EFFECTS OF TEMPERATURE ON AMYLOID PRECURSOR PROTEIN (APP) METABOLISM IN CULTURED ASTROCYTES

By

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ABSTRACT

Alzheimer's disease (AD), the most common type of senile dementia, is characterized by the presence of β-amyloid (Aβ)-containing neuritic plaques and tau-positive neurofibrillary tangles and the loss of neurons in specified regions of the brain. Evidence suggests that increased levels/aggregation of Aβ peptides can contribute to neuronal loss and subsequent development of AD pathology. These Aβ peptides are generated from their precursor, amyloid precursor protein (APP), which is processed proteolytically by either the amyloidogenic β-secretase or the non-amyloidogenic α-secretase pathways. Some recent studies emphasize that ambient temperature may influence disease progression and/or pathology in animal models and AD patients. However, very little is known how the temperature can influence Aβ metabolism in different cell types in the brain. In the present study I used astrocytes, the most abundant glial cells in the brain, to evaluate how temperature can influence APP processing leading to the generation of Aβ-related peptides. My data show that viability of cultured astrocytes was significantly affected in hypothermic (27°C) compared to normal (37°C) and hyperthermic (40°C) conditions. Accompanying these changes, the levels of APP and its cleaved products are increased in the hyperthermic condition, whereas APP- processing enzymes are differentially altered in hypothermic, normal and hyperthermic conditions. The secretory levels of $A\beta_{1-40}$ are markedly increased but its degrading enzyme neprilysin is found to be decreased in the hyperthermic condition in a time-dependent manner. This study also indicates that markers of the autophagic lysosomal system, a major site of APP metabolism, are also significantly altered following exposure to different temperature conditions. These results, taken together, indicate that ambient temperature may influence AD pathology by regulating APP metabolism in astrocytes.

PREFACE

This thesis is an original work by Mr. Mohammad Murshedul Alam. The research described in the thesis was designed by me in collaboration with my supervisor, Dr. S. Kar and co-supervisor, Dr. G. Baker. I conducted the experiments, collated the results and analyzed the data. I wrote the thesis, with feedback from Drs. Kar and G. Baker. Two members of our laboratory, Drs. D. Vergote and G. Karthivashan, helped me in analysing some of the data included in the thesis. Some of the results from the experiments described in this thesis were presented by me at the 2019 Annual Meeting of the Society of Neuroscience in Chicago. The abstract (M. Alam *et al.,* Effects of temperature on APP processing in cultured astrocytes and its potential implication in Alzheimer's Disease pathology) was published in the meeting's e-book of abstracts (# 126.10).

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ABBREVIATIONS

Aa	Amino acid
Aβ	Amyloid-β peptide
AD	Alzheimer's disease
ADAM10	A Disintegrin and Metalloprotease 10
AMPA	alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APH1	Anterior pharynx-defective phenotype1
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
ATG5	Autophagy protein 5
BACE1	β-site APP cleaving enzyme1
BSA	Bovine serum albumen
CNS	Central nervous system
α/βCTF	C-terminal fragment-alpha/beta
DMEM	Dulbecco's modified Eagle medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbence assay
ER	Endoplasmic reticulum
GFAP	Glial fibrillary acidic protein

IDE	Insulin degrading enzyme
LC3	Microtubule-associated protein 1A/1B light-chain 3
MTT	3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide
NEP	Neprilysin
NFT	Neurofibrillary tangles
PBS	Phosphate-buffered saline
PEN2	Presenilin enhancer 2
PS1/PS2	Presenilin 1/Presenilin 2
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate

INTRODUCTION

1.1 Overview on Alzheimer's Disease (AD)

Alzheimer's disease (AD), the most common form of dementia of the elderly, is characterized by progressive loss of memory and cognitive impairment (Santos et al., 2016). AD affects more than 36 million people worldwide (WHO, 2013) and represents ~60-70% cases of all age-related dementia (Burns and Iliffe, 2009; WHO, 2015). In Canada, AD afflicts ~7% of the population over 65yrs of age and its prevalence doubles every 5yrs thereafter. Etiologically, most AD cases are sporadic, whereas only a minority (<10%) of cases segregate with mutations in three known genes: amyloid precursor protein (*APP*) gene on chromosome 21, presenilin 1 (*PSEN1*) gene on chromosome 14 and *PSEN2* gene on chromosome 1. Other factors that increase the risk of AD include age, inheritance of apolipoprotein E4 genotype (APOE4), head injury and stress (Holmes, 2002; St George-Hyslop and Petit, 2005; Selkoe, 2011; Bertram and Tanzi, 2012; Mayeux and Stern, 2012; Karch and Goate, 2015; Leduc et al., 2015).

The neuropathological features associated with AD include the presence of amyloid-β (Aβ) peptide-containing extracellular neuritic plaques, intracellular tau protein-positive neurofibrillary tangles (NFT) and loss of neurons in selected brain regions (Santos et al., 2016). Difficulty in remembering recent events is the most common early symptom (Burns and Iliffe, 2009), and with the progression of disease, symptoms advance to language problems, disorientation, mood swings, poor self-care, loss of motivation and behavioral issues (Burns and Iliffe, 2009; WHO, 2015). As the person's condition deteriorates, they often withdraw from the family and society (Burns and Iliffe, 2009). A probable diagnosis is based on the history of the illness and cognitive testing along with brain imaging and blood tests to rule out other possible causes (NICE, 2017). No treatments

are available to stop or reverse its progression, although some drugs such as cholinesterase inhibitors (i.e., Donepezil, Rivastigmine and Galantamine) and glutamatergic NMDA receptor antagonists (i.e., Memantine) may slow down the progression of dementia in a subset of AD patients (WHO, 2015). The affected people increasingly rely on others for assistance, often placing a burden on their network of caregivers; the pressure expands to include social, psychological, physical and economic elements (Thompson et al., 2007). Executive functions such as attentiveness, planning, flexibility and abstract thinking or impairments in semantic memory are also subsequently affected adversely in AD patients (Backman et al., 2004). Depressive symptoms, irritability and reduced awareness of subtle memory difficulties are also quite common (Murray and Price, 2012).

