although it is produced at an elevated level in neurons, with a suite of post-transcriptional modifications which include *N*- and *O*-linked glycosylation, sialylation, and phosphorylation. APP is distributed throughout the cell body, along axons and at the dendrites of neurons, but also resides within intracellular membrane bound vesicles such as

endosomes and lysosomes. There are many endogenous functions attributed to APP apart from its association with AD, such as cell adhesion, synaptogenesis, intracellular signalling, and apoptosis (Chen et al., 2017; Muller and Zheng, 2012). Proteolysis of APP is essential for some of these roles and is a key process in the context of AD pathogenesis, which involves the amyloidogenic pathway of APP processing **(Fig. 1.1)**. This process is initiated by β-secretase (BACE1, β-APP-site cleaving enzyme-1) cleavage of APP, resulting in the release of N-terminal soluble APPβ (sAPPβ), while the β-C-terminal fragment (β–CTF) is retained on the plasma or vesicular membrane. Alternatively, non-amyloidogenic processing of APP is initiated by α-secretase enzymes such as A disintigrin and metalloprotease domain-containing 10 (ADAM10), which produces both soluble APPα (sAPPα) and the α-C-terminal fragment (α–CTF). A sequential proteolytic step is catalyzed by the γ-secretase complex, which is a multi-subunit intramembrane protease complex comprised of the aspartyl protease presenilin 1 or 2 (PS1 or PS2) and three cofactors: the proteins nicastrin, presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1) (Bergmans and De Strooper, 2010). The γ-secretase complex can cleave both the α–CTF and β–CTF to produce a variety of APP related peptides, with the β–CTF amyloidogenic route producing Aβ. These Aβ peptides vary in size from 37-49 amino acids due to flexibility in the γ-secretase cleavage site with the most common products being $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (Bergmans and De Strooper, 2010; Chen et al., 2017; Olsson et al., 2014). Various oligomeric states from monomers, dimers, and trimers to full amyloid plaque forms of these Aβ peptides are believed to be key mediators of disease pathogenesis, causing gliosis, oxidative stress and inflammatory responses (Cizas et al., 2010; Jung Lee et al., 2017; Reiss et al., 2018). Aβ peptides are produced under normal physiological conditions, with $A\beta_{1\text{-}40}$ making up approximately 90% of peptide species produced and $A\beta_{1\text{-}42}$ constituting the remaining 10% (Haass et al., 1992). Representative of the disease state, $A\beta_{1\text{-}42}$ aggregates more readily into amyloid plaques and is more neurotoxic, with a key pathological feature being the $A\beta_{1\text{-}40}$:$A\beta_{1\text{-}42}$ ratio (Jan et al., 2008). Pathogenic accumulation of Aβ peptides may be driven by an increase in production or a decrease in cellular clearance mechanisms (Wang et al., 2017). Apart from amyloidogenesis and production of Aβ peptides, the other key molecular process driving the progression of AD involves the microtubule binding protein tau.

Figure 1.1: APP processing

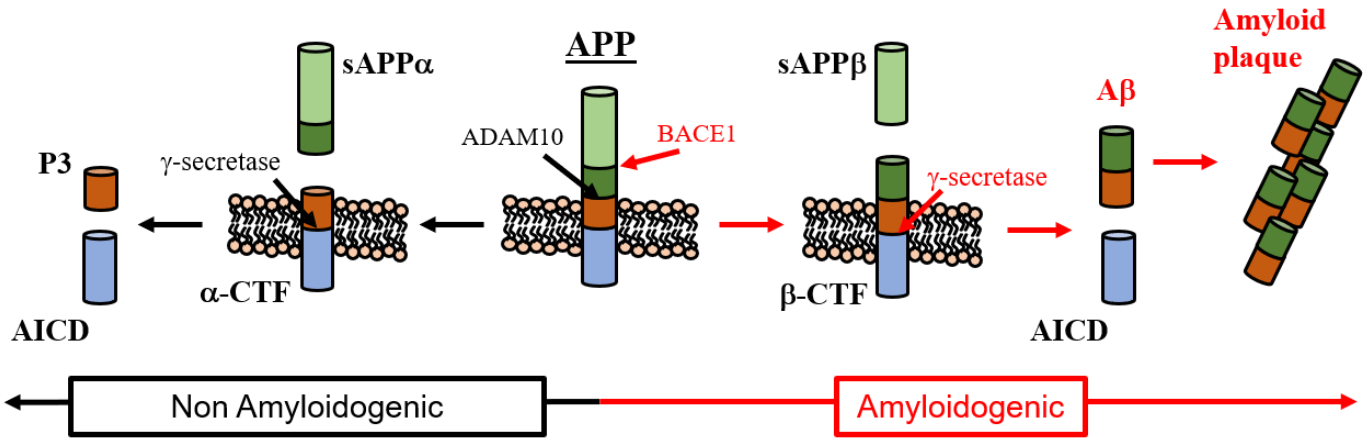

Figure 1.1: Schematic diagram of APP processing to α/β-CTFs and Aβ along amyloidogenic and non-amyloidogenic pathways mediated by α-, β- and γ-secretase enzymes.

Tau is an endogenous phosphoprotein that is highly expressed in neurons throughout the central nervous system (CNS) and at lower levels in glia. Alternative splicing of the microtubule associated protein tau (*MAPT*) gene results in the production of 6 tau isoforms with either 0, 1, or 2 N-terminal inserts and 3 or 4 C-terminal repeating regions. These C-terminal repeats govern the main function of tau, which is to bind to and stabilize microtubules as well as to facilitate axonal transport (Wang and Mandelkow, 2015). Reversible phosphorylation regulates tau's ability to bind microtubules, as it has 85 putative phosphorylation sites (Hanger et al., 2009). Various kinase enzymes, such as glycogen synthase kinase 3 β (GSK3β), cyclin-dependent kinase-5 (CDK5), and extracellular regulated kinases 1/2 (ERK1/2) phosphorylate tau to induce its disassociation from microtubules, while phosphatases such as protein phosphatase 2A and 2B (PP2A and PP2B) dephosphorylate tau to permit microtubule binding (Gong et al., 1993; Hanger et al., 2009). In the disease state, collectively referred to as tauopathies, imbalances in kinase/phosphatase activities and phospho-tau turnover cause a buildup of hyperphosphorylated tau which is prone to aggregation into insoluble neurofibrillary tangles (NFTs) – a characteristic associated with AD pathology (Guo et al., 2017). This process leads to loss of structural integrity of the neuron and neurotoxicity, although the mechanism is still unclear (Wang and Mandelkow, 2015).

While the presence of both Aβ and tau aggregates are characteristic of AD, the predominant view in the field is that Aβ production precedes tau hyperphosphorylation (Bloom, 2014). Amyloid deposits are first typically seen in the basal neocortex before spreading to neighboring hippocampal regions and eventually encompassing most of the cortex. Tau tangles, on the other hand, most often appear first within the temporal lobe, then spread through the entorhinal region before sweeping throughout the neocortex (Braak and Braak, 1997; Thal et al., 2002). However, since the spatiotemporal pattern of deposition varies between individuals, the severity of cognitive decline correlates with the NFT load rather than Aβ deposition, and that individuals may develop these insoluble aggregates with age without the onset of dementia, there is a clear need to understand the true nature of how AD begins (Nelson et al., 2012).

AD can arise by one of two mechanisms that are related in presentation and symptoms, yet distinct in their impetus, age of onset and rate of progression. First of these is early-onset familial AD (FAD), which accounts for approximately 5% of total diagnoses of the disease (Bird, 1993; Ertekin-Taner, 2007). FAD is also referred to as early-onset AD, as individuals are typically diagnosed between the ages 50 to 65, with diagnoses as young as 35 years of age also being reported, depending on the causal mutation (Ryan and Rossor, 2010). As implied by the name, FAD is an inherited form of Alzheimer's which is genetically transmitted *via* heritable mutations in the *APP* gene or those encoding the γ-secretase enzyme constituent *PS1* or *PS2*. The *APP* gene is located on chromosome 21 and can be mutated in a variety of ways that promote AD development, most of which are clustered around secretase enzyme cut sites. Specifically, the Swedish mutation (KM670/671NL) is adjacent to the BACE1 cut site on APP, and results in an increase in total Aβ generation (Citron et al., 1992; Johnston et al., 1994). Other mutations exist within the Aβ domain of APP or proximal to the γ-secretase cut site and are capable of enhancing levels of Aβ (Fraser et al., 1992; Goate et al., 1991; Nilsberth et al., 2001). FAD causing mutations in *PS1*/*2* also can enhance levels of Aβ peptides, in particular $A\beta_{1\text{-}42}$, with some also increasing NFT deposition (Gómez-Isla et al., 1999). However, while mutant tau isoforms (e.g. P301L tau) are utilized to study tau aggregation and tauopathies, there are no known mutations in the *MAPT* gene which are associated with AD. The far more prevalent course in which AD manifests is

sporadically (SAD), which accounts for approximately 95% of cases, and is associated with a variety of risk factors that can enhance the risk of developing the disease (Masters et al., 2015).

1.2 Risk factors for developing sporadic Alzheimer's disease

Sporadic AD has a later age of onset of around 65 years or older, with the incidence and risk of developing the disease increasing as individuals age (Hebert et al., 2013, 2010). This increased rate at advanced age is likely due to the longitudinal effects which the varied risk factors exert on the body, combined with age-related decline of normal functioning bodily systems (Hou et al., 2019). One of the most predictive risk factors for developing SAD is the inheritance of the apolipoprotein E ε4 (*APOEε4*) allele. Inheritance of the *ε4* allele increases the risk of developing SAD in a dose-dependent manner, with heterozygotic inheritance increasing risk by approximately threefold, and individuals homozygotic for the *ε4* allele are 8-12 times more likely to develop the disease (Alzheimer's Association, 2019; Corder et al., 1993; Farrer, 1997). Alternatively, inheritance of the *ε2* allele is protective against AD (Corder et al., 1994; Liu et al., 2013). Comorbidities such as type 2 diabetes mellitus and vascular abnormalities such as hypertension, heart disease, and smoking have also been proposed as risk factors for AD (Leibson et al., 1997; Luchsinger et al., 2005). Alternatively, maintaining an active and socially engaging lifestyle has been associated with less mild cognitive impairment and AD diagnoses (Fratiglioni et al., 2004; Kramer et al., 2006; Lautenschlager et al., 2008). Amid the multitude of potential risk factors that have been implicated to date, the most direct relationship is between AD and advanced age, with a steady increase in the number of cases with each year past 65 (Alzheimer's Association, 2019).

The human body is typically capable of withstanding non-lethal challenges to its internal and external environments by robust adaptive responses that are carried out by a variety of systems and work to establish and preserve bodily homeostasis, which is a state of dynamic equilibrium attuned to optimal functioning. These homeostatic mechanisms occupy a wide range of functions, from the maintenance of ion gradients to preserve neuronal function to the storage and mobilization of glucose that maintains steady state blood levels. Unfortunately, as the body ages, our ability to maintain homeostasis declines, and fitness to maintain balance decreases (Davies, 2016; Pomatto and Davies, 2017). For example, age-related decline in cellular proteostasis

network can contribute to accumulation of misfolded and aggregated proteins and lead to disease pathogenesis. During aging, the expression of chaperone heat shock proteins (HSPs) 60 and 70 is reduced, contributing to the misfolding of various proteins. HSPs typically become active at temperatures below those which cause irreversible protein misfolding to promote the correct structure of their various targets (Hipp et al., 2019; Pomatto and Davies, 2017). The age-related decline in the ability to maintain body temperature has been implicated as not only a risk factor for elderly to develop hypothermia, but also as a risk factor for AD (Almeida and Carrettiero, 2018).

1.3 Thermal homeostasis, aging and AD

Maintenance of thermal homeostasis through changes in environmental temperature is a vital process that helps ensure correct biochemical and physiological function of enzymatic, cellular, and organ systems. Human core body temperature is sustained between 35.5-37.5°C through a variety of mechanisms, including metabolic rate, non-shivering brown adipose thermogenesis (BAT), skeletal muscle shivering, and vasodilation/constriction of blood vessels (Selman et al., 2002; Tansey and Johnson, 2015). Unfortunately, with age, these regulatory mechanisms begin to decline as metabolism slows, BAT and skeletal muscle are lost, and vasculature alterations cause a reduction in cerebral blood flow (Li et al., 2018; Pomatto and Davies, 2017). These combined changes have been shown to result in a decreased thermogenic capacity in the elderly when faced with acute core body temperature cooling, as well as progressively lower orally measured temperatures as they continue to advance in age (Frank et al., 2000; Gomolin et al., 2005). Systemically, the risk for the elderly (>60 years of age) to develop chronic hypothermia has been a prevalent issue for more than 30 years, with clinical observations of this susceptibility dating back to the 1970s (Brody, 1994; Fox et al., 1973; Keilson et al., 1985; Wongsurawat et al., 1990). In addition to the progressive decline in ability to defend core body temperature, several other factors such as diabetes mellitus, apnea-related hypoxia, malnutrition, alcohol intake and anesthesia can increase the risk of hypothermia development in the elderly (Almeida and Carrettiero, 2018). More recently, this decreased ability to regulate body temperature has been tied to the age-related susceptibility to AD, making hypothermia a newly identified risk factor for AD

(Almeida and Carrettiero, 2018; Carrettiero et al., 2015; Holtzman and Simon, 2000; Whittington et al., 2010).

While there have been many reports detailing the risk of hypothermia to the elderly, there is less information comparing individuals with and without AD. Holtzman and Simon (2000) describe how individuals with Down syndrome (DS) have approximately a 0.5-1°C lower body temperature than age-matched controls without DS (Holtzman and Simon, 2000). Since individuals with DS develop AD-like neuropathology by 50 years of age, this reduced body temperature may have an effect on the development of these features (Head et al., 2012; Wisniewski et al., 1985). Additionally, individuals with AD have been shown to have abnormalities in their circadian rhythm, which is a process tied with both body temperature and tau phosphorylation (Harper et al., 2004). These circadian variations in tau phosphorylation have recently been shown to be driven by body temperature in a mouse model (Guisle et al., 2019). Body temperature abnormalities in AD patients may be connected to neuronal damage caused by the presence of amyloid plaques in the hypothalamus, a key brain region responsible for temperature regulation (Simpson et al., 1988; Zhao et al., 2016). A prospective study following a group of approximately 2,300 Finnish men demonstrated that individuals who regularly used high-temperature sauna baths were less likely to develop AD and other dementias as they age. Interestingly, this study also showed that the frequency and average duration of sauna bathing are proportional to a lower risk of developing dementia and AD (Heinonen and Laukkanen, 2018; Laukkanen et al., 2017). Data that describe the molecular mechanisms behind these apparent effects of temperature is gradually being brought to light as research on this association continues.