Formation of NFTs and extracellular neuritic plaques are considered hallmarks of AD pathology. NFTs are generated due to the hyperphosphorylation of the microtubule-associated protein tau which leads to the formation of paired helical filaments (PHFs) – an insoluble and aggregated form of the tau protein. In the normal physiological condition, the ability of tau to bind microtubules is regulated through the reversible processes of phosphorylation and dephosphorylation mediated by multiple kinases and phosphatases, respectively. Tau is expressed in abundance in the human central nervous system as six distinct isoforms (ranging from 352 to 441 amino acids) with each isoform being a product of alternative splicing of a single gene located on chromosome 17 (Goedert et al., 1989). NFTs are detected in abundance in human AD brains, mainly in the cortex, hippocampus, amygdala and certain subcortical regions (Dickson, 1997; Selkoe, 2001).

The extracellular neuritic plaques, unlike intracellular NFTs, contain a central deposit of Aβ-related peptides which are derived from APP, a large transmembrane protein encoded by a gene

located on human chromosome 21. For more than two decades in AD research the "Amyloid-cascade hypothesis" has been the central dogma, and states that accumulation and aggregation of Aβ peptides lead to alterations in synaptic plasticity, activation of microglia, oxidative stress and release of inflammatory mediators, culminating in neurodegeneration associated with AD pathology (Selkoe, 2008; Hardy, 2009). Although this hypothesis has positioned aggregated and insoluble Aβ as the central mediator of brain damage in AD, recent developments within the field indicate that the soluble oligomeric form of Aβ may be the dominant cause of toxicity (Hardy, 2009; Pimplikar et al., 2010; Larson and Lesne, 2012). It has been reported that these soluble oligomers and intermediate Aβ species are the most neurotoxic forms (Walsh and Selkoe, 2007), and that it is the level of oligomers, and not the total Aβ, which correlates with the severity of the cognitive symptoms in AD (Lue et al., 1999). It has been reported that the extracellular neuritic plaques containing Aβ peptides are evident predominantly in the entorhinal cortex, neocortex, hippocampus and certain subcortical regions of the brain in AD patients (Dickson, 1997; Selkoe, 2011). There is also substantial evidence that Aβ peptides and amyloid plaques, especially intracellular Aβ deposits, appear in the brain ahead of the manifestation of the NFTs and AD symptoms (Tanzi, 1996). These findings, taken together, suggest that Aβ peptides play a critical role in initiating/contributing directly to the loss of neurons and subsequent development of AD pathology.

1.2 Amyloid Precursor Protein (APP) metabolism and synthesis of Aβ

The Aβ peptides are a group of hydrophobic peptides containing 39-43 amino acid (aa) residues, but the two most prevalent isoforms found in the brain are $A\beta_{1-40}$ and $A\beta_{1-42}$. While $A\beta_{1-42}$ constitutes ~10% of the total Aβ secreted from cells, it aggregates faster and is more toxic to neurons than $A\beta_{1-40}$ (Koo et al., 1990; O'Brien and Wong, 2011; Haass et al., 2012; Penner et al.,

2013). Evidence suggests that either an over-production and/or lack of degradation may result in an increase of Aβ levels which, in turn, may contribute to neuronal loss and development of AD. Aβ peptides are generated from the APP, which is proteolytically cleaved either by the amyloidogenic β-secretase or by the non-amyloidogenic α-secretase pathways. The β-secretase pathway is mediated by β-site APP cleaving enzyme (BACE1) which cleaves APP to produce soluble N-terminal APPβ and an Aβ-containing C-terminal fragment (β-CTF) that can be processed further by γ-secretase to generate full-length $A\beta_{1-40}$/$A\beta_{1-42}$ peptides. The α-secretase pathway, on the other hand, is mediated by tumor necrosis factor-α converting enzyme (TACE/ADAM17), ADAM10 or ADAM9 which cleaves APP within the Aβ domain, yielding soluble APPα and a C-terminal fragment (α-CTF) which is subsequently processed by γ-secretase to generate $A\beta_{17-40}$/$A\beta_{17-42}$ fragments. Whereas the α-secretase processing occurs mostly at the plasma membrane and the late secretory pathway, the endosomal-lysosomal (EL) system plays a critical role in the production of Aβ-related peptides. The β-secretase BACE1 is a single transmembrane domain aspartyl protease which localizes predominantly in the late-Golgi/trans-Golgi network and endosomes, consistent with amyloidogenic processing of APP. Unlike β-secretases, γ-secretase is a tetrameric complex comprising the aspartyl protease presenilin 1 or 2 (PS1/PS2) and three cofactors, i.e., nicastrin, presenilin enhancer 2 (PEN2) and anterior pharynx defective 1 (APH1) (Thinakaran and Koo, 2008; Haass et al., 2012).

1.3 Familial AD

A small percentage of all AD cases (<10%) are the result of an autosomal dominant trait which exhibits early-onset Familial AD (FAD) manifested at <65 years of age (Blennow et al., 2006). At present, as mentioned earlier, mutations in three genes, i.e., *APP*, *PSEN1* and *PSEN2* have been recorded as the genetic causes behind early onset FAD (Selkoe, 2011; Bertram and Tanzi, 2012;

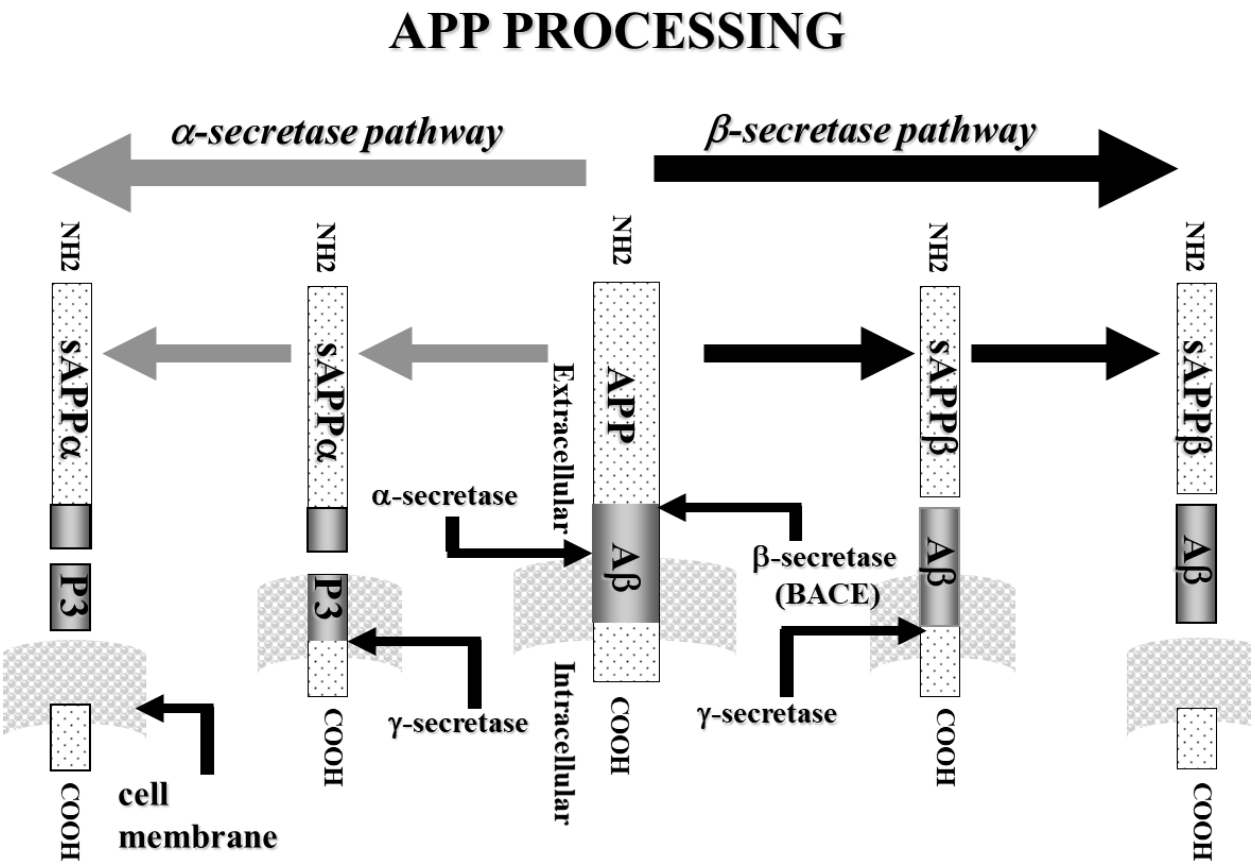


Figure. 1: Diagram showing APP Processing pathways. (modified from S. Kar et al. (2004) *J. Psychiatry Neurosci.* 29:427-441)

Karch et al., 2014). Mutations in *PSEN1* and *PSEN2* genes change the γ-secretase-mediated cleavage of APP, leading to an elevation of the $A\beta_{42}/A\beta_{40}$ ratio and increased Aβ plaque load in AD patients (Duff et al., 1996). Several AD-related mutations have been recorded within and around the Aβ domain in the *APP* gene which also either increase the production of Aβ peptides or alter the ratio of $A\beta_{42}$:$A\beta_{40}$ in the brain (Chartier-Harlin et al., 1991; Selkoe, 2001). Many of these mutations are capable of accelerating AD progression through assorted pathways and mechanisms. Some common and well-studied mutations include: the Swedish mutation (APP KM670/671NL) at the amino terminus of Aβ domain (Mullan et al., 1992) which leads to increased Aβ production (Citron et al., 1992; Cai et al., 1993); the Florida mutation (APP 1716V) at the carboxy end of APP which results in elevated pathogenic $A\beta_{1\text{-}42}$ levels (Suzuki et al., 1994); the Arctic mutation (APP E693G) within the Aβ region which increases the production of fibrillar Aβ peptides (Nilsberth et al., 2001) and the Dutch mutation (APP E693Q) (Levy et al., 1990) which influences Aβ production and fibril formation. Interestingly, many transgenic mouse models overexpressing mutant APP and/or PS1 are found to recapitulate certain features of AD pathology including cognitive behavioral deficits, presence of senile plaques, gliosis, dystrophic neuritis and occasionally loss of neurons in selected brain regions (Games et al., 1995; Hsiao et al., 1996; Borchelt et al., 1997; Sturchler-Pierrat et al., 1997; Holcomb et al., 1998; Chen et al., 2000).

1.4 Sporadic AD

While a small portion of AD occurs due to genetic variants, the major category of AD is late onset and sporadic in origin (~90-95%). The underlying cause of sporadic AD is presumed to be multifactorial, where biological and genetic susceptibility are linked with external variables to accelerate the development of AD symptoms. The major known genetic risk factor is the inheritance of the ε4 allele of the APOE (Strittmatter et al., 1993; Mahley et al., 2006) gene which

encodes proteins involved in cholesterol transportation. The APOE gene is located in chromosome 19 and has three distinct alleles: ε2, ε3 and ε4. At least one APOE ε4 is carried by 40-80% of people with AD (Mahley et al., 2006). The APOE ε4 allele enhances the risk of the disease by 15 times in homozygotic and by 3 times in heterozygotic individuals (Mahley et al., 2006). By the age of 85, individuals having one or two copies of the APOE ε4 allele develop AD at the level of 45% and 50-90%, respectively (Reiss and Voloshyna, 2012). The APOE ε2 allele has been reported to play a protective role in AD (Corder et al., 1994). Blocking the interaction between Aβ and APOE ε4 leads to the reduction of Aβ fibril formation *in vitro* and lowering of Aβ plaque deposition in an *in vivo* paradigm (Sadowski et al., 2004). Furthermore, mice which overexpress mutant APP along with APOE ε4 exhibit an elevated level of Aβ production compared with mice expressing either no or alternative types of the APOE allele (Holtzman and Simon, 2000; Carter et al., 2001).

1.5 Clearance and degradation of Aβ

Evidence suggests that impaired clearance of Aβ peptides, apart from increased production, has a role in the development of AD pathology. Both neurons and glial cells (i.e., microglia and astrocytes) can clear Aβ by regulating multiple processes including transport across the blood-brain barrier, internalization followed by degradation in lysosomes and by activation of various Aβ-degrading enzymes such as insulin-degrading enzyme (IDE), neprilysin (NEP), angiotensin converting enzyme, endothelin converting enzymes and matrix metalloproteinases-2 (MMP-2) and MMP-9 (Saido and Leissring, 2012; Baranello et al., 2015). It is reported that most of the Aβ-degrading enzymes are expressed in astrocytes under normal conditions. However, activated astrocytes exhibit impaired clearance of Aβ, possibly due to altered levels/activity of Aβ-degrading enzymes or impaired functioning of the endosomal-lysosomal system (Koistinaho et al., 2004;

Pihlaja et al., 2011; Xiao et al., 2014). This overall observation leads to a notion that alteration in rates of Aβ synthesis as well as clearance may play a role in AD pathogenesis. Some of the notable enzymes which are active in degrading Aβ include IDE and nepilysin (Marr et al., 2004; Baranello et al., 2015). For example, neprilysin knockout mice showed increased deposition of $A\beta_{1-40}$/$A\beta_{1-42}$ which indicates its influence on cellular Aβ levels (Huang et al., 2006). IDE is a zinc-endopeptidase found in the cytosol, peroxisomes and at the cell surface which can cleave small peptides of different categories (Baranello et al., 2015). Selective deletion of the *IDE* gene in a transgenic mouse manifested chronic elevated levels of cerebral Aβ (64%) compared to wild type mice (Farris et al., 2003; Miller et al., 2003).