Regarding the AD-associated neuropathological “hallmarks” of Aβ and tau, the effect of temperature on tau hyperphosphorylation has been studied by various groups. In the first series of reports detailing a relationship between hypothermia and tau phosphorylation, Korneyev, et al. showed a rapid and reversible increase of hyperphosphorylated tau within rat cortex, cerebellum and hippocampus after exposing the animals to cold water stress (Korneyev et al., 1995; Korneyev, 1998). Later studies provided more insight as to the mechanism behind tau phosphorylation, beginning with observations made in mouse brain sections acutely exposed to room temperature (23°C). These metabolically active sections displayed robust tau hyperphosphorylation that

increased over time, an effect determined to be due to a hypothermia-induced inhibition of PP2A (Planel et al., 2004). Later observations in db/db diabetic mice showed that aged (24 months) mice have a significantly lower body temperature than young (4 months) mice, and also display a higher degree of tau hyperphosphorylation (El Khoury et al., 2016). This effect was later attributed to the observed hypothermia and impaired thermoregulation in the aged mice, rather than deficiencies in glucose metabolism (Gratuze et al., 2017). The difference in temperature sensitivity between young and aged mice may be attributed to a protective inhibition of GSK3β that is lost during ageing, which leads to higher kinase activity and more significant tau phosphorylation in aged mice (Tournissac et al., 2017). Other research has described a memory deficit phenotype concurrent with the accumulation of amyloid plaques along with hyperphosphorylation and aggregation of tau into NFTs in rats challenged by cold water swimming induced hypothermia (Ahmadian-Attari et al., 2015). Conversely, exposing 20-month old hypothermic *3xTgAD* mice to a warm environment for seven days normalized core body temperature and reduced the observed increase in Aβ peptides and tau phosphorylation as well as ameliorated cognitive deficits (Vandal et al., 2016). Other molecular changes may be induced in cells exposed to a hypothermic environment. For example, a set of cold shock proteins (CSPs) are induced at temperatures considered to be mildly hypothermic (25°C) including cold-inducible RNA binding protein (Cirp) and RNA binding motif 3 (Rbm3), which are capable of inducing transcriptomic changes associated with a lower environmental temperature (Pomatto and Davies, 2017).

The use of anesthetics in various procedures poses a risk for aged individuals when considering their effect on body temperature. For instance, aged and AD-afflicted individuals have been observed to both recover less well and decline in condition following the use of anesthetic (Fernandez et al., 2003). Anesthesia has been recognized to induce tau phosphorylation, which may be the driving factor in the debilitating effect of anesthetic on the elderly (Whittington et al., 2013). This effect has been studied using mice, where anesthetic-induced tau hyperphosphorylation was shown to be caused by a decrease in body temperature. Phosphorylation was blocked by controlling animals' body temperature, and there was no notable effect on APP metabolism (Planel et al., 2007). Later studies show that anesthetic causes dissociation of tau from microtubules, also likely due to a change in body temperature (Planel et al., 2008). There is a

sizeable body of literature describing the effect of hypothermia on tau phosphorylation, however, whether there is an effect on APP processing, and the mechanisms behind any potential alteration in amyloidogenic processes remains unknown.

1.4 Endolysosomal dynamics, APP trafficking and AD

The endolysosomal (EL) system is a complex and interconnected network comprised of membrane-bound intracellular vesicles. These vesicles transport cargo throughout the cell along synthetic and degradative pathways from various sites of origin including the plasma membrane and trans-Golgi network (TGN) (Hu et al., 2015). Trafficking along the EL system is essential for the post-translational modification and maturation of proteins as they are synthesized. For example, APP is synthesized in the endoplasmic reticulum (ER), then receives its *N*- and *O*- linked glycans and phosphorylation motifs as it is trafficked from the ER to the plasma membrane through the TGN. Approximately 10% of APP is localized to the plasma membrane, with the majority distributed throughout the EL system, Golgi apparatus and TGN. This is due in part to a YENPTY amino acid motif at the carboxy terminus of APP, which mediates the rapid internalization of plasma membrane localized APP to endosomal compartments (Lai et al., 1995; Marquez-Sterling et al., 1997). APP is then either processed within the EL system, trafficked back to the plasma membrane, retrogradely transported to the TGN or degraded in lysosomes (Thinakaran and Koo, 2008).

The subcellular localization of α-, β- and γ-secretase enzymes is also important in order to understand the EL system in the context of APP processing. ADAM10, the chief α-secretase, has been shown to primarily reside on the plasma membrane, where α-secretase cleavage increases the shedding of sAPPα into the extracellular space (Kuhn et al., 2010). The cleavage of APP by α-secretase may occur within endosomes, as ADAM10 also resides within those compartments, although most of the enzyme here is in the immature and inactive state (Lammich et al., 1999; Sisodia, 1992). BACE1 is more widely distributed throughout the cell, localizing to the TGN, endosomal compartments, and the plasma membrane, so there are many compartments where the enzyme may cross paths with APP (Kinoshita et al., 2003). Since BACE1 is an aspartyl protease, its activity is greatest at an acidic pH; thus the site of β cleavage is likely within acidic endosomes

(Vassar et al., 1999). The γ-secretase complex acts largely within the EL system, localizing to the ER and TGN to produce Aβ peptides, but has also been shown to produce Aβ peptides at the plasma membrane (Wai-Yan Choy et al., 2012). Therefore, amyloidogenic processing of APP to Aβ peptides occurs primarily within the EL system, rather than shedding at the plasma membrane (Koo and Squazzo, 1994; Perez et al., 1999).

Diverse vesicles make up the EL system, including early endosomes (EEs), late endosomes (LEs), recycling endosomes (REs), autophagosomes and lysosomes. The degradative pathway within the EL system targets cargo carrying autophagosomes and endosomes for eventual fusion with lysosomes to form autolysosomes, which have a pH of approximately 4.5-5.0 to facilitate the activity of multiple acid hydrolase enzymes that can break down various molecules, including APP and its metabolites. This process of maturation and fusion is accompanied by molecular changes in proteins resident on the vesicular membranes, such as the cleavage of the membrane-associated protein LC3-I to LC3-II, which is a crude measure of the number of autophagosomes, the main constituents of the autophagic pathway. Autophagy is the principal mechanism for the degradation of long-lived normal and aggregated proteins as well as cellular organelles (Ghavami et al., 2014; Xilouri and Stefanis, 2012). Macroautophagy, usually referred to as autophagy, is the predominant mechanism that engulfs macromolecules and cellular organelles before fusing with lysosomes to degrade its contents. Interestingly, certain ubiquitinated proteins that are conjugated to the adaptor protein p62 are constitutively degraded by autophagy (Dikic and Elazar, 2018; Eskelinen, 2004; Glick et al., 2010). Since the EL system is a major site of Aβ metabolism, it is important to assess the potential alterations of this pathway using various markers when studying the effect of temperature on amyloidogenic processes (Bajaj et al., 2019; Nixon, 2017; Wang et al., 2018).

1.5 Hypothesis and objectives

Considering the current landscape of knowledge and research on AD and thermal homeostasis, we have developed the hypothesis that a hypothermic environment causes elevated levels of APP metabolites in neuron-like cells. In order to pursue this research, we utilized wild-type mouse neuroblastoma neuro-2a (N2a) and N2a cells overexpressing Swedish mutant human APP (N2a-APP). These cells were grown at normal temperature conditions (37°C) until approximately 70%

confluency was attained, then each growth chamber was either transferred to a hypothermic (27°C) or a hyperthermic (40°C) incubator, or maintained at the normothermic (37°C) temperature for 6, 12, or 24 hours. We then evaluated these cells along these specific experimental objectives:

I. determine how hypothermic (27°C) and hyperthermic (40°C) conditions can influence the levels of APP holoprotein and its processing enzymes (ADAM10, BACE1, PS1 and nicastrin), α/β–CTFs and $A\beta_{1\text{-}40/42}$ in cultured N2a and N2a-APP cells.

II. determine how hypothermic (27°C) and hyperthermic (40°C) conditions can influence the cellular distribution of APP and its metabolites in the endolysosomal-autophagic system in cultured N2a and N2a-APP cells.

2. MATERIALS AND METHODS

2.1 Reagents

Cell culture media and supplements including Dulbecco's Modified Eagles Medium (DMEM), Opti-MEM, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). NuPAGE 4-12% gels, MES SDS running buffer, enhanced chemiluminescence kit, fluorescently conjugated secondary antibodies and bicinchoninic acid (BCA) protein assay kits were purchased from Thermo Fischer (Waltham, MA, USA). Sources of primary antibodies and their dilutions are listed in **Table 1**. Horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA), whereas enzyme-linked immunosorbent assay (ELISA) kits for mouse and human Aβ peptides were purchased from Fischer Scientific and EMD Millipore, respectively (MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for cell viability was purchased from Sigma-Aldrich (St. Louis, MO, USA), the redox cell viability kit was obtained from Abcam (Cambridge, UK), while the lactate dehydrogenase (LDH) assay kit was purchased from Promega (Madison, WI, USA). The activity assay kit for ADAM10 was purchased from AnaSpec (Fremont, CA, USA), and the BACE1 activity assay kit was purchased from Abcam (Cambridge, UK). All other chemicals were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher (Waltham, MA, USA).

2.2 Cell culture and treatments

Wild-type murine neuroblastoma Neuro-2a cells (N2a) and Swedish-mutant human APP (KM670/671NL) harboring N2a cells (N2a-APP) were generous gifts from Dr. G. Thinakaran (University of Chicago, IL). N2a cells were maintained in normal growth medium composed of a 1:1 mixture of DMEM:Opti-MEM, 5% FBS and 100 units/mL of penicillin/streptomycin, while N2a-APP cells were maintained in normal growth medium supplemented with 0.4% Geneticin as described before (Maulik et al., 2015). Briefly, cells were grown at 37°C in a humid 5% CO_2 incubator, with their respective culture media replaced every 2-3 days. Cells were seeded at 13,000 cells/cm^2 and harvested at 90% confluency. In our experimental paradigm, cultured N2a and N2a-APP cells were grown at 37°C until approximately 70% confluency, then were transferred to 27°C,

37°C, or 40°C incubators for different periods of time (6, 12 and 24 hours) before being harvested for further analysis. In a separate series of experiments, cultured N2a-APP cells exposed to 27°C, 37°C, or 40°C for 24 hours were treated with protein synthesis inhibitor cycloheximide (30µg/ml) for the final 1, 3, or 6 hours of their incubation period, as described previously (Ourdev et al., 2019; Rebelo et al., 2009). For each experimental paradigm, cultured cells were collected after washing with cold phosphate-buffered saline (PBS, pH 7.4) and then harvested in radioimmunoprecipitation assay (RIPA) buffer. Lysis was performed by sonication and debris was pelleted and removed by centrifugation at 10,000RPM for 10 minutes at 4°C and the supernatant was used either immediately or stored at -80°C until further processing.

2.3 Cell viability assays

Viability of N2a and N2a-APP cells was determined by the MTT assay, which utilizes a tetrazolium salt to measure mitochondrial dehydrogenase activity. Briefly, cells that had been exposed to 27°C, 37°C, or 40°C for 24 hours were treated with a solution of MTT reagent diluted 1:10 into cell culture media for 4 hours at 37°C. The reaction was terminated by removing the reaction media, then 200mL dimethyl sulfoxide (DMSO) was added and absorbance was measured spectrophotometrically at 570nm. In parallel, we utilized a cellular redox state sensitive cytotoxicity assay (Abcam, ab112118) to further assess viability of cultured N2a and N2a-APP cells following a 24-hour exposure to 27°C, 37°C or 40°C as per manufacturer's instructions. We also performed an LDH assay with our N2a and N2a-APP along the same experimental paradigm. Cells in control wells were lysed to measure total LDH at each environmental temperature, and cells exposed to 27°C, 37°C, or 40°C were normalized as a percentage of maximum LDH leakage. Each experiment was performed in quadruplicate and repeated 3 to 4 times.

2.4 Western blotting

Cultured N2a and N2a-APP cells collected from various experimental paradigms were processed for western blotting as described earlier (Ourdev et al., 2019). In brief, the protein quantity of each lysate was determined by BCA assay and then normalized to a common working concentration with RIPA buffer or culture media without FBS. Samples were then resolved by 7-17%, 8% or 12% sodium polyacrylamide or Invitrogen NuPAGE 4-12% Bis-Tris gels, transferred to

polyvinylidene fluoride (PVDF) membranes, blocked in 5% milk and incubated with various primary antibodies overnight at 4°C (dilutions listed in **Table 1**). The following day, membranes were incubated with HRP-conjugated secondary antibodies diluted in 5% milk (1:5,000) and immunoreactive proteins were detected with enhanced chemiluminescence reagent. All membranes were re-probed with an anti-β-actin antibody to monitor protein loading and each blot was quantified using a high-quality image scanner and BioRad Quantity One.

2.5 ELISA for human and mouse $A\beta_{1-40}$ and $A\beta_{1-42}$

Cellular and secretory levels of Aβ peptides in N2a and N2a-APP cells were assessed following exposure to 27°C, 37°C, or 40°C for different periods of time (6, 12, 24 hours) according to each manufacturer's instructions. In brief, cells were homogenized in ice-cold RIPA buffer and then levels of human (N2a-APP) and mouse (N2a) $A\beta_{1-40}$ and $A\beta_{1-42}$ were measured using their respective ELISA kits, as described earlier (Chung et al., 2018; Maulik et al., 2015). To assay secreted Aβ peptides, we incubated both cell types in serum free Opti-MEM for the last hour of each experimental time point, then collected the media. Subsequently, each media sample was concentrated using Amicon 3K cut-off spin columns and then processed to measure human and mouse $A\beta_{1-40}$ and $A\beta_{1-42}$ using their respective ELISA kits, as described earlier (Chung et al., 2018; Maulik et al., 2015). Data from each ELISA assay was normalized to total protein in each sample (determined by BCA assay) and represented as pg Aβ/μg protein. All samples were assayed in duplicate, and each experiment was repeated 3 to 5 times.

2.6 Activity assays

Activity assays for ADAM10 and BACE1 were performed on our N2a and N2a-APP cells after 24 hours temperature exposure as per manufacturer's instructions with independent experiments and protein extraction protocols. In brief, protein extraction was performed using each assay kits extraction buffer, then each lysate was quantified by BCA assay. The protein was normalized to a working concentration and added to kit reagents (e.g. fluorogenic substrate) before incubating at 37°C for 1-4 hours. Plates were read using a multi-well plate reader at wavelengths specified by each manufacturer's protocols, as described earlier (Chung et al., 2018; Maulik et al., 2015). Data

was normalized as fluorescent units per μg protein as determined by BCA quantification. All samples were assayed in duplicate, and each experiment was repeated 3 times.

2.7 Immunocytochemistry

For subcellular localization of APP and its cleaved products, N2a and N2a-APP cells were grown in 8-well chamber slides until ~80% confluency before exposing cells to 27°C, 37°C, or 40°C for 24 hours. The cells were then washed with PBS, fixed using 4% paraformaldehyde for 15 minutes, permeabilized with a 0.25% Triton X-100 PBS solution for 15 minutes, and then blocked overnight at 4°C with a 3% BSA in PBS solution. Primary antibody incubation utilized anti-APP (22C11, N-terminal) or anti-Aβ antibodies in combination with either lysosomal-autophagic marker anti-LAMP1 or anti-LC3 antibodies diluted in 3% BSA in PBS (listed in **Table 1**), and was done overnight at 4°C as described earlier (Chung et al., 2018; Maulik et al., 2015). Each well was then exposed to Alexa Fluor 488/594 conjugated secondary antibodies (1:1,000) for 1-2 hours at room temperature, washed and mounted with prolong gold anti-fade medium. Immuno-stained cells were visualized using a Zeiss LSM700 Confocal Microscopy System under an oil-immersion 63X magnification lens.

2.10 Statistical analyses

All data is expressed as mean ± SEM. Statistical significance was determined with one-way ANOVA utilizing Tukeys post-hoc multiple comparisons test. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

2.11 Table 1: Antibodies and their dilutions

Antibody	Dilution WB	Dilution ICC	Host	Source	Catalogue Number
APP (Y-188)	1:5,000	-	Rabbit	Abcam	ab32136
APP (22C11)	1:2,000	1:1,000	Mouse	Millipore	MAB348
Aβ (4G8)	-	1:500	Mouse	Covance Corp.	SIG-39220
LC3	1:1,000	1:500	Rabbit	MBL	PM036
LAMP1	1:5,000	1:1,000	Rabbit	Abcam	ab24170
p62	1:500	-	Mouse	Millipore	MABC32
ADAM10	1:500	-	Mouse	Millipore	MAB1427
BACE1	1:500	-	Mouse	Millipore	MAB931
PS1-NT	1:500	-	Rabbit	-	Gift from Dr. Thinakaran
Nicastrin	1:1,000	-	Goat	Santa Cruz	Sc376513
β-actin	1:5,000	-	Mouse	Sigma	A5441

3. RESULTS

3.1 Cell survival and viability are affected by changes in temperature

N2a and N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C for 24 hours and processed for cell viability by either the well-established MTT mitochondrial activity assay, a redox state cellular viability assay or the LDH assay. Both N2a **(Fig. 3.1A)** and N2a-APP **(Fig. 3.1D)** cells exhibited an approximate 40% reduction in MTT after incubation at 27°C compared to 37°C for 24 hours, with no apparent change in MTT absorbance between 37°C and 40°C. A similar pattern is also observed in N2a **(Fig. 3.1B)** and N2a-APP **(Fig. 3.1E)** cells assayed for viability with a cellular redox state sensitive kit, where the reduction in optical density was approximately 50% in cells exposed to 27°C compared to those exposed to 37°C or 40°C for 24 hours. LDH data reveal a 30-40% increase in the LDH leakage (percentage of maximum LDH) from N2a **(Fig. 3.1C)** and N2a-APP **(Fig. 3.1F)** cells exposed to 27°C for 24 hours, when compared to those exposed to 37°C for 24 hours. N2a-APP cells exposed to 40°C for 24 hours also displayed an approximate 10% increase in LDH leakage (percentage of maximum LDH) compared to those exposed to 37°C for 24 hours **(Fig. 3.1F)**.

3.2 Effects of temperature on the levels of APP and its cleaved products

Cultured N2a and N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C for either 6, 12, or 24 hours and then assayed for levels of full-length APP holoprotein and its cleavage products by western blot. Neither N2a **(Fig. 3.2A, 3.2B)** or N2a-APP **(Fig. 3.2C, 3.2D)** cells displayed a change in full-length APP holoprotein levels over time within each temperature group as evidenced by both C-terminal Y-188 **(Fig. 3.2A, 3.2C)** and N-terminal 22C11 **(Fig. 3.2B, 3.2D)** APP antibodies. There was also no significant difference in APP holoprotein levels between temperature groups at each time point in both N2a **(Fig. 3.2A, 3.2B)** and N2a-APP **(Fig. 3.2C, 3.2D)** cells. As for APP cleavage products, both N2a **(Fig. 3.3A, 3.3B)** and N2a-APP **(Fig. 3.3C, 3.3D)** cells displayed a significant time-dependent decrease in levels of both α-CTF **(Fig. 3.3A, 3.3C)** and β-CTF **(Fig. 3.3B, 3.3D)** after exposure to 37°C and 40°C. However, N2a cells exposed to 27°C displayed a significant increase in α-CTF levels over time **(Fig. 3.3A)**, while β-CTF levels

were maintained over time **(Fig. 3.3B)**. Additionally, both α/β-CTFs were significantly elevated in N2a cells exposed to 27°C for 24 hours when compared to those exposed to 37°C for 24 hours **(Fig. 3.3A, 3.3B)**. In N2a-APP cells, those exposed to 27°C showed a significant increase in β-CTF levels as a function of time **(Fig. 3.3D)**, while the α-CTF levels were maintained over time **(Fig. 3.3C)**. N2a-APP cells also showed significantly higher levels of both α/β-CTFs in cells exposed to 27°C for 24 hours compared to those exposed to 37°C for 24 hours **(Fig. 3.3C, 3.3D)**.

3.3 Temperature-based effect on APP secretase enzymes

N2a and N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C, for either 6, 12, or 24 hours and assayed for steady-state levels of α-secretase, β-secretase and two components of the γ-secretase enzyme complex by western blot. There were no observed changes in the levels of the α-secretase ADAM10 **(Fig. 3.4A, 3.4C)**, β-secretase BACE1 **(Fig. 3.4B, 3.4D)** in either N2a **(Fig. 3.4A, 3.4B)** or N2a-APP **(Fig. 3.4C, 3.4D)** cells over time at each temperature, or at each time point assayed between temperature groups. There was also no marked change in levels of the γ-secretase complex components nicastrin **(Fig. 3.5A, 3.5C)** and PS1 **(Fig. 3.5B, 3.5D)**, in either N2a **(Fig. 3.5A, 3.5B)** or N2a-APP **(Fig. 3.5C, 3.5D)** cells over time at each temperature, or at each time point assayed between temperature groups.

3.4 Temperature-based effects on ADAM10 and BACE1 enzyme activity

N2a and N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C for 24 hours and assayed for enzymatic activity of ADAM10 and BACE1 using their respective kits. ADAM10 showed no temperature-dependent alteration in activity in N2a cells **(Fig. 3.6A)**, however there was a significant increase in enzyme activity in N2a-APP **(Fig. 3.6C)** cells exposed to 27°C for 24 hours compared to cells exposed to 37°C for 24 hours. In contrast to ADAM10 activity, we did not observe any temperature-dependent change in BACE1 enzyme activity after 24 hours exposure to different temperature conditions in N2a **(Fig. 3.6B)** or N2a-APP **(Fig. 3.6D)** cells.

3.5 Effects of temperature on cellular and secreted levels of Aβ peptides

N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C, for either 6, 12, or 24 hours and then processed to measure the levels of intracellular and secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ using their respective ELISA kits. The intracellular levels of $A\beta_{1-40}$ **(Fig. 3.7A, 3.8A)** and $A\beta_{1-42}$ **(Fig. 3.7B, 3.8B)** remained unaltered in both N2a **(Fig. 3.7A, 3.7B)** and N2a-APP **(Fig. 3.8A, 3.8B)** cells, with respect to each temperature group over time, and at each time point in between temperature groups. However, levels of secreted $A\beta_{1-40}$ peptides were significantly reduced in media collected from N2a cells exposed to a hypothermic temperature (27°C) for 6 and 12 hours when compared to media collected from N2a cells exposed to normothermic temperature (37°C) for 6 and 12 hours. There was no change in secreted $A\beta_{1-40}$ within each temperature group over time **(Fig. 3.7C)**. In media collected from N2a-APP cells, both secreted $A\beta_{1-40}$ **(Fig. 3.8C)** and $A\beta_{1-42}$ **(Fig. 3.8D)** levels were significantly reduced after 6 and 12 hours exposure to hypothermic temperature (27°C) when compared to media collected from cells exposed to normothermic temperature (37°C) for 6 and 12 hours. There also was no change in secreted $A\beta_{1-40}$ **(Fig. 3.8C)** or $A\beta_{1-42}$ **(Fig. 3.8D)** within each temperature group over time in cultured N2a-APP cells.

3.5 Effects of temperature on endolysosomal-autophagic markers

N2a and N2a-APP neuroblastoma cells were exposed to 27°C, 37°C, or 40°C, for either 6, 12, or 24 hours and then processed to assess changes in endolysosomal constituent markers by western blot. In both N2a and N2a-APP cells, there was no change in the steady-state levels of the lysosomal proteins LAMP1 **(Fig. 3.10A, 3.10E)** or p62 **(Fig. 3.10C, 3.10G)** over time at each temperature condition, and at each time point between temperatures. However, there was an approximate two-fold increase in LC3-II at hypothermic temperature (27°C) for all time points in both N2a **(Fig. 3.10B)** and N2a-APP **(Fig. 3.10F)** cells when compared to both normal (37°C) and elevated temperature (40°C) conditions. There was no time-dependent increase in LC3-II in either N2a **(Fig. 3.10B)** or N2a-APP **(Fig. 3.10F)** cells.

3.6 Effects of temperature on the localization of APP and Aβ and endolysosomal-autophagic compartments

N2a **(Fig. 3.13, 3.14)** and N2a-APP neuroblastoma cells **(Fig. 3.11, 3.12)** were incubated for 24 hours at 27°C, 37°C, or 40°C before being processed for immunocytochemistry to determine potential localization of APP and Aβ within lysosomal (LAMP1) and autophagic (LC3) compartments. Immunoreactive APP showed partial colocalization with LAMP1 **(Fig. 3.11, 3.13)** at each temperature condition, indicating its presence in lysosomes, as well as a broad distribution throughout the cell body, extending to the plasma membrane. Similarly, a subset of immunoreactive APP is found to colocalize with LC3 positive puncta **(Fig. 3.11, 3.13)**, while remaining APP is distributed throughout the cell in those exposed to each temperature condition. Interestingly, the LAMP1 and LC3 immunoreactivities appear to become more punctate and focal at lower temperatures **(Fig. 3.11-3.14)**. The pattern displayed by Aβ seems to become more focal and punctate at hypothermic (27°C) temperatures than that observed at higher temperature (37°C and 40°C) conditions **(Fig. 3.12, 3.14)**. This change in distribution manifests as an apparent increase in the colocalization between Aβ and both LAMP1 and LC3 at 27°C when compared to both 37°C and 40°C **(Fig. 3.12, 3.14)**.

3.7 Changes in APP cleavage products may be due to an inhibition of clearance mechanisms

N2a-APP neuroblastoma cells incubated at 27°C, 37°C, or 40°C for 24 hours were exposed to 30μg/mL cycloheximide for the last 0, ½, 3, or 6 hours of the 24-hour incubation period to inhibit total protein synthesis. Cells were then processed to measure the levels of APP and α/β-CTFs by western blot. N2a-APP cells exposed to hypothermic (27°C) temperatures appear to retain both α- and β-CTFs for a longer period after cycloheximide treatment **(Fig. 3.9C, 3.9D)**, indicating an attenuation of clearance pathways associated with these molecules at hypothermic conditions. However, there was no alteration in full-length APP turnover in N2a-APP cells exposed to each 27°C, 37°C, or 40°C temperature conditions **(Fig. 3.9B)**.

Figure 3.1: Cell viability at different ambient temperatures

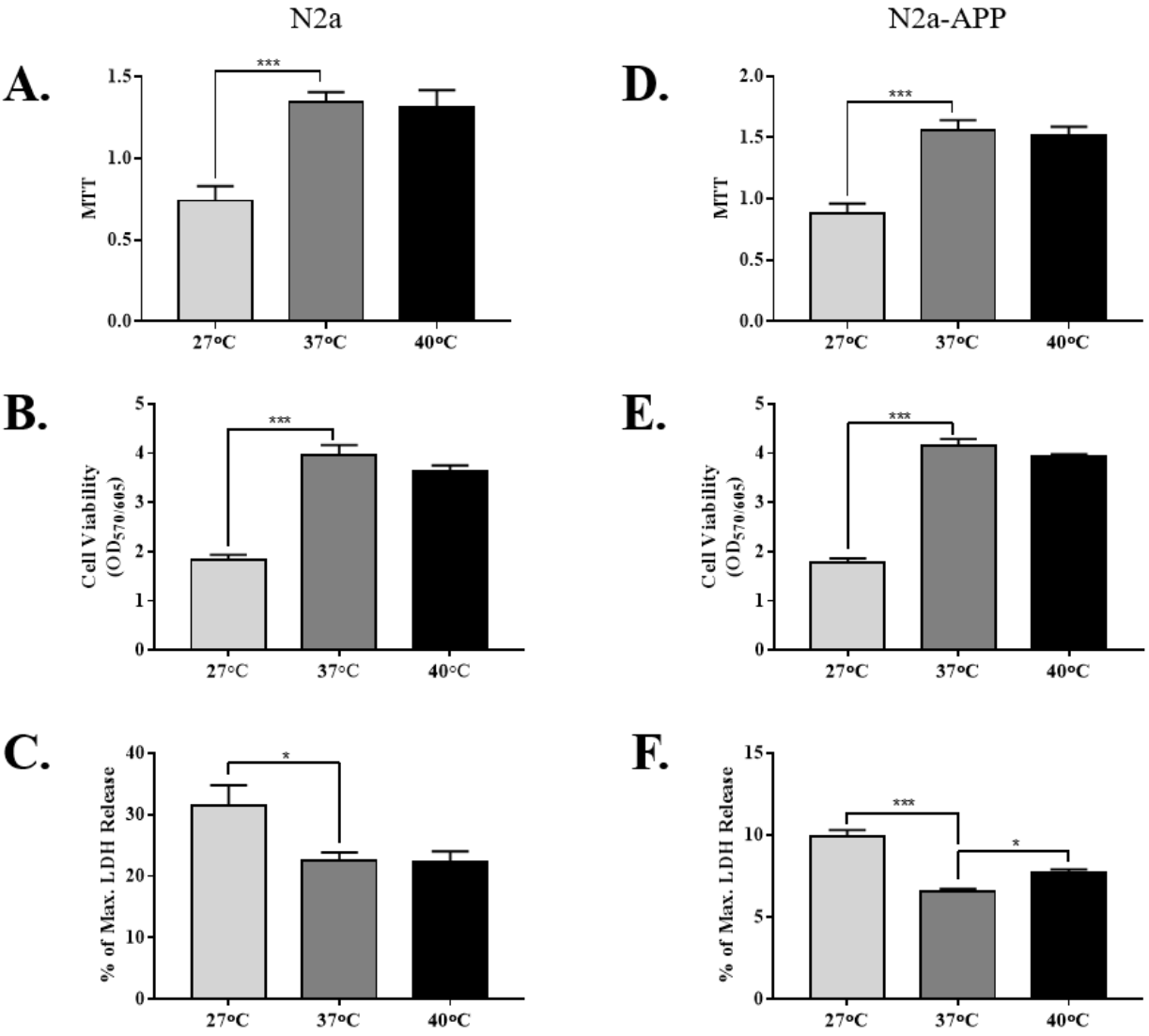

Figure 3.1: N2a **(Fig. 3.1A, 3.1B, 3.1C)** and N2a-APP **(Fig. 3.1D, 3.1E, 3.1F)** were incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 24 hours. Cells were assayed for viability by mitochondrial activity MTT assay **(Fig. 3.1A, 3.1D),** a cytotoxicity kit purchased from Abcam **(Fig. 3.1B, 3.1E)**, or by the LDH assay **(Fig. 3.1C, 3.1F)**. ***: $p < 0.001$.