Lysosomes are designated as multipurpose heavy-duty degradation points for large proteins, aggregates and different organelles (De Duve and Wattiaux, 1966). Cargo is shipped to the lysosomes through different routes such as endocytosis, autophagy and phagocytosis, referred to in sum as the endosome-autophagosome-lysosome pathway (EALP) (Koh et al., 2019). In recent years, attention has been drawn to the area of active participation of autophagy in APP turnover and Aβ metabolism (Zare-Shahabadi et al., 2015). Autophagy is the dominant cellular pathway through which waste protein is shuttled through intracellular autophagosomes and transported to lysosomes for degradation (Menzies et al., 2015). Key proteins involved in autophagy include the autophagy protein 5 (ATG5), microtubule-associated protein 1A/1B light-chain 3 (LC3), Beclin-1 and p62 (Glick et al., 2010). In the case of AD, studies have shown that the mechanistic target of rapamycin kinase (mTOR) is the rate limiting point controlling autophagy and that the activation of mTOR is reciprocally related to the level of autophagy (Caccamo et al., 2010). Beclin-1 is a crucial protein involved in autophagosome formation, and when it is deleted there is an elevation in the Aβ levels as shown in cell and animal models (Pickford et al., 2008; Swaminathan et al.,

2016). The transcription factor EB (TFEB), an important regulatory protein in the autophagy pathway, synchronizes the general operation of autophagy in different systems and directly influences Aβ metabolism in normal as well as pathological conditions (Xiao et al., 2015; Zhang and Zhao, 2015). As aging is the greatest risk factor of AD, normal lysosomal operation may become compromised to initiate the foundation for the deposition of aggregated proteins like Aβ and tau (Mawuenyega et al., 2010).

1.6 Astrocytes and their role in APP metabolism

Astrocytes are the most abundant glial cells in the central nervous system. They play vital roles in maintaining brain homeostasis by regulating trophic/metabolic support, neurotransmitter milieus, the blood-brain barrier, synaptic activity and synapse formation/remodeling (Barres, 2008; Belanger and Magistretti, 2009; Nag, 2011; Sidoryk-Wegrzynowicz et al., 2011; Pekny et al., 2014; Osborn et al., 2016). Upon activation, which may result from injury or development of diseases such as AD, astrocytes undergo specific modifications resulting in "reactive gliosis" – characterized by hypertrophy of cellular processes and upregulation of intermediate filament proteins including glial fibrillary acidic protein (GFAP). Consequently, activated astrocytes lose some of their normal homeostatic functions and participate in inflammatory reactions that contribute to a variety of pathological changes (Belanger and Magistretti, 2009; Rodríguez et al., 2009; Sofroniew, 2009; Fuller et al., 2010; Allaman et al., 2011; Nag, 2011; Sidoryk-Wegrzynowicz et al., 2011; Jo et al., 2014; Pekny et al., 2014; Batarseh et al., 2016). A role for activated astrocytes in AD is supported by the evidence that i) they increase neuronal vulnerability to toxicity by impairing metabolic support and glutamate recycling (Belanger and Magistretti, 2009; Steele and Robinson, 2012), ii) they generate reactive oxygen/nitrogen species that can influence cell death (Akama et al., 1998; Lüth et al., 2002; Dzamba et al., 2016), iii) they express

pro-inflammatory molecules such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6 and interferon-γ (IFNγ) that can increase Aβ production (Blasko et al., 2000; Medeiros and LaFerla, 2013; Batarseh et al., 2016), iv) they exacerbate Aβ-mediated toxicity (Domenici et al., 2002; Garwood et al., 2011) and v) they are unable to efficiently regulate Aβ clearance (Nagele et al., 2004; Wyss-Coray et al., 2003; Mulder et al., 2012). Although activated astrocytes associated with neuritic plaques in AD brains have been shown to accumulate Aβ which correlates positively with the severity of AD-associated tissue damage, very little is currently known regarding APP/Aβ metabolism in activated astrocytes and their role in AD pathology.

Evidence suggests that nine different APP isoforms, produced from a single gene by alternative splicing, encode proteins ranging from 365 to 770 amino acids. The APP695 isoform is expressed predominantly in neurons, whereas APP751 and APP770 are found mostly in astrocytes. Under normal conditions, neurons are the major source of Aβ, while astrocytes play an important role in its clearance (Zhao et al., 1996, 2011; Calhoun et al., 1999; Thal, 2012; Avila-Munoz and Arias, 2014). However, given the evidence that astrocytes, which outnumber neurons in the brain, can express APP upon activation and AD may take decades to develop, it is likely that astrocytic Aβ could contribute to cerebral Aβ levels to influence AD pathology over time (Abbott, 2002; Haseloff et al., 2005; Siddharthan et al., 2007; Garwood et al., 2017). Thus, it is important to study APP metabolism in activated astrocytes and its potential contributions to the development of AD pathology.

1.7 Hypothermia and AD

Our body regulates exposure to higher ambient temperature by directing the blood, and thus heat, to the skin, where heat is released by sweating allowed by nervous innervation and control of skin

blood flow. This is accompanied by reduced blood pressure and increased heart rate. On the other hand, when exposed to a cool environment, our body tries to maintain heat balance by reducing heat loss and/or increasing heat production. Heat loss is minimized by cutaneous vasoconstriction, which decreases heat transfer from the core to the skin and other distal parts of the body. Conversely, heat production is increased by non-shivering and shivering thermogenesis. Shivering especially is a very efficient way to produce heat (Kenney and Munce, 2003; Kelly, 2006; Lu et al., 2010; Waalen and Buxbaum, 2011; Blatteis, 2012).

Old age (>60 years), the most important risk factor for AD pathology, is known to be associated with a decrease in body temperature compared to young age (<20-40 years). This is likely a consequence of impaired function of thermoregulatory processes including an age-related decline in cardiovascular, respiratory, neuromuscular and gastrointestinal functions (Kelly, 2006; Waalen and Buxbaum, 2011; Blatteis, 2012). Moreover, elderly humans defend their core temperature less efficiently than younger adults when exposed to hot environments and have a greater risk of hypothermia and morbidity than young adults during extended periods of exposure to cold ambient temperatures. This may be due to age-related changes in autonomic mechanisms, such as a reduction of peripheral isolation resulting from a loss of body fat and reduced vasoconstriction, and a reduced metabolic heat production as a consequence of less intense shivering, decreased muscle mass, and a lower basal metabolism (Kenney and Munce, 2003; Kelly, 2006; Waalen and Buxbaum, 2011). In addition to a progressive decline in ability to defend core body temperature, a number of 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). Supporting this notion, one study using an animal model of AD (i.e.,

3xTgAD mice) reported this model to have a lower body temperature at 12-14 months of age compared to age-matched control mice (Vandal et al., 2016).

Multiple experimental paradigms have shown that hypothermia induced during hibernation, anesthesia and other paradigms can increase the phosphorylation of tau protein. A decrease in temperature between 3°C and 10°C below 37°C (considered to be the normothermic condition for most homeothermic animals) has been shown to increase the phosphorylation of tau both in animal models of AD as well as cultured cells (Korneyev et al., 1995; Korneyev, 1998; Planel et al., 2004, 2007b, 2009; Whittington et al., 2013). Additionally, exposing 20-month-old hypothermic 3xTgAD mice to a warmer environment for 7 days not only increases core body temperature but has also been found to reduce phosphorylation of tau protein and cognitive deficits (Vandal et al., 2016). A recent prospective study of 2300 middle age Finish men followed over 20 years reported that repeated heat exposure through sauna bathing is inversely associated with dementia and AD. Interestingly, this study also showed frequency of sauna bathing is proportional to lower risk of developing dementia and AD (Laukkanen et al., 2017). These results, taken together, suggest a potential role for temperature in the development of AD, while the cellular sites and/or mechanisms by which temperature can influence tau and APP metabolism remain unclear.

1.8 Background work related to this project

Earlier studies by our group and others have shown that activated astrocytes following a variety of experimental paradigms and in animal models of neurodegeneration can express enhanced levels of APP and its processing enzymes such as BACE1 as well as the γ-secretase complex, but their functional significance in relation to the development of disease pathology remains unclear (Siman et al., 1989; Banati et al., 1995; Nihashi et al., 2001; Nadler et al., 2008; Kodam et al., 2010, 2018;

Avila-Munoz and Arias, 2015; Yang et al., 2017). Recently, some studies have reported that alterations in ambient temperatures can influence AD-related pathological features such as phosphorylation of tau protein and altered APP level/processing both in *in vitro* and *in vivo* paradigms (Hartig et al., 2007; Planel et al., 2007a; Arendt et al., 2015; Vandal et al., 2016; Tournissac et al., 2017). However, in the context of astrocytes and APP processing, absolutely no information is currently available about their response to either hypo- and/or hyper-thermic environments.

1.9 Hypothesis and objectives

On the basis of the aforementioned information and given the significance of activated astrocytes in APP metabolism, we raised the hypothesis that alteration in ambient temperature may influence APP processing in astrocytes. To address this hypothesis, we performed the following two sets of experiments:

i) determine how hypothermic (27°C) and hyperthermic (40°C) conditions can influence APP levels and its processing enzymes in rat primary cultured astrocytes compared to cells grown at normal (37°C) ambient temperature.

ii) determine how hypothermic (27°C) and hyperthermic (40°C) conditions can influence the steady-state levels of the Aβ-degrading enzymes IDE and neprilysin along with lysosomal and autophagic markers in rat primary cultured astrocytes compared to cells grown at normal (37°C) ambient temperature.

MATERIALS AND METHODS

2.1 Materials: Rat primary astrocytes as well as Astrocyte Medium-animal (AM-a) and Astrocyte-Medium-phenol red free (AM-prf) were purchased from ScienceCell (Carlsbad, CA, USA). NuPAGE 4–12% Bis-Tris gels, 3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide (MTT), and an Enzyme-linked immunosorbent assay (ELISA) kit for the detection of mouse $A\beta_{1-40}$ were obtained from Invitrogen (USA). The bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence kit were purchased from Thermo Fisher Scientific (Montreal, QC, Canada). Vivaspin filtration columns were from GE Healthcare (Mississauga, ON, Canada). Sources and dilutions of all primary antibodies are listed in Table-1. Horseradish peroxidase-conjugated secondary antibodies were procured from Santa Cruz Biotechnology (Paso Robles, CA, USA). All other chemicals were from Sigma-Aldrich or Thermo Fisher Scientific.

2.2 Cell Culture: Rat primary astrocytes were cultured in AM-a media containing 2% fetal bovine serum, 1% penicillin (10K U/ml)/streptomycin (10K μg/ml) solution and 1% astrocyte growth supplement-animal (AGS-a) according to the protocol recommended by the company. Cells were grown at 37°C in a humid 5% CO_2 incubator, and the culture medium was changed every 2 or 3 days. Cells were seeded at $1x10^4$ cells/cm^2 and harvested at 90% confluency. In our experimental paradigms, cultured astrocytes were maintained at 27°C, 37°C or 40°C for different periods of time (6, 12 and 24hr) and then cells were harvested for further analysis. In brief, cells exposed to different temperatures were washed with phosphate-buffered saline (PBS, pH 7.4) and collected in ice-cold PBS followed by centrifugation at 600*g* for 5min. Collected pellets were lysed in ice-cold radioimmunoprecipitation lysis assay (RIPA) buffer (1% protease inhibitor) and then centrifuged at 600*g* for 10min. The supernatant and the pellets were harvested and used either immediately or stored at -80°C until further processing.

2.3 Viability of cultured astrocytes: The viability of cultured astrocytes exposed to different temperatures (27°C, 37°C and 40°C) was evaluated using the colorimetric MTT assay as described earlier (Wang et al.,2015). In brief, cells after 24hr exposure were treated with 10µl of 0.25% MTT and then incubated for 4hr at 37°C. The reaction was terminated by removing the reaction media and absorbance was measured spectrophotometrically at 570nm. Each experiment was conducted in triplicate and was repeated three times. Cell viability was assessed and compared across three different temperature conditions.

2.4 Western blotting: Cultured astrocytes from different experimental paradigms homogenized in RIPA lysis buffer were processed for immunoblotting as described earlier (Maulik et al., 2015). In brief, all cultures were first processed to measure the amount of protein using a BCA kit and then equal amounts of protein (10-15µg) were separated on 7-17% gradient polyacrylamide or 4-12% NuPAGE Bis-Tris gels. The proteins were then transferred onto Polyvinylidene Fluoride (PVDF) membranes, blocked with 5% skimmed milk in Tris-buffered saline (0.1% Tween) and then incubated overnight at 4°C with anti-APP, anti-BACE1, anti-ADAM10, anti-nicastrin, anti-PS1, anti-IDE, anti-neprilysin, anti-microtubule-associated protein 1 light chain 3 (LC3) and anti-lysosomal-associated membrane protein 1 (LAMP1) antibodies at dilutions listed in Table-1. The membranes were incubated with horseradish peroxidase-tagged secondary antibodies (1: 5000) for 1hr at room temperature and then immunoreactive proteins were detected using an enhanced chemiluminescence detection kit as described earlier (Maulik et al., 2015). All blots were re-probed with β-actin antibody to verify the protein loading and were quantified using a Microcomputer Imaging Device (MCID) image analysis system (Imaging Research, Inc., St Catherines, ON, Canada) as described earlier (Kodam et al., 2010). All experiments were conducted at least 3–4 times.

2.5 ELISA for $A\beta_{1-40}$: Cultured astrocytes from different experimental paradigms were processed to measure the secretory levels of rat $A\beta_{1-40}$ using an ELISA kit as reported earlier (Maulik et al., 2015; Wang et al., 2015). For secretory $A\beta_{1-40}$, astrocytes were cultured in phenol-red free basal astrocyte media for 6, 12 and 24hr at 27°C, 37°C or 40°C. Subsequently, the media collected from different experimental conditions were concentrated using Vivaspin filtration, centrifuged at 4000*g* for 5hr and then processed to measure rat $A\beta_{1-40}$ levels using a specialized high sensitivity ELISA kit. The OD value was converted to pg/ml according to a standard curve. All samples were assayed in duplicate and the data were obtained from three independent experiments.

2.6 Statistical analysis: Data were expressed as mean $\pm$ SEM. Statistical significance of differences was determined by one-way ANOVA followed by Bonferroni's post-hoc analysis for multiple comparisons with a significance threshold of $p<0.05$. All analyses were conducted using GraphPad Prism (GraphPad Software, Inc., CA, USA).

TABLE-1: List of primary antibodies

Antibody	Type	WB dilution	Source
APP (clone Y188)	Monoclonal	1:5000	Abcam Inc.
APP-KPI	Polyclonal	1:1000	Abcam Inc
APP (22C11)	Monoclonal	n/a	EMD Millipore
ADAM10	Polyclonal	1:1000	EMD Millipore
BACE1	Monoclonal	1:1000	R & D Systems
β-actin	Monoclonal	1:5000	Sigma-Aldrich
GFAP	Polyclonal	1:1000	ThermoFisher
LAMP1	Polyclonal	1:1000	Abcam Inc.
LC3	Polyclonal	1:1000	Medical & Biological Lab
PS1	Polyclonal	1:1000	Gift from Dr. Gopal Thinakaran
IDE	Polyclonal	1:1000	Abcam Inc.
Nicastrin	Polyclonal	1:1000	Chemicon Intl
TFEB	Polyclonal	1:1000	Abcam Inc
Neprilysin	Polyclonal	1:1000	EMD Millipore

Abbreviations (WB: Western blotting)

RESULTS

3.1 Effects of temperature on the viability of cultured astrocytes: To determine if hypothermic or hyperthermic conditions can influence cell survival ability, I measured viability of cultured astrocytes following 24hr exposure to 27°C, 37°C and 40°C using the established MTT assay. The results clearly indicate that viability of the cultured astrocytes decreased significantly (to 57%) in the hypothermic condition (27°C) compared to the normal condition (37°C, $p<0.05$). Interestingly, the hyperthermic (40°C) temperature markedly improved the viability of cultured astrocytes (i.e., to 120%) compared to the normal condition (37°C, $p<0.05$) (Fig. 1). Thus, it appears that astrocytes survive better in a hyperthermic environment compared to normal as well as hypothermic conditions.

3.2 Effect of temperature on GFAP: GFAP, an intermediate filament protein, is an established marker of astrocytes. Its expression is known to increase markedly following activation of astrocytes (Belanger and Magistretti, 2009; Rodríguez et al., 2009). To evaluate if temperature can influence GFAP expression, I measured steady-state levels of GFAP using Western blotting following 6, 12 and 24hr exposure of the cultured astrocytes to different temperature conditions (i.e., 27°C, 37°C and 40°C) (Fig. 2). The results clearly revealed no significant alterations in the expression of GFAP at any time across the three different temperatures. Additionally, the level of GFAP did not alter significantly as a function of time at any given temperature conditions (Fig. 2).

3.3 Effects of temperature on APP and APP-CTFs: To evaluate the influence of temperature on APP metabolism in rat astrocytes, I first measured APP holoprotein levels using two different antibodies (i.e., Y188 and APP-KPI) following exposure of cultured astrocytes at 27°C, 37°C and 40°C for 6, 12 and 24hr (Fig. 3). While the Y188 is an established monoclonal antibody developed

against a synthetic peptide corresponding to the C-terminus of human APP, APP-KPI is a polyclonal antibody known to recognize APP expressed predominantly in astrocytes. The steady-state levels of APP, as recognized by both antibodies, were significantly (p<0.05) increased in a time-dependent manner at both 37°C and 40°C. However, no alteration in APP levels was apparent as a function of time at the hypothermic condition (27°C) (Fig. 3A, B). It is also of interest to note that APP levels in cultured astrocytes were markedly (p<0.05) increased at 37°C and 40°C compared to 27°C following 24hr exposure, although no significant alteration was observed between 37°C and 40°C at 24hr (Fig. 3A, B).