Figure 3.2: Full length APP level at different ambient temperatures

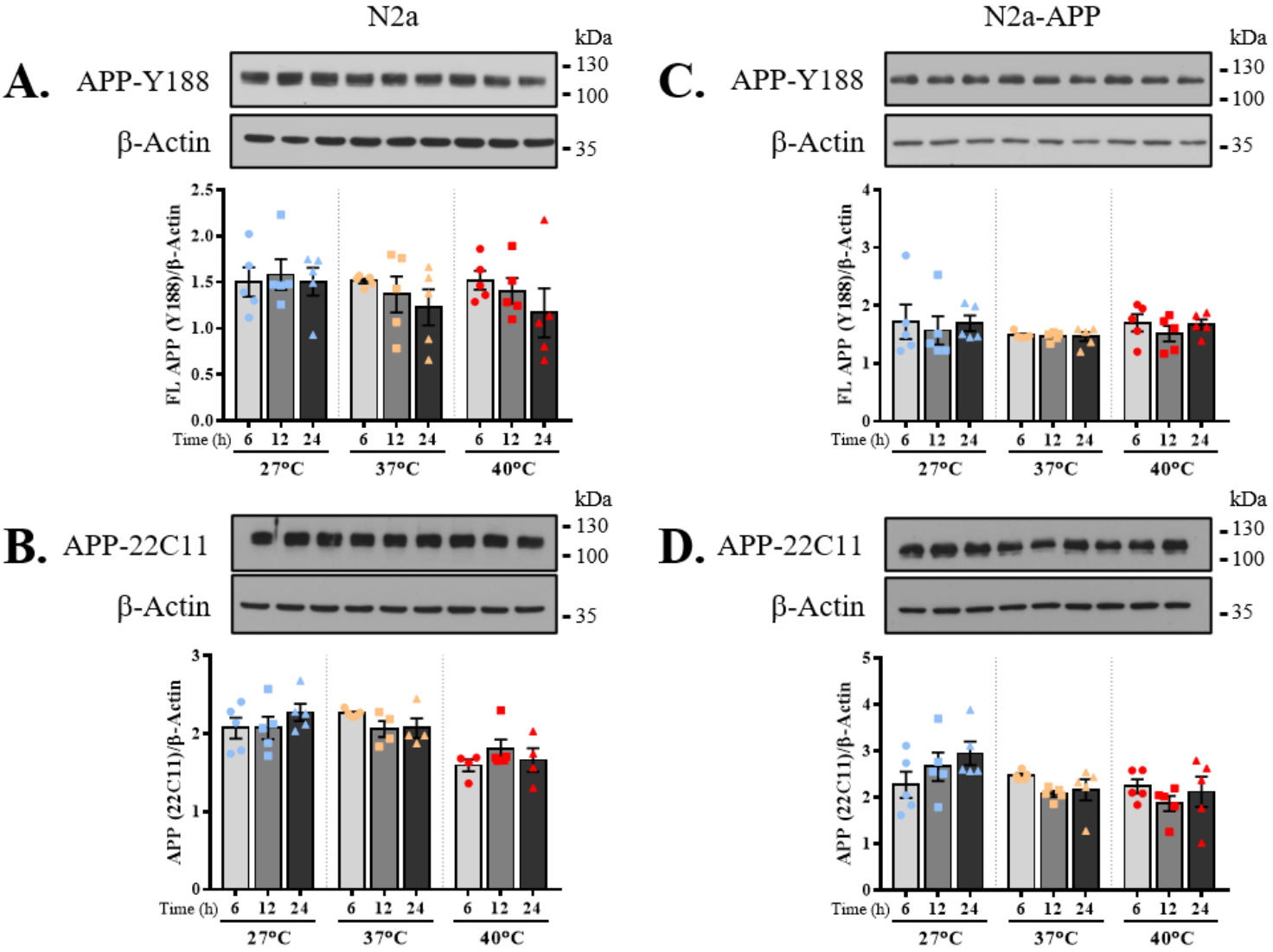

Figure 3.2: Western blots and corresponding histograms of N2a **(Fig. 3.2A, 3.2B)** and N2a-APP **(Fig. 3.2C, 3.2D)** cells grown at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**.

Figure 3.3: α/β-CTF levels at different ambient temperatures

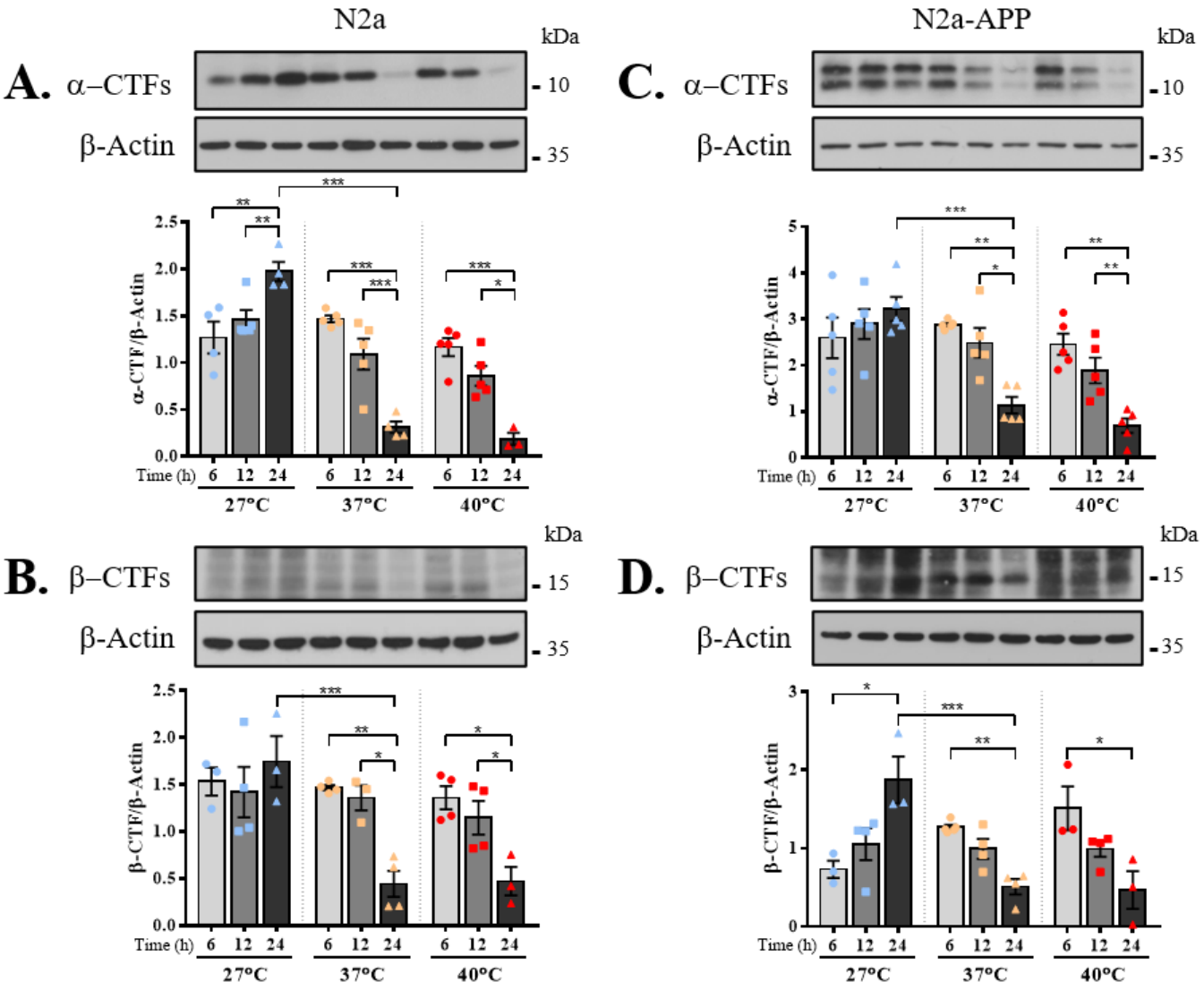

Figure 3.3: Western blots and corresponding histograms of N2a **(Fig. 3.3A, 3.3B)** and N2a-APP **(Fig. 3.3C, 3.3D)** cells grown at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3.4: α and β secretase enzyme levels at different ambient temperatures

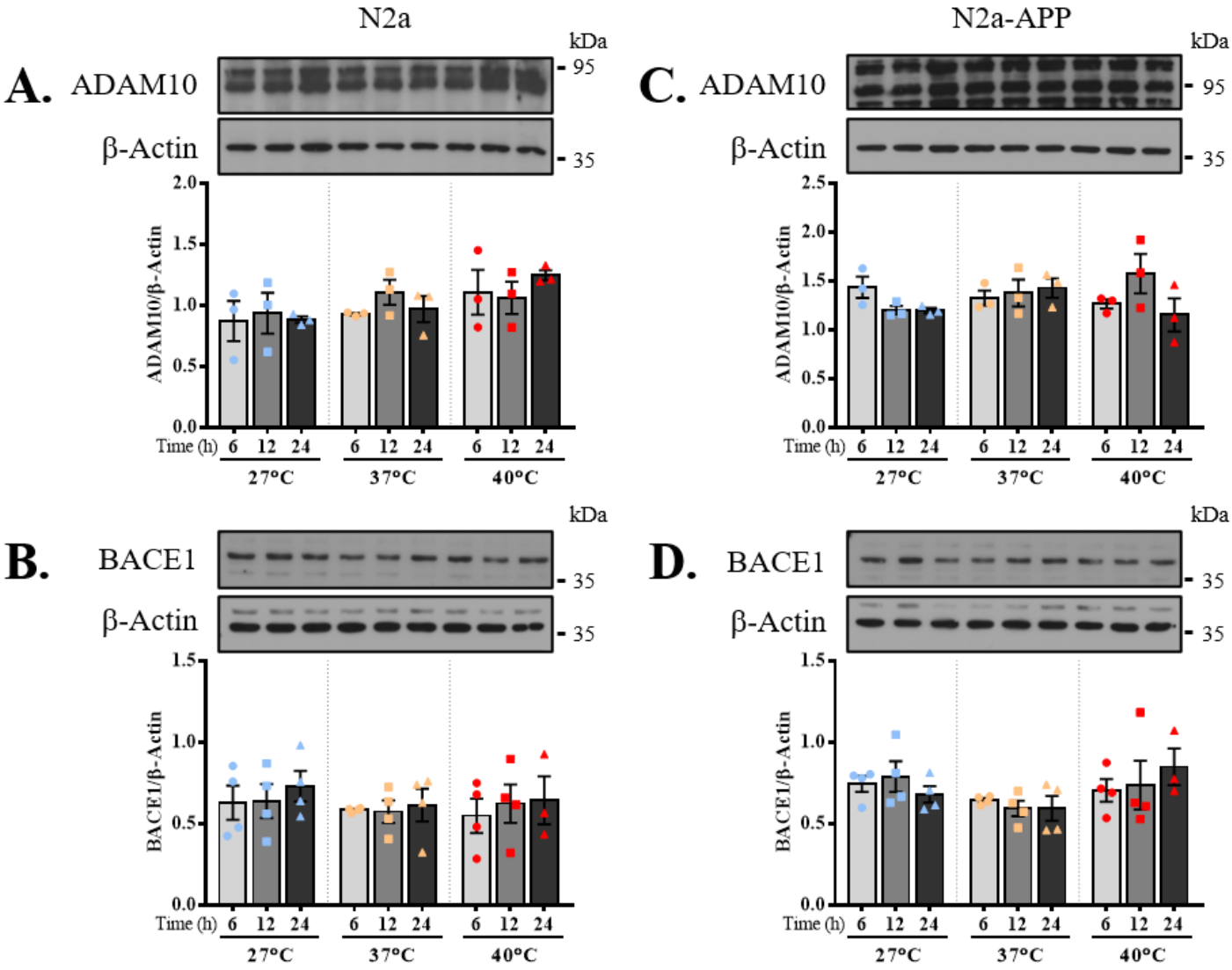

Figure 3.4: Western blots and corresponding histograms of N2a **(Fig. 3.4A, 3.4B)** and N2a-APP **(Fig. 3.4C, 3.4D)** cells grown at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**.

Figure 3.5: γ-secretase enzyme component levels at different ambient temperatures

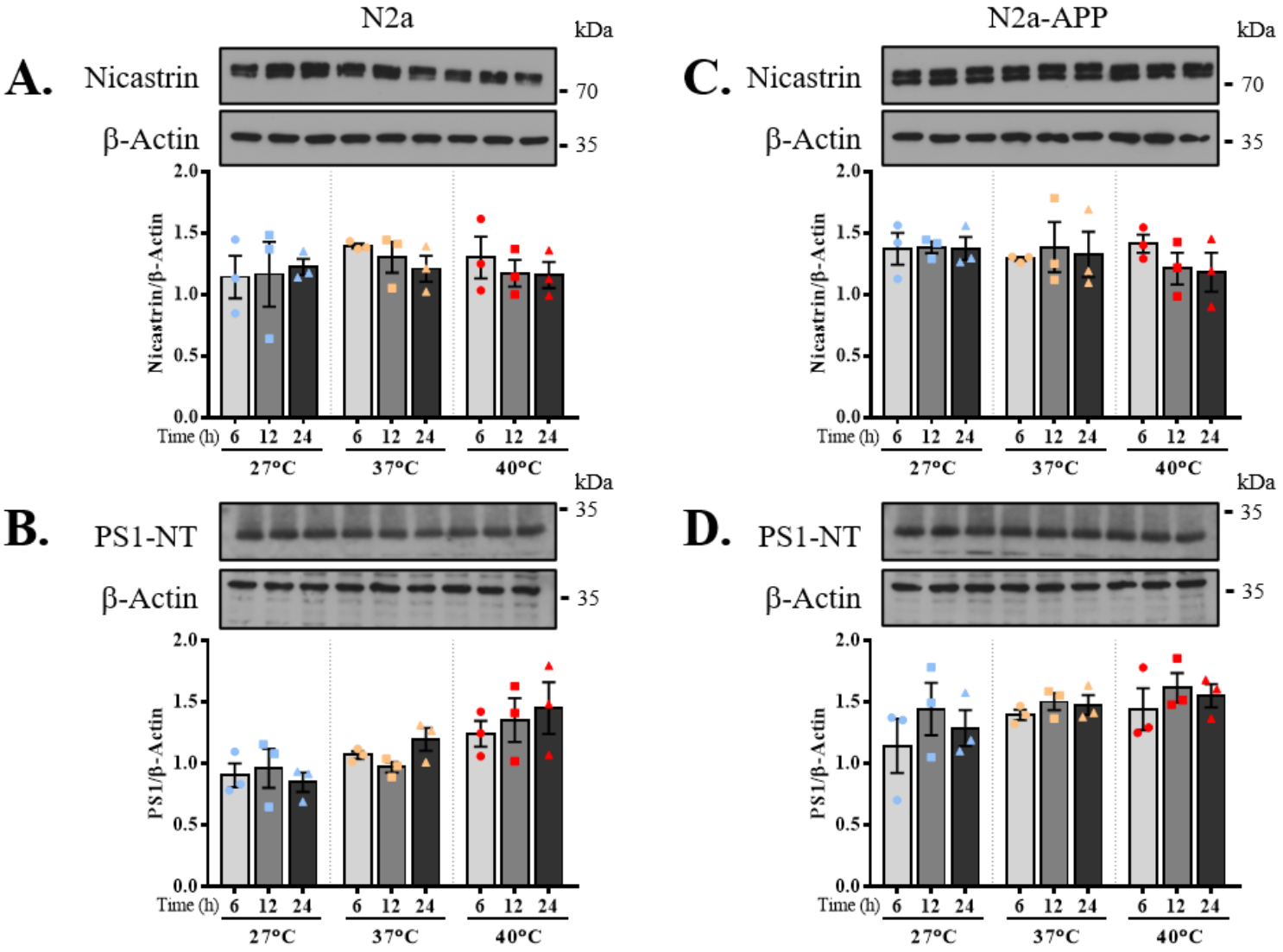

Figure 3.5: Western blots and corresponding histograms of N2a **(Fig. 3.5A, 3.5B)** and N2a-APP **(Fig. 3.5C, 3.5D)** cells grown at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**.

Figure 3.6: ADAM10 and BACE1 secretase enzyme activities at different ambient temperatures

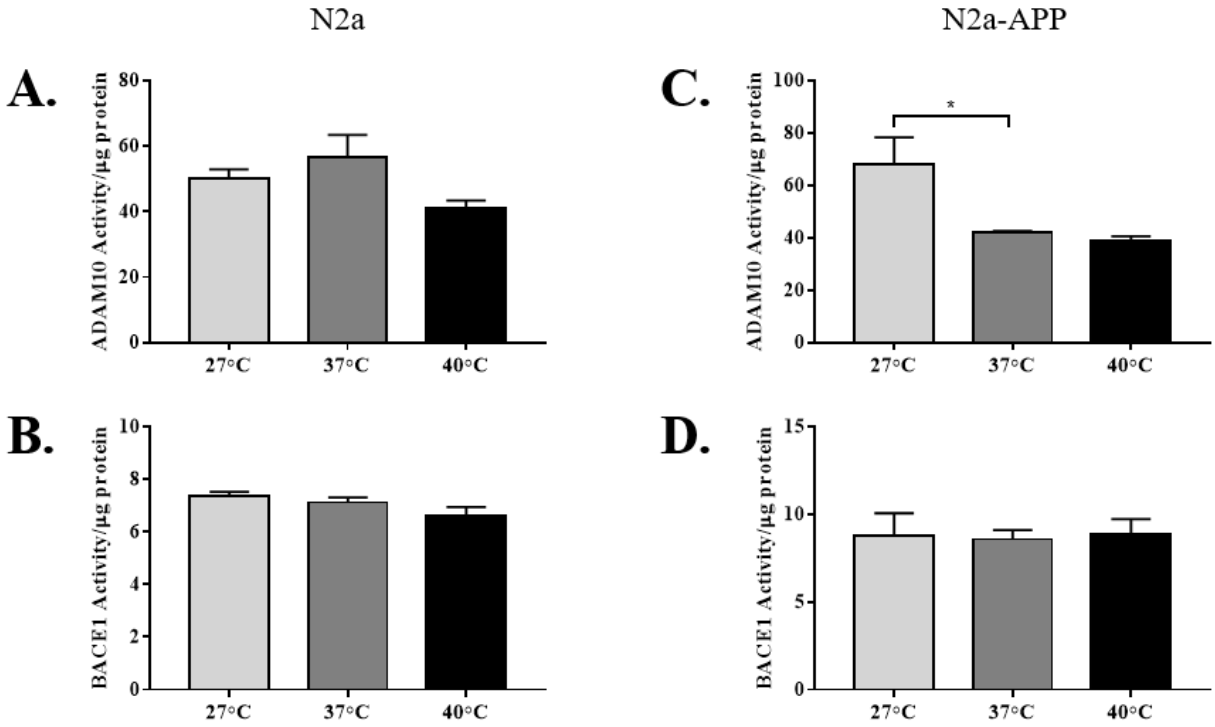

Figure 3.6: N2a **(Fig. 3.6A, 3.6B)** and N2a-APP **(Fig. 3.6C, 3.6D)** were incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 24 hours. Protein was extracted and cells were analyzed for ADAM10 **(Fig. 3.6A, 3.6C)** and BACE1 **(Fig. 3.6B, 3.6D)** activity by their respective kits. *: $p < 0.05$

Figure 3.7: Cellular and secreted Aβ in N2a cells at different ambient temperatures

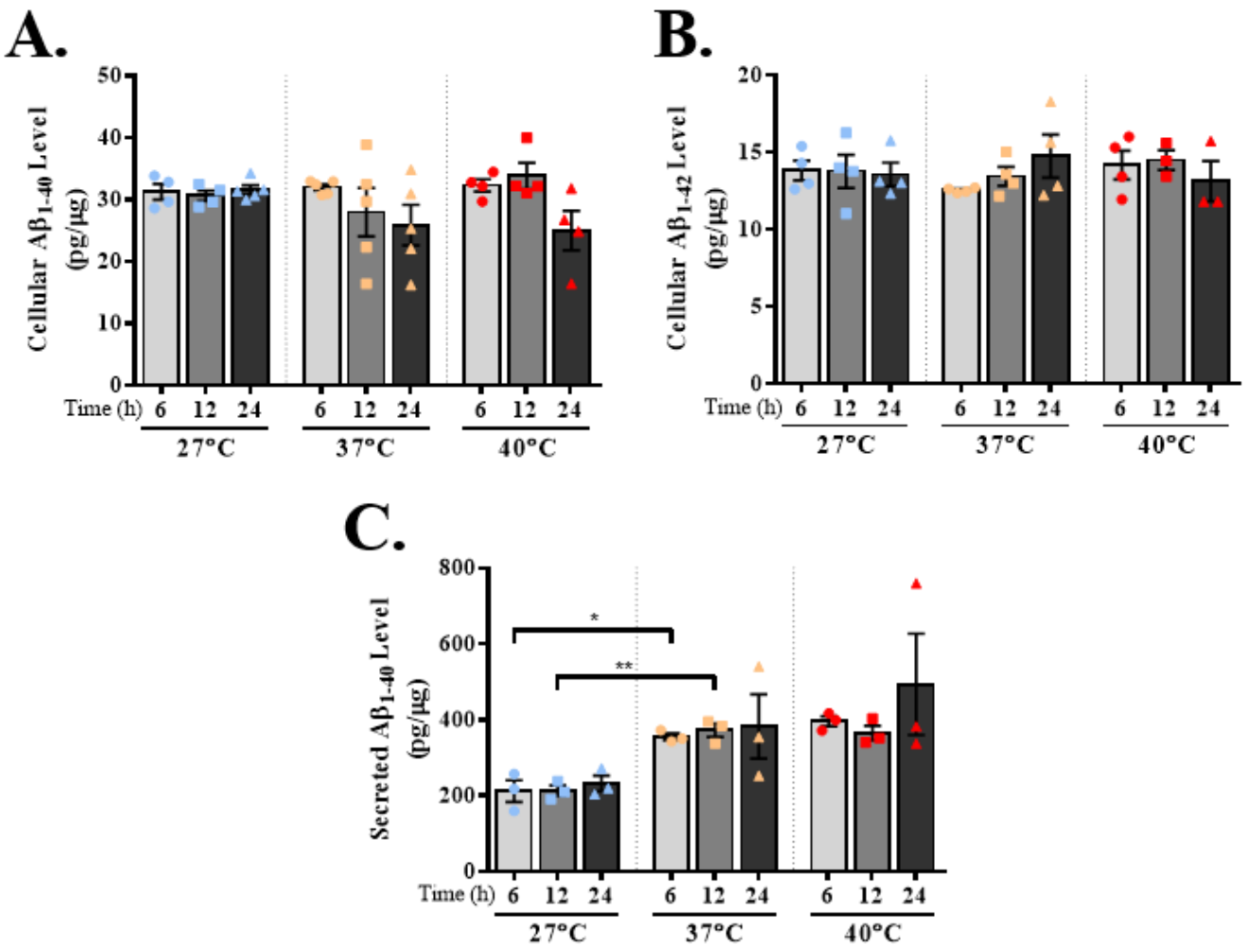

Figure 3.7: N2a **(Fig. 3.7A-C)** cells were grown to approximately 70% confluency at normothermic temperature (37°C) before incubation at hypothermic (27°C), normothermic, or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Cells **(Fig. 3.7A, 3.7B)** and media **(Fig. 3.7C)** were collected and analyzed for $A\beta_{1\text{-}40/42}$ by ELISA kits. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3.8: Cellular and secreted Aβ in N2a-APP cells at different ambient temperatures

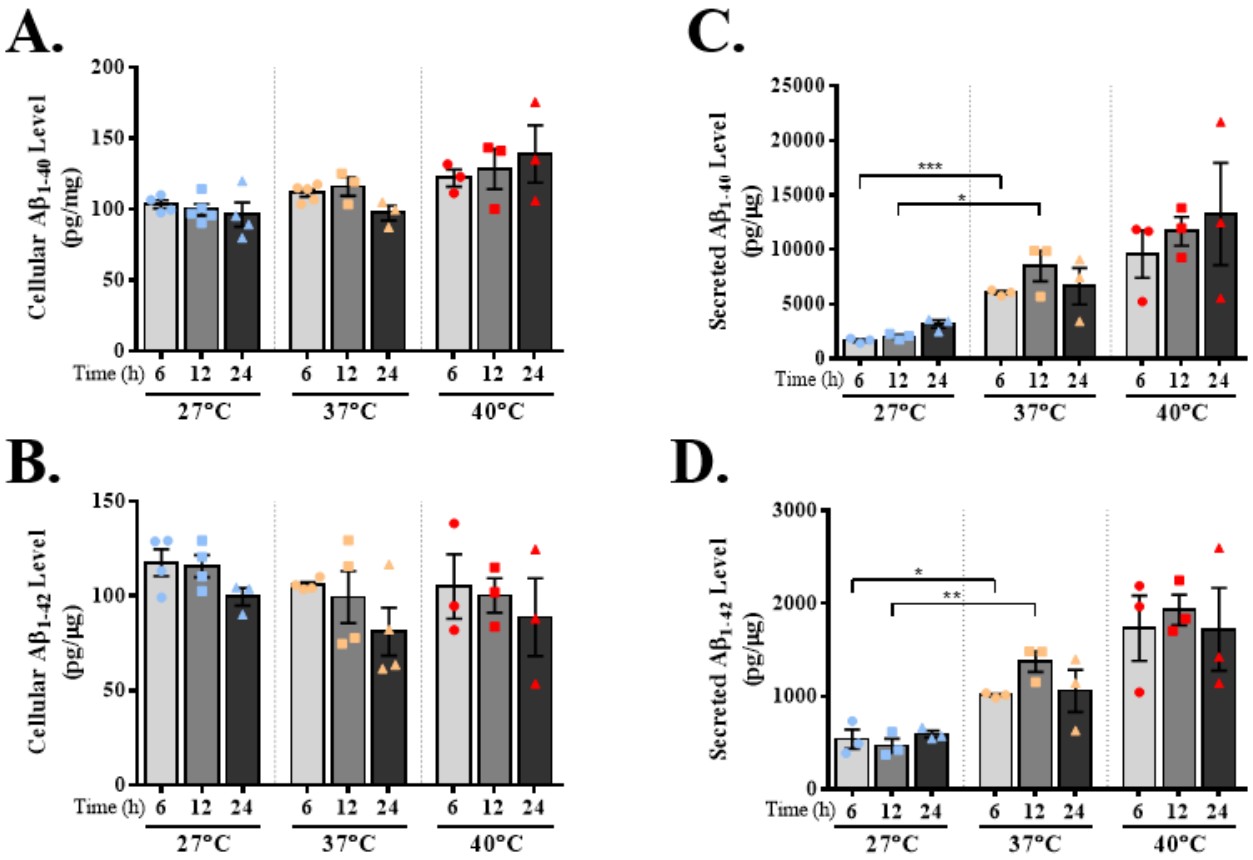

Figure 3.8: N2a-APP **(Fig. 3.8A-D)** cells were grown to approximately 70% confluency at normothermic temperature (37°C) before incubation at hypothermic (27°C), normothermic, or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Cells **(Fig. 3.8A, 3.8B)** and media **(Fig. 3.8C, 3.8D)** were collected and analyzed for $A\beta_{1\text{-}40/42}$ by ELISA kits. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3.9: Cycloheximide pulse chase assay of N2a-APP cells at different ambient temperatures