Analysis of APP cleavage products revealed that the levels of α-CTF and β-CTF, generated by α- and β-secretases, respectively, were altered as a function of time in cultured rat astrocytes across the hypothermic (27°C), normal (37°C) and hyperthermic (40°C) conditions. With regard to α-CTF, a time-dependent significant increase (p<0.05) was observed at 37°C and 40°C, whereas a non-significant decrease was apparent at 27°C (Fig. 4A). Additionally, the levels of α-CTF in cultured astrocytes were markedly increased (p<0.05) following 24hr exposure to 37°C and 40°C compared to 27°C (Fig. 4A). In contrast to α-CTF, the levels of β-CTF increased significantly (p<0.05) with time at hypothermic (27°C) and to some extent at hyperthermic (40°C) but not at normal (37°C) conditions. In parallel, we observed that β-CTF levels in astrocytes also increased significantly (p<0.05) across the temperatures at the following time points; 6hr exposure at 37°C *vs* 6hr exposure at 27°C, 12hr exposure at 37°C and 40°C *vs* 12hr exposure at 27°C and finally 24hr exposure at 37°C *vs* 24hr exposure at 27°C (Fig. 4B). These data suggest that temperature, depending on the exposure time, can differentially affect α-CTF and β-CTF levels in astrocytes (Fig. 4A, B).

3.4 Effects of temperature on APP secretases: As the α-/β-CTF levels are markedly altered in astrocytes in response to different temperatures, I measured the levels of α-secretase ADAM10 and β-secretase BACE1 following exposure of rat cultured astrocytes at 27°C, 37°C and 40°C for 6, 12 and 24hr (Fig. 5). While the levels of ADAM10, as a function of time, did not exhibit any significant alterations at 37°C, they were found to be markedly enhanced ($p<0.05$) at 27°C and decreased ($p<0.05$) at 40°C in cultured astrocytes. Additionally, ADAM10 levels were significantly increased ($p<0.05$) following 6hr exposure to 37°C as well as 40°C compared with 27°C. In parallel, I observed increased levels ($p<0.05$) of ADAM10 after 12hr exposure at 37°C compared with 27°C. Consistent with the ADAM10 result, I did not observe any significant alteration in β-secretase BACE1 levels over time at 37°C. The levels of the enzyme, however, were found to be significantly increased ($p<0.05$) at 27°C and decreased to some extent at 40°C with time. Across the three temperature conditions, BACE1 levels did not exhibit any marked variation excepting a decreased level of the enzyme following 24hr exposure to 40°C compared to 27°C.

Accompanying ADAM10 and BACE1, I evaluated the levels of two important components of the γ-secretase complex, i.e., PS1 and nicastrin after 6, 12 and 24hr exposure of rat cultured astrocytes at three different temperatures (27°C, 37°C and 40°C) conditions. The steady-state levels of PS1 showed a time-dependent decrease at 27°C but no significant alteration at either 37°C or 40°C (Fig. 6A). The levels of the enzyme complex subunit were also found to markedly ($p<0.05$) decrease in the cultured astrocytes following 6hr exposure at 37°C as well as 40°C compared with 27°C. Conversely, PS1 levels increased significantly ($p<0.05$) after 24hr exposure to the hyperthermic (40°C) condition compared to hypothermic (27°C) or normal (37°C) conditions (Fig. 6A). While the levels of nicastrin exhibited a time-dependent decrease at 27°C and 40°C, increased levels of the

enzyme were apparent at 37°C. The decrease of nicastrin levels reached significance ($p<0.05$) at 24hr compared to 6hr exposure in the hypothermic (27°C) condition, and at 12hr and 24hr compared to 6hr at the hyperthermic (40°C) condition (Fig. 6B). The levels of the enzyme were found to be significantly enhanced ($p<0.05$) at 24hr compared to 6hr exposure at the normal (37°C) condition in cultured astrocytes. Additionally, nicastrin levels were markedly upregulated ($p<0.05$) after 6hr exposure at 27°C as well as 40°C compared to the corresponding exposure at 37°C. Conversely, the enzyme levels were enhanced ($p<0.05$) after 24hr exposure at 37°C compared to the corresponding exposure time at 27°C and 40°C conditions (Fig. 6B).

3.5 Effects of temperature on $A\beta_{1-40}$ secretion: To define whether different temperatures are able to regulate the secretion of Aβ levels, I measured $A\beta_{1-40}$ by ELISA in the conditioned media of cultured astrocytes following 6, 12 and 24hr exposure at 27°C, 37°C and 40°C conditions (Fig. 7). The results clearly indicate a significant ($p<0.05$) time-dependent increase in the secretory levels of $A\beta_{1-40}$ at 37°C and 40°C, whereas at 27°C the increase was evident only between 6 and 24hr time points. It is also of interest to note that the secretory levels of $A\beta_{1-40}$ did not alter significantly at any time across the three different temperatures (Fig. 7).

3.6 Effects of temperature on Aβ-degrading enzymes IDE and neprilysin: Neprilysin and IDE are important Aβ-degrading enzymes which are known to be expressed in astrocytes (Mulder et al., 2012; Baranello et al., 2015). To determine whether temperature can alter steady-state levels of IDE or neprilysin, I measured their levels in cultured astrocytes following 6, 12 and 24hr exposure at 27°C, 37°C and 40°C. The results clearly revealed that IDE levels did not alter as a function of time at any given temperature condition. Additionally, no alteration was evident at either 6, 12 or 24hr

exposure time points among three different temperature conditions (i.e., 27°C, 37°C and 40°C) (Fig. 8A). With regard to steady-state levels of neprilysin, I observed a time-dependent increase in hypothermic (27°C) and a decrease in hyperthermic (40°C) conditions. In parallel, I observed that neprilysin levels were significantly increased ($p<0.05$) following 6hr exposure to both 37°C as well as 40°C compared with 27°C experimental conditions. Additionally, I observed that the levels of the enzyme were significantly decreased ($p<0.05$) at 40°C compared to 27°C as well as 37°C following 24hr exposure (Fig. 8B). These data clearly indicate that Aβ-degrading enzymes in cultured astrocytes are differentially regulated by temperature (Fig. 8A, B).