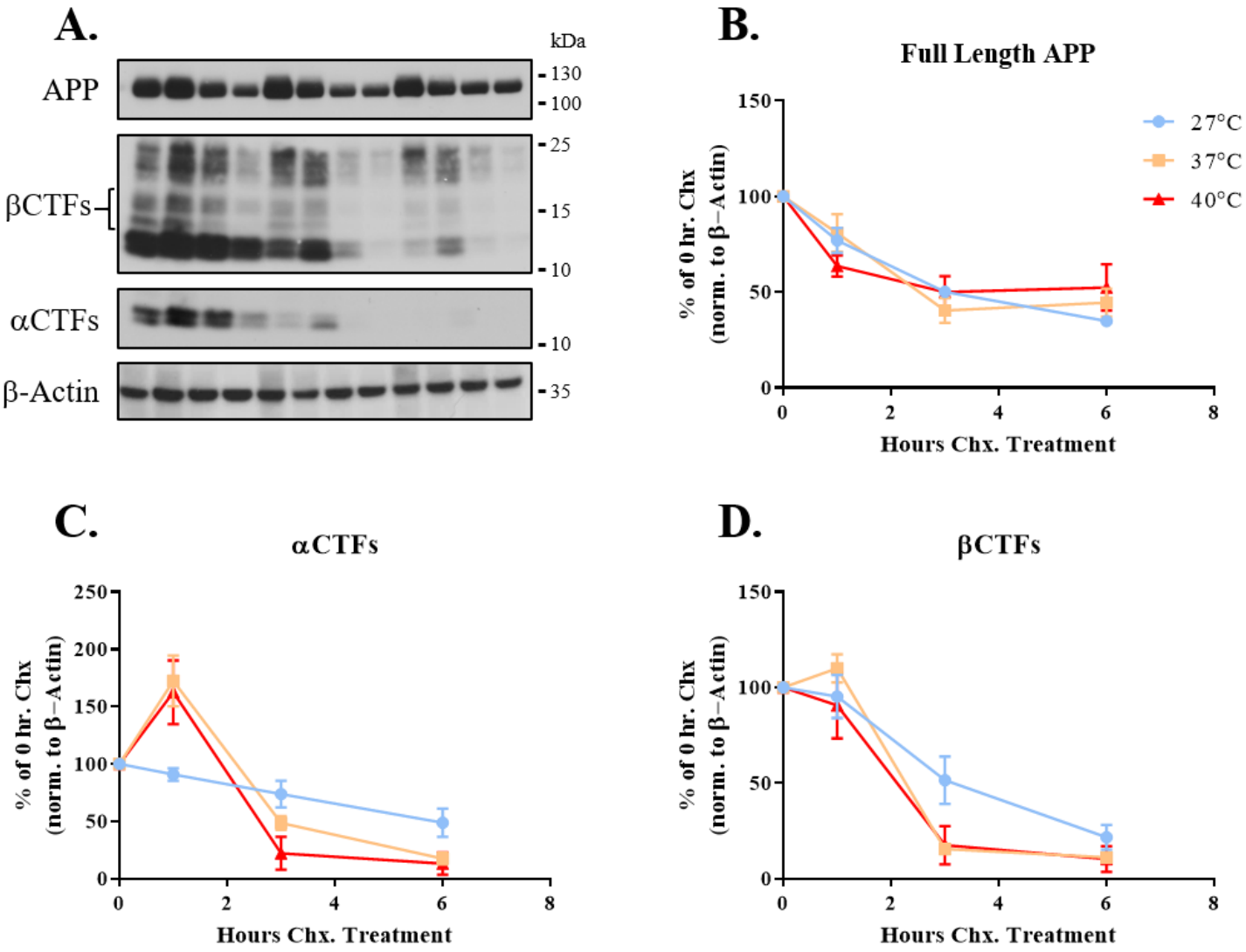

Figure 3.9: Western blot and corresponding histograms of N2a-APP cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 24 hours. For the final ½, 3, or 6 hours of incubation, cells were exposed to 30µg/mL cycloheximide before being collected for western blot. Representative immunoblots are shown in **Fig. 3.9A**, and quantifications **(Fig. 3.9B-D)** are the result of 3 independent experiments. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**.

Figure 3.10: Endolysosomal constituents at different ambient temperatures

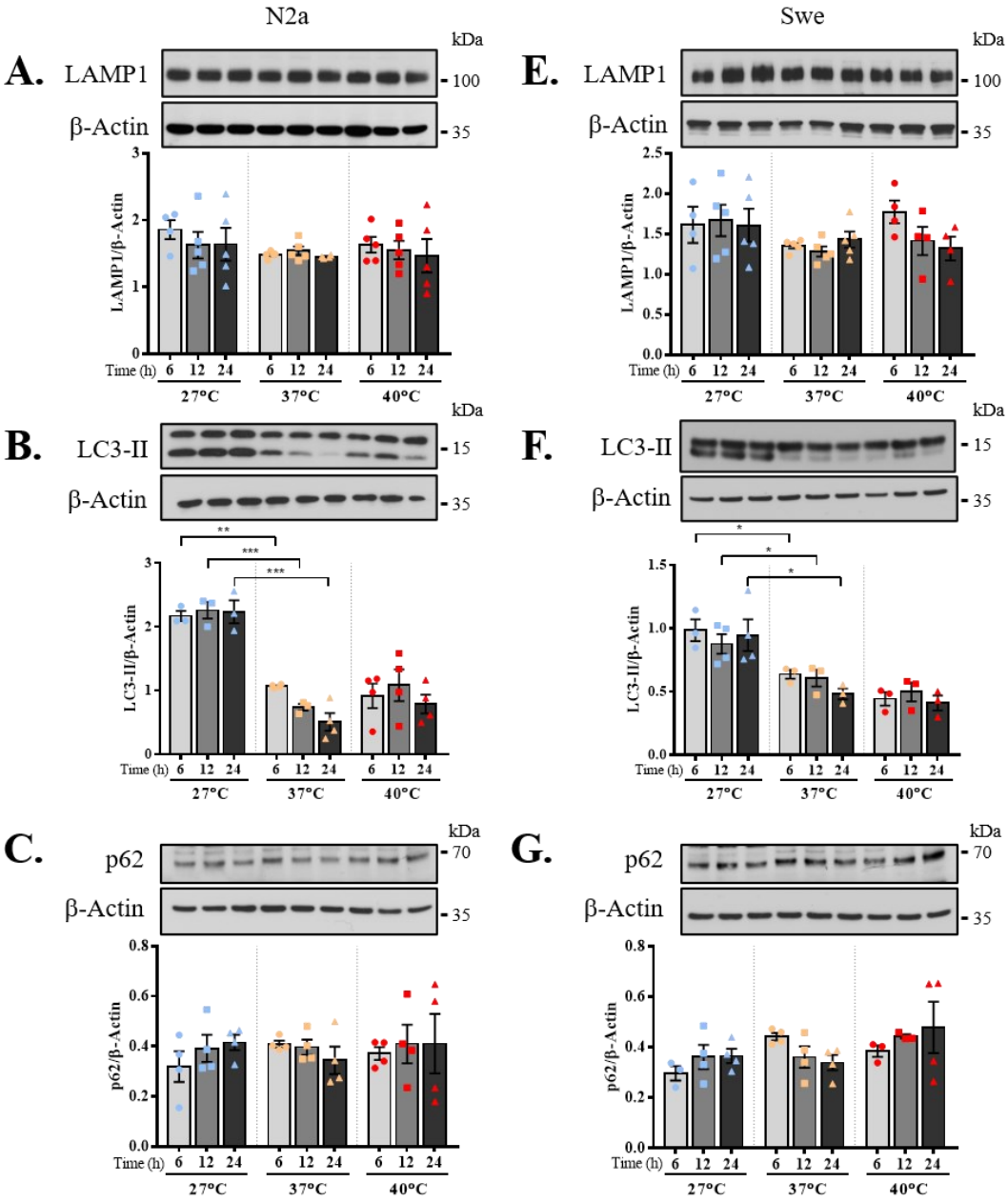

Figure 3.10: N2a **(Fig. 3.10A-C)** and N2a-APP **(Fig. 3.10D-G)** cells were grown to approximately 70% confluency at normothermic temperature (37°C) before incubation at hypothermic (27°C), normothermic, or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Each data point shown represents an independent experimental replicate. Blots were re-probed with β-actin to monitor protein loading. Antibodies and dilutions for each western blot are listed in **Table 1**. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3.11: Localization of APP and endolysosomal constituents in N2a-APP cells at different ambient temperatures

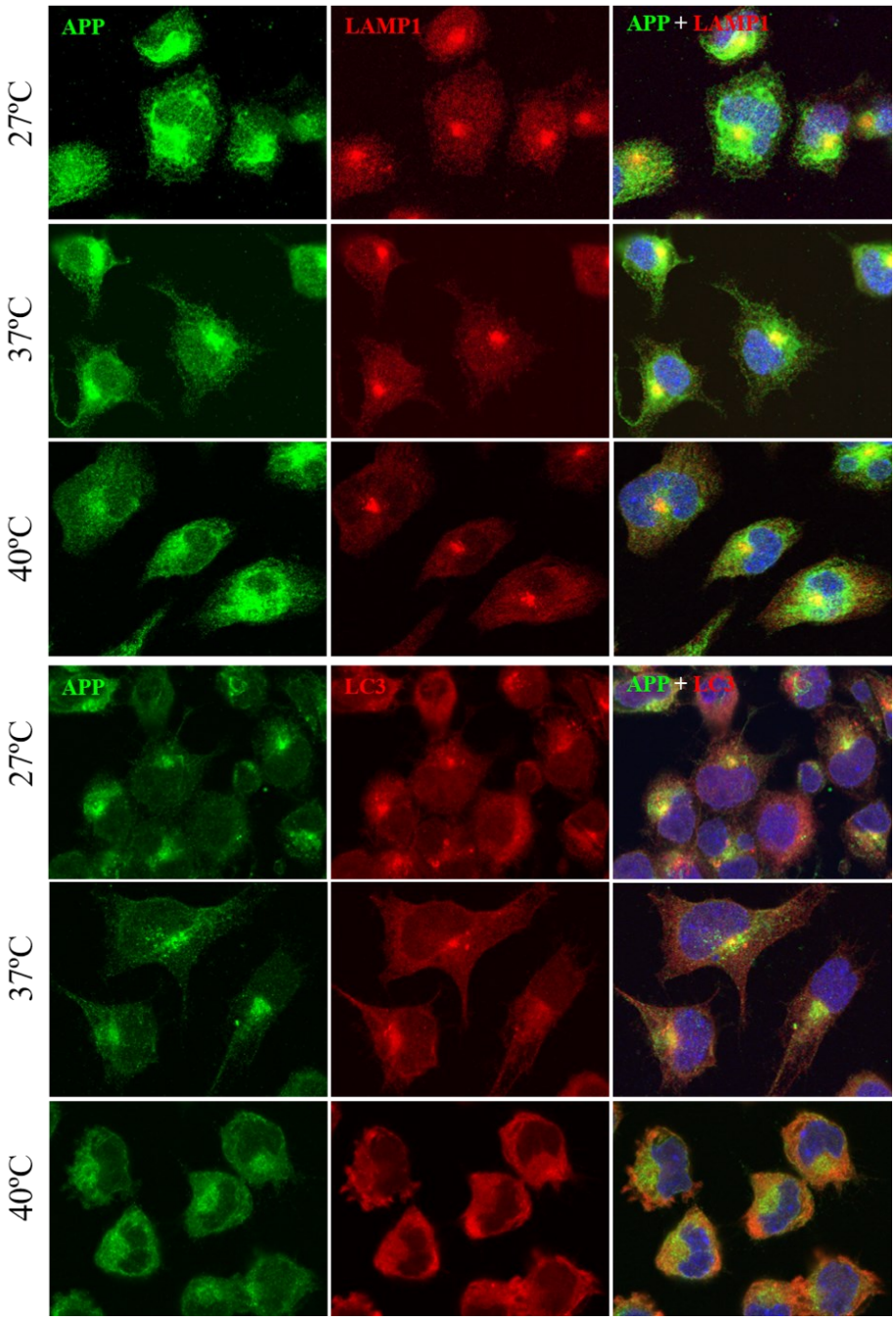

Figure 3.11: Representative confocal microscopy images depicting the subcellular localization of APP with LAMP1 and LC3 in N2a-APP cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures. Antibodies and dilutions are listed in **Table 1**.

Figure 3.12: Localization of Aβ and endolysosomal constituents in N2a-APP cells at different ambeint temperatures

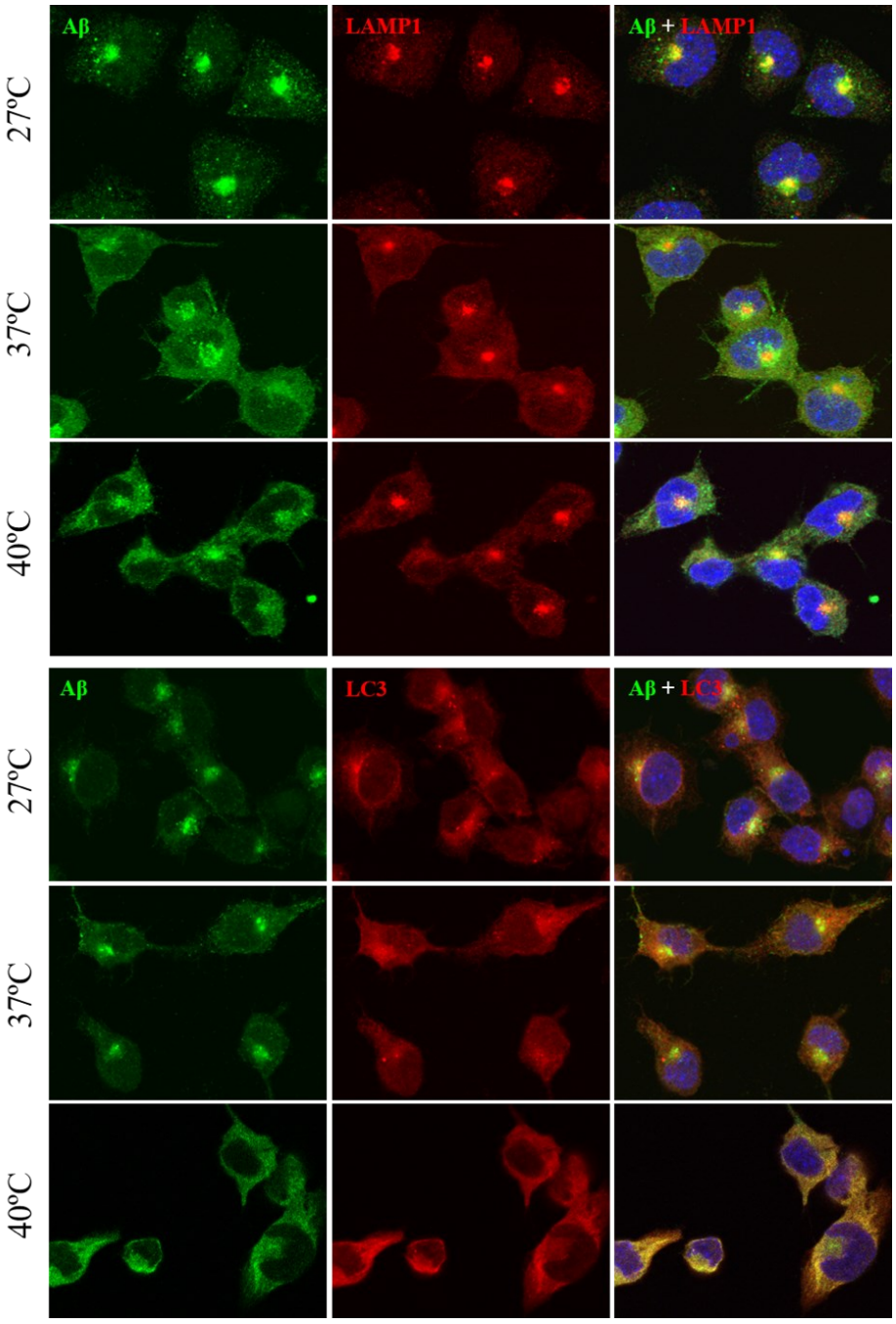

Figure 3.12: Representative confocal microscopy images depicting the subcellular localization of Aβ with LAMP1 and LC3 in N2a-APP cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures. Antibodies and dilutions are listed in **Table 1**.

Figure 3.13: Localization of APP and endolysosomal constituents in N2a cells at different ambient temperatures

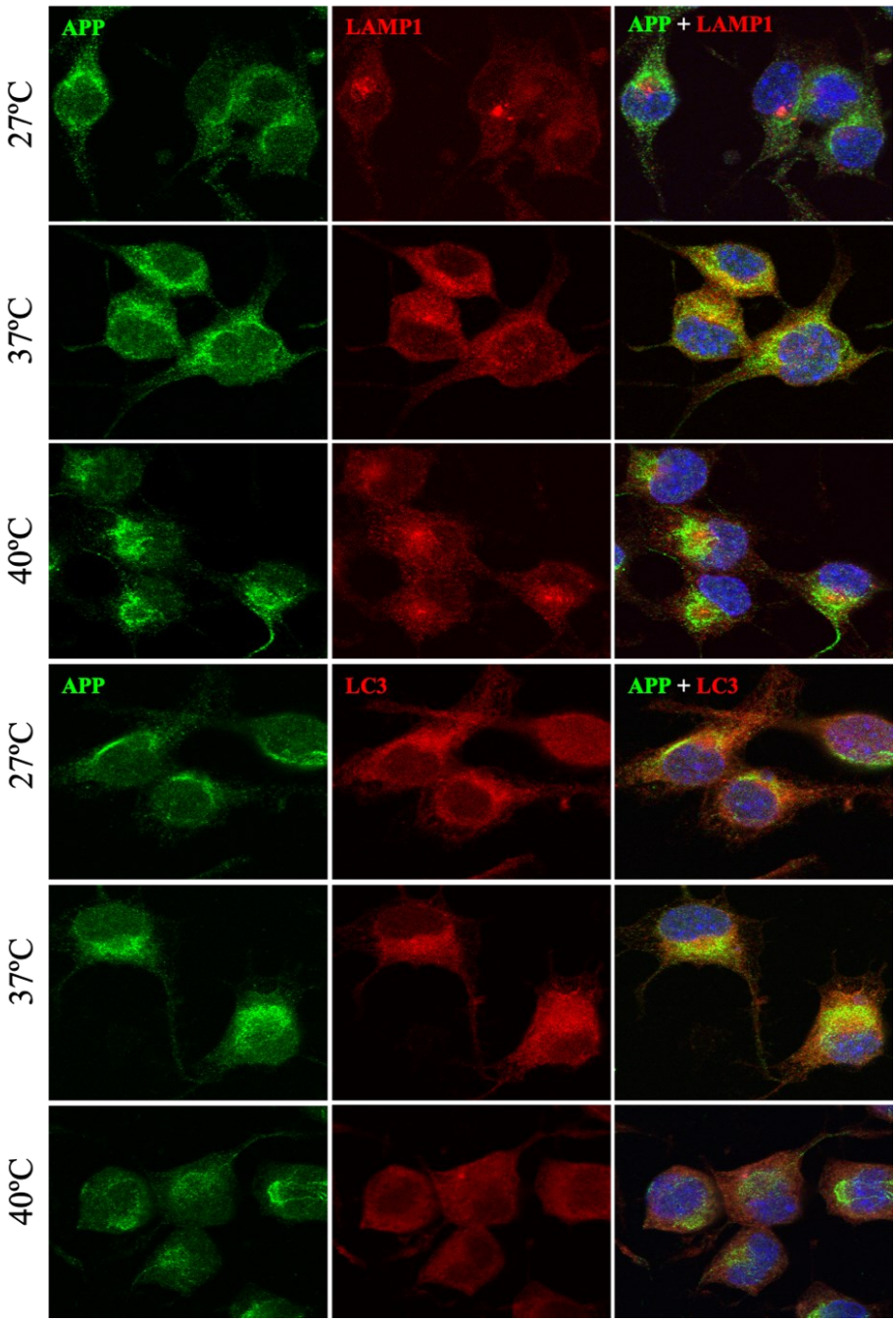

Figure 3.13: Representative confocal microscopy images depicting the subcellular localization of APP with LAMP1 and LC3 in N2a cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures. Antibodies and dilutions are listed in **Table 1**.

Figure 3.14: Localization of Aβ and endolysosomal constituents in N2a cells at different ambient temperatures

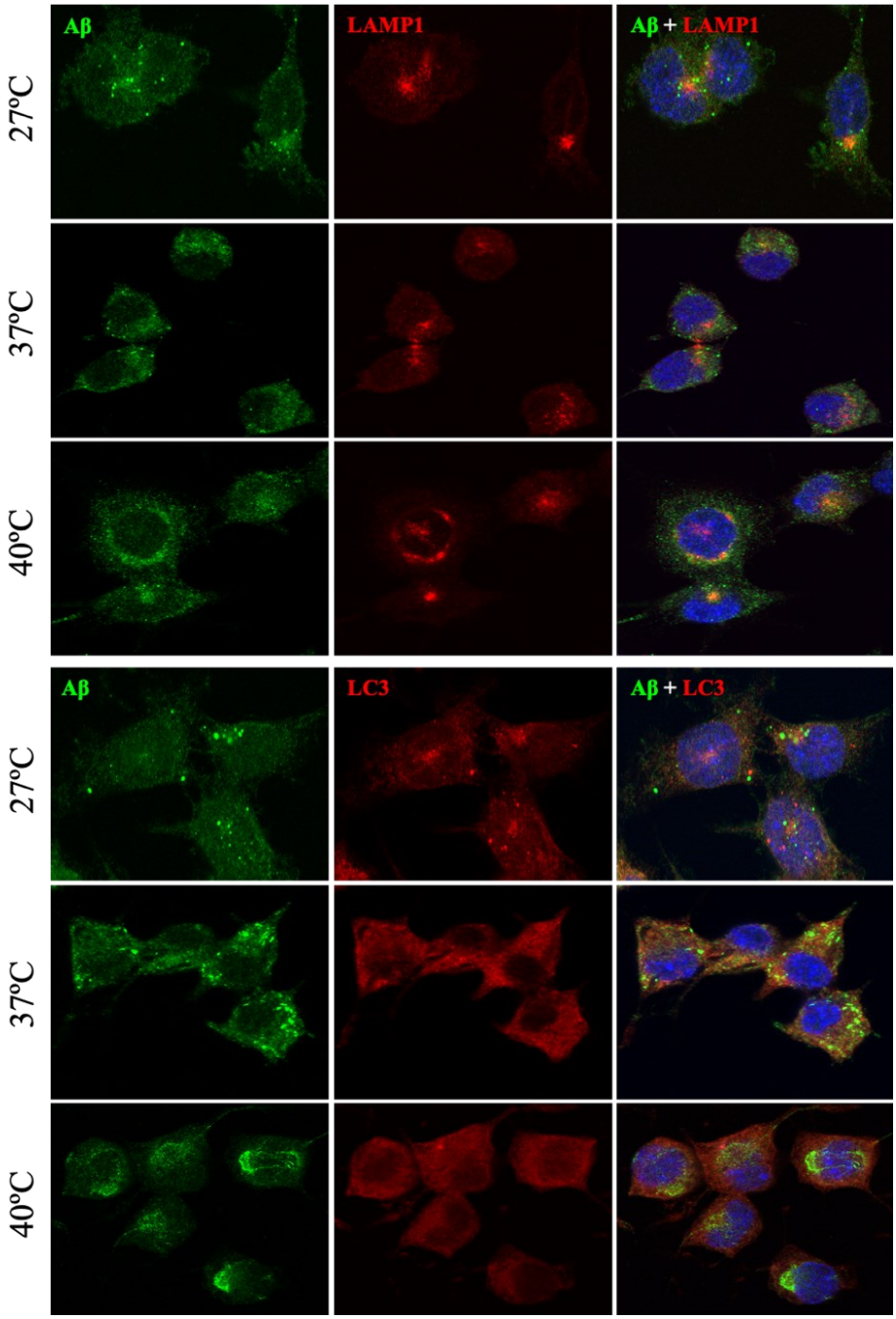

Figure 3.14: Representative confocal microscopy images depicting the subcellular localization of Aβ with LAMP1 and LC3 in N2a-APP cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures. Antibodies and dilutions are listed in **Table 1**.

Figure 3.15: Tau phosphorylation at different ambient temperatures

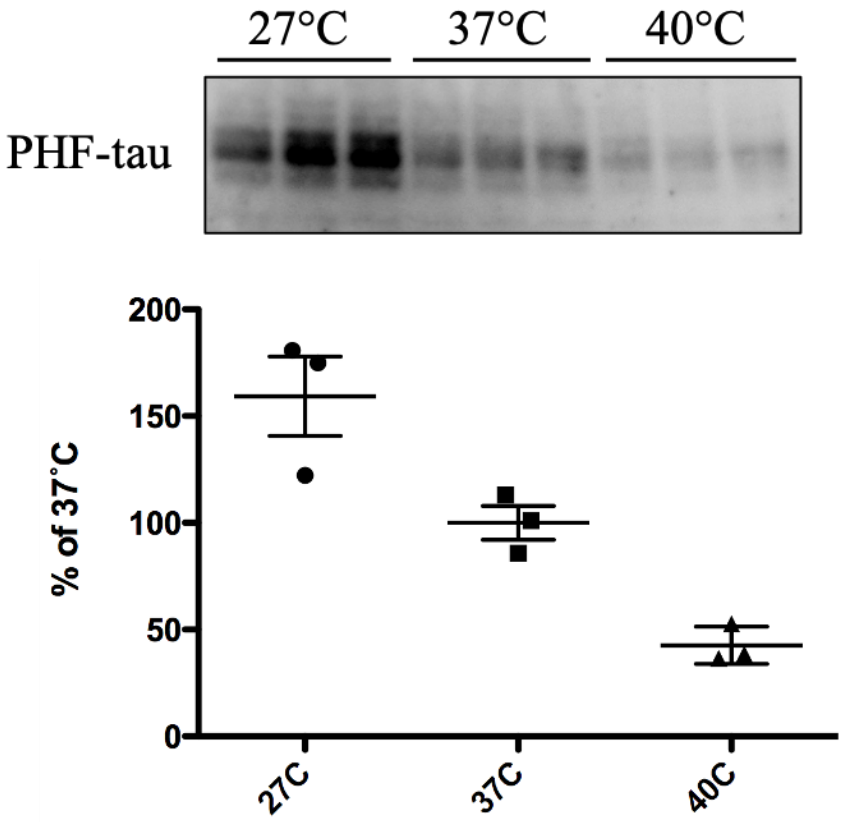

Figure 3.15: Western blot and corresponding histogram of N2a-APP cells incubated at hypothermic (27°C), normothermic (37°C), or hyperthermic (40°C) temperatures for 6, 12, or 24 hours. Data provided by Dr. Emmanuel Planel from the Université Laval.

4. DISCUSSION

4.1 Summary of results

Our results obtained so far indicate that exposure of cultured N2a and N2a-APP cells to 27°C instigates an accumulation of α/β-CTFs without any alteration in APP holoprotein levels when compared to both 37°C and 40°C. This is likely due to a decrease in CTF clearance mechanisms, as we see a decreased rate of degradation of both α/β-CTFs in a cycloheximide pulse chase assay. We also observe a decrease in the secretion of $A\beta_{1-40}$ and $A\beta_{1-42}$ without any change in the cellular content of these Aβ peptides. These alterations in APP metabolism are not due to any temperature related change in the expression of α-, β- or γ-secretase enzymes ADAM10, BACE1, PS1 or nicastrin, or in the activity of ADAM10 or BACE1, with the exception of an increase in ADAM10 activity in N2a-APP cells exposed to 27°C for 24 hours. Immunocytochemistry also shows that at low temperature there is a relative increase in the colocalization between Aβ and the lysosomal markers LAMP1 and LC3 without a change in the localization of full-length APP.

4.2 A cell culture model of hypothermia

Studying the response of neuron-like cells to hypothermic insults in a cell culture model, while difficult to extrapolate to AD pathogenesis, provides experimental flexibility to assess the potential mechanisms underlying the hypothermic response. Our choice of N2a and N2a-APP cell lines was made to have an approximation of sporadic (N2a – no mutations) and familial (N2a-APP – Swedish mutation in APP) AD phenotypes at the cellular level. While there are alternatives that may be more biologically relevant, such as primary mouse cortical neurons, induced pluripotent stem cells (iPSCs), or mutant APP-transgenic mice, these cells were easy to use and understand basic mechanisms by which temperature can influence APP metabolism. Before we conducted any experiments, we believed it would be prudent to utilize the N2a-APP cells in case we could not detect any Aβ in the N2a cells. This is because BACE1 has a greater affinity for the Swedish mutant APP than wild type resulting in greater Aβ production, so it was likely for us to detect an appreciable amount in the N2a-APP cells (Citron et al., 1994; Li et al., 2015). We believe this N2a and N2a-APP cell culture model to be appropriate for our investigations for the aforementioned

reasons, but we performed an additional validation by measuring total phosphorylated tau in our N2a-APP cells with the help of Dr. Emmanuel Planel from the Université Laval. The data produced aligns with the phenomenon established in the literature as described, with hypothermic temperature (27°C) inducing the hyperphosphorylation of tau **(Figure 15)**. This collaboration is ongoing, and we hope to measure more tau phosphorylation sites with specific antibodies and to determine whether turnover of tau is affected by temperature.

Temperature will have an influence on cell growth in culture, as the normothermic environment of 37°C is established as the most optimal condition for cell growth. Our data shows that decreasing the temperature to 27°C decreases the viability of both our N2a and N2a-APP cells. It is important to note that both the MTT and cytotoxicity kit do not measure cell death *per se*, rather mitochondrial integrity and cellular metabolism, which are indirect measures of cell viability. Since a low temperature may decrease cell proliferation, and skew our results, we supplemented these data by completing an LDH assay which corroborated our findings with a more relevant measure of cell death. These observations give us some confidence in concluding that the hypothermic environment has a negative effect on cell viability, rather than just a general effect of decreasing metabolism.