3.7 Effects of temperature on autophagic-lysosomal markers: The autophagic-lysosomal system which plays a critical role in Aβ metabolism is known to be altered in AD pathology (Zhang and Zhao, 2015; Swaminathan et al., 2016). To determine if temperature conditions can influence the functioning of the autophagic-lysosomal system, I measured the levels of the three established markers of the pathway, i.e., LAMP1 (a marker of the lysosomes), LC3II (a marker of autophagosomes) and TFEB (a master regulator of lysosomal biogenesis) in cultured astrocytes following 6, 12 and 24hr exposure at 27°C, 37°C and 40°C conditions. The data reveal that LAMP1 levels increased with time in all three temperature conditions, but it reached statistical significance ($p<0.05$) only between 6hr *vs* 24hr time points at both 27°C and 40°C experimental conditions (Fig. 9A). In keeping with LAMP1, the levels of LC3-II increased with time in all three temperature conditions, although the changes were more obvious at 27°C and 40°C than at 37°C. A significant increase ($p<0.05$) in LC3-II levels in cultured astrocytes was noted following 12hr and 24hr compared to the 6hr time point at 27°C as well as 40°C. Interestingly, the levels of LC3-II were also significantly increased ($p<0.05$) following 12hr and 24hr exposure to 27°C conditions compared to

the respective time points at 37°C. Furthermore, LC3-II levels following 24hr exposure to 40°C were found to be markedly upregulated ($p<0.05$) compared to the corresponding exposure time at 37°C (Fig. 9B). In contrast to LAMP1 and LC3-II, I observed a time-dependent decrease in TFEB levels at all three temperatures (i.e., 27°C, 37°C and 40°C). The decrease of TFEB levels reached significance ($p<0.05$) at 24hr compared to 6hr exposure in all experimental paradigms. Additionally, the levels of TFEB were found to be markedly decreased ($p<0.05$) after 24hr exposure at 40°C compared to the corresponding exposure at 37°C (Fig. 10).

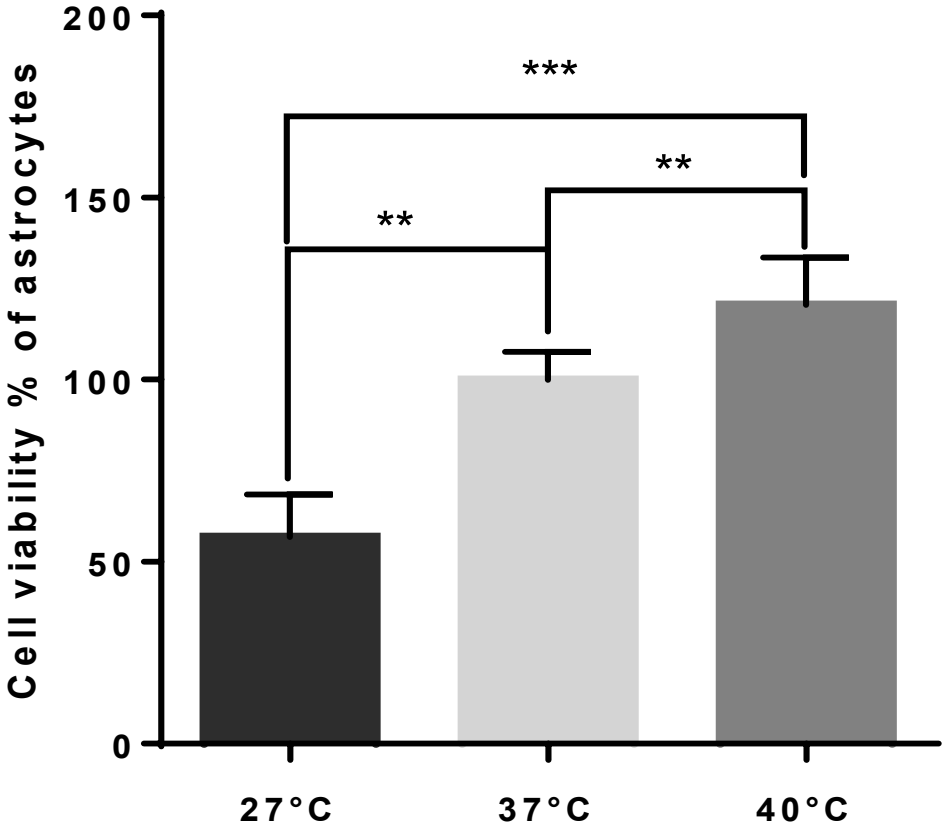


Figure 2: Histogram of cell viability measured with the MTT assay of cultured astrocytes at different temperature conditions for the depicted 24-hour incubation periods. Data represent means ± SEM from 3 independent experiments. ***$p<0.001$.

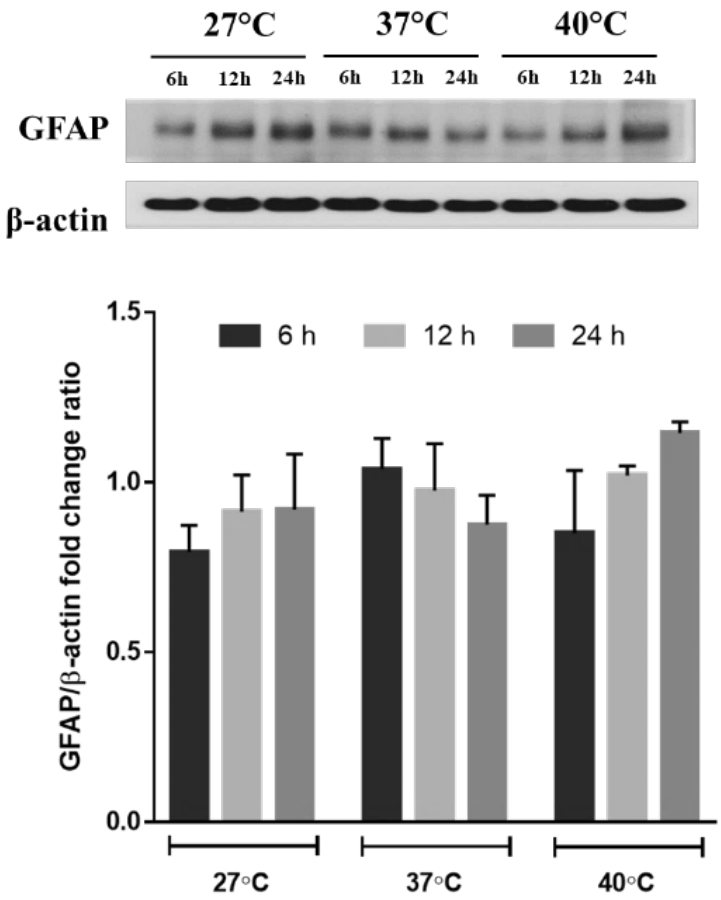


Figure 3: Western blot and parallel histograms showing time- and temperature-dependent effects on the expression levels of GFAP in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments.