Others have utilized similar paradigms to study the effect of temperature on tau phosphorylation, exposing metabolically active mouse brain slices (normally maintained at 37°C) to various environmental temperatures from 30°C to room temperature (23°C) and down to 1°C (Planel et al., 2004). Additionally, hypothermia has been utilized as a platform to study tau kinase inhibitors (e.g. LiCl and roscovitine) in both metabolically active mouse brain slices and SH-SY5Y cells, although this group only reduced the ambient temperature to 30°C (Bretteville et al., 2012). Another group studied how hypothermia inhibits the co-chaperone BAG2 (B-cell lymphoma-associated athanogene 2) ability to mediate ubiquitin-independent phospho-tau degradation by exposing SH-SY5Y cells to slightly hypothermic temperature (34°C) (de Paula et al., 2016). From surveying established *in vitro* protocols, we believe that our choice to utilize 27°C as a hypothermic temperature may be acceptable, as we want our findings to have the potential to be placed in the context of the established relationship between hypothermia and tau phosphorylation.

4.3 Significance of hypothermia in AD pathology

For our findings to be applied to human disease, it is important to reconcile our observed effects of hypothermia in our cell culture model with established clinical practices of acute hypothermia. For example, hypothermia has been shown to reduce primary and secondary injuries resulting from ischemic stroke. This is interesting, since the premise of this treatment is hypothermic temperatures preserve neuronal viability, a concept that has been prevalent in stroke research for many years (Busto et al., 1987). It is believed that low temperatures prevent many of the causes for neuronal death in stroke, such as excitotoxicity, apoptosis, inflammation and generation of free radicals (Kurisu and Yenari, 2018; Yenari and Soo Han, 2012). Hypothermia has also been utilized to minimize neuronal damage caused by cardiac arrest and epilepsy (Motamedi et al., 2013; Silverman and Scirica, 2016). However, hypothermia has also been shown to be ineffective at preventing neuronal damage in the case of non-penetrating severe brain injury (Clifton et al., 2011). While there are different results with each clinical context, these practices provide ample reason for caution in the interpretation of our results. Distinctions between the physiological context and pathogenesis of each condition leaves the potential for the drastically different conclusion that we interpret from our data: that hypothermia enhances pathogenic markers of AD.

To reiterate a fundamental principal in this field of hypothermia and AD, it is merely a risk factor, rather than a causative agent (Almeida and Carrettiero, 2018; Carrettiero et al., 2015; Holtzman and Simon, 2000; Whittington et al., 2010). In this respect, it combines with other factors to increase or decrease the likelihood of developing the disease. With the wide variety of risk factors which can potentially exert an influence, and the myriad of ways in which they can interact with one another, it is unsurprising that this is a complicated issue (Bartolotti and Lazarov, 2016). Perhaps the most relevant distinction is that the role of hypothermia in AD is more longitudinal in its effect, rather than an acute instance. In the case of each stroke, cardiac arrest and epilepsy, regardless of global surface cooling or targeted endovascular infusions as the chosen method, the cooling is transient and temporary (Song and Lyden, 2012). For example, some recent clinical studies show that a hypothermic intervention soon after stroke (from 6 to 12 hours) for approximately 24 hours show a decreased diffusion weighted imaging (DWI) lesion size with minimal induction of side effects like pneumonia (De Georgia et al., 2004; Hemmen et al., 2010).

It is more likely that hypothermia exerts its effects on AD pathogenesis over its protracted disease course, with the effects of a reduced body temperature compounding over time, especially as mean body temperature declines further with advanced age (Gomolin et al., 2005).

This can potentially be extended to the observations made in Finland, with men who are regular sauna users (Heinonen and Laukkanen, 2018; Laukkanen et al., 2017). The authors describe sauna as integral part of Finnish culture, where individuals are typically lifetime users and begin from a young age. Temperatures in the sauna range from 80-100°C and is semi humid. This is different than the typical dry sauna, which usually is not as warm or as humid. Finnish sauna bathing is also unique in that it is often combined with short exposures to very cold water, in a circuit where the individual repeatedly exposes their body to very hot and very cold conditions. Their observations draw correlations between both the frequency of sauna use and the duration of each session with improved health outcomes, inclusive of decreased rates of cardiovascular and all-cause mortality, incidence of hypertension, respiratory diseases, and AD and other dementias (Heinonen and Laukkanen, 2018). Further research into how body temperature can influence disease progression may become more relevant in the future, as a recently observed trend of a whole-population decrease in body temperature by 0.03°C per birth decade has been established from studying populations dating back to the 1860s (Protsiv et al., 2020). By framing the difference between established conventions in the fields of stroke, cardiac arrest and epilepsy and findings regarding AD with knowledge about the type of thermal affect being introduced, it is possible to reconcile our data with population-based observations about elderly thermosensitivity and the therapeutic benefit of long-term sauna usage.

4.4 Influence of hypothermia on APP metabolism

Our major findings from the cell culture model reveal that there is a decreased clearance of α/β-CTF peptides at hypothermic temperatures, causing a buildup of these APP cleavage products. The elevated levels of α/β-CTFs in cells exposed to 27°C may be due to a temperature-dependent inhibition of clearance mechanisms of these peptides. This is supported by our cycloheximide pulse chase assay, where we treated N2a-APP cells with 30µg/mL cycloheximide for the last ½, 3, or 6 hours of the total 24-hour temperature exposure. As seen in the representative western blot

and in the corresponding histograms, there is a preservation of both α/β-CTFs in cells exposed to hypothermic temperature, as most evident at 3 hours cycloheximide treatment. Interestingly, the CTFs each appear to increase at ½ hour cycloheximide treatment, which may be due to a time delay between when cycloheximide inhibits protein synthesis and when degradative pathways begin to remove protein. This pattern has been seen before in cycloheximide experiments conducted using N2a-APP cells (Chung et al., 2018; Maulik et al., 2015). Our experimental approach did not seek to determine which pathway in particular is inhibited by hypothermia, rather we were more focused on whether the increased CTF levels we see in N2a and N2a-APP cells at hypothermic temperature was due to an increased synthesis or decrease in clearance pathways in general.

The observed time-dependent increase in α/β-CTFs in N2a and N2a-APP cells may be related to the reduction in cellular viability at hypothermic temperatures. While Aβ is typically viewed as the key agent of neurotoxicity, as its effects of Ca^{++} disturbances, tau mislocalization and phosphorylation and eventual axon degeneration are well documented, our data show that low temperature increases CTF levels, rather than an effect on cellular Aβ levels (Zempel et al., 2010). In research presented by Lauritzen et al., the β-CTF is shown to aggregate within the membranes of EL vesicles both *in vitro* and *in vivo*. They go on to describe how this results in dysfunction of lysosomal proteolysis and autophagy, causing cellular toxicity by apoptosis (Lauritzen et al., 2016, 2012). APP metabolites have also been shown to act as transcription factors, inducing the expression of genes such as GSK3β (Kim et al., 2003). This is interesting as a potential connection between our data and established knowledge on tau hyperphosphorylation at low temperatures (Carrettiero et al., 2015). However, more research is necessary to determine whether CTFs are causing toxicity, or whether the effect on transcription is induced at hypothermic temperatures.

Another interesting trend we note in both N2a and N2a-APP cells is that at normothermic and hyperthermic temperatures we see a time-dependent decrease in both α/β-CTF without any alteration in full-length APP holoprotein levels. This phenomenon can be rationalized with our other data as we have implicated cellular clearance mechanisms, rather than synthesis pathways, in the observed changes in APP metabolism. With a constant rate of APP production, we would not see any alteration in its levels, however, since clearance appears to be inhibited at low

temperatures, it makes sense to see a change in the APP cleavage products. The effect of hypothermia on α/β-CTF levels has not previously been described in the literature, so this result is novel in its potential implication in AD pathology.

We also note a decrease in the secretion of Aβ peptides at hypothermic temperature. This was illustrated by $A\beta_{1-40}$ in N2a cells and $A\beta_{1-40}$ and $A\beta_{1-42}$ in N2a-APP cells. As described earlier in the introduction, most of the β-cleavage of APP occurs within the EL system and sequentially, most Aβ generation also occurs within these compartments. Since there does not appear to be any alteration in the cellular content of $A\beta_{1-40/42}$ in N2a or N2a-APP cells, it is likely the process of secretion that is inhibited, rather than the production of Aβ peptides themselves. Our data showing constant levels of secretase enzymes also supports this, as a change in enzyme level may indicate a change in the rate of APP processing. However, it is known that the quantity of enzyme does not necessarily correlate with activity (Thinakaran et al., 1997). Our enzyme activity assays so far show no change in activity in BACE1 in either N2a or N2a-APP cells, however we observe an increase in activity of ADAM10 in cells exposed to hypothermic temperature, but only in N2a-APP and not N2a cells. It is unlikely for this increase in activity to be solely responsible for the elevated levels of α-CTFs in N2a-APP cells, as we see similar changes in β-CTF levels with no alteration in BACE1 activity. Also, since there is no change in ADAM10 activity in N2a cells, it is not likely to be the sole mechanism behind increased α-CTF levels. It is important to recognize the limitations of the methodological choices that we made while assessing α/β-secretase enzyme activity. Cells were incubated at 27°C, 37°C, or 40°C for 24 hours, before protein was extracted and the activity assay was performed at 37°C. We chose to perform all the assays at the same normothermic temperature (37°C), rather than at 27°C and 40°C to best streamline our experimental workflow and minimize confounds introduced by completing both the incubation and the assay at different temperatures. An alternative solution would be to perform both methods, this would allow us to more accurately determine whether the change in activity was due to the assay being performed at different temperatures, or due to the incubation at different temperatures.

Apart from APP processing by secretase enzymes, the effect of low temperature on exocytosis in general has been studied in the past, which can also affect cellular and secreted Aβ levels.

Independent studies utilizing rabbit alveolar macrophages and mouse hair cells have shown that there is a linear relationship between temperature and rate of exocytosis in general (Nouvian, 2007; Tomoda et al., 1989). So, a potential explanation for the decreased secretion of Aβ may be a general inhibition of exocytosis in our N2a and N2a-APP cells at hypothermic temperatures. Interestingly, previous *in vitro* studies utilizing cold water induced hypothermia have found that hypothermic temperatures induce an increase in Aβ plaque formation. The authors also show an increase in tau phosphorylation as well as an induction of apoptotic pathways *via* Bax and cleaved-caspase 3 (Ahmadian-Attari et al., 2015). We can reconcile this with our data along the reduction in cellular viability of N2a and N2a-APP cells that we observe at a hypothermic temperature. However, as we see a decrease in $A\beta_{1\text{-}40/42}$ secretion, more experiments would be needed to discover how our data may be applied in this context. This discrepancy can potentially be explained by the fact that Ahmadian-Attari et al. measured number of plaques, rather than Aβ generation and secretion specifically.

In this vein, we have also gathered data that describes an approximately two-fold increase in levels of LC3-II in both our N2a and N2a-APP cells exposed to hypothermic temperature, with no corresponding change in p62 levels. The elevated LC3-II levels may be related to enhanced α/β-CTFs, as LC3-II plays an important role in targeting the CTFs from endosomes to autophagosomes through an interaction with an adaptor protein (AP) 2 and phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia (PICALM) complex (Tian et al., 2013). Autophagy has been shown to be involved in the synthesis, degradation and secretion of Aβ peptides, so the temperature-dependent effects that we observe may be due to a dysfunction of this system (Nilsson et al., 2013; Nilsson and Saido, 2014). Our observations so far indicate that APP is localized to LAMP1 positive lysosomal and LC3 positive autophagosome compartments, as well as broadly distributed throughout N2a and N2a-APP cell bodies extending to the plasma membrane. Interestingly, Aβ also localizes readily to LAMP1 and LC3 positive puncta in both N2a and N2a-APP cells, a focal distribution that becomes more prominent in cells grown at 27°C relative to those grown at 37°C or 40°C. We may be seeing an increase in LC3-II positive autophagosomes, as we see a temperature-dependent increase in this protein in our western blot data as well. At this point, it is difficult to determine where the deficiency within the EL system lies, however, we can hypothesize

that there is a decreased turnover of autophagosomes, as we have a build-up of LC3-II with no turnover of p62, which is typically degraded as cargo is broken down and the autophagosome is recycled (Ichimura et al., 2008). Alternatively, increased levels of LC3-II can be due to an increased synthesis of autophagosomes. This buildup can be what is inhibiting the secretion of Aβ peptides, causing an inhibition that manifests as increased levels of α/β-CTFs. Much of this interpretation needs more experiments to tease out the molecular details, but at this point it is clear there are abnormalities within the EL system at hypothermic temperatures.

4.5 Future directions

This research begins to describe a how hypothermia effects amyloidogenic processes within a cell culture model of AD. So far, our experimental data describe:

I. A time-dependent inverse relationship between environmental temperature and α/β-CTF levels in both N2a and N2a-Swe cells, with no corresponding change in full length APP holoprotein level. We also describe an inverse relationship between the secretion of both $A\beta_{1\text{-}40}$ in N2a cells, and $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in N2a-APP cells with temperature, with no temperature-dependent change in the cellular content of $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ in either cell type. We also utilized a cycloheximide pulse chase assay which indicated that the accumulation of α/β-CTFs is likely due to a decreased clearance of the CTF peptides rather than an increase in their production.
II. That the difference in APP metabolism is not due to a change in protein quantity of any of the α-, β-, or γ-secretase enzymes assayed (ADAM10, BACE1, nicastrin, PS1) at each temperature condition. We also describe an increase in ADAM10 activity at 27°C in N2a-APP cells, while there was no corresponding change in N2a cells. The activity of BACE1 remained unaltered at different temperatures in both N2a and N2a-APP cells.
III. An increased punctate and focal immunolocalization between Aβ and the lysosomal markers LAMP1 and LC3 at hypothermic temperature that becomes dispersed at normothermic and even more diffuse at hyperthermic temperatures. However, there did not appear to be a major shift in the localization of APP over each temperature.

We can continue to pursue this research by evaluating:

I. Specific clearance mechanisms responsible for the turnover of α/β-CTFs.
II. Evaluating cellular and secretory levels of sAPPα/β.
III. Extending our study to more disease-relevant models.
IV. Assess transcriptional changes associated with different temperatures.
V. Using more protein markers or genetic or pharmacological methodologies to further assess mobilization and activity of the EL system.

It is our hope that further exploration into the mechanisms behind this observed temperature-dependent amyloidogenic response in our cultured cell system will yield a deeper understanding of the pathogenesis of AD, with potential to mitigate risk factors and contribute to alternative therapeutic strategies. This research begins to describe a potential rationale for perturbations of APP processing and amyloidogenesis at hypothermic temperatures, which is a valuable accompaniment to the established knowledge of tau phosphorylation under these conditions.

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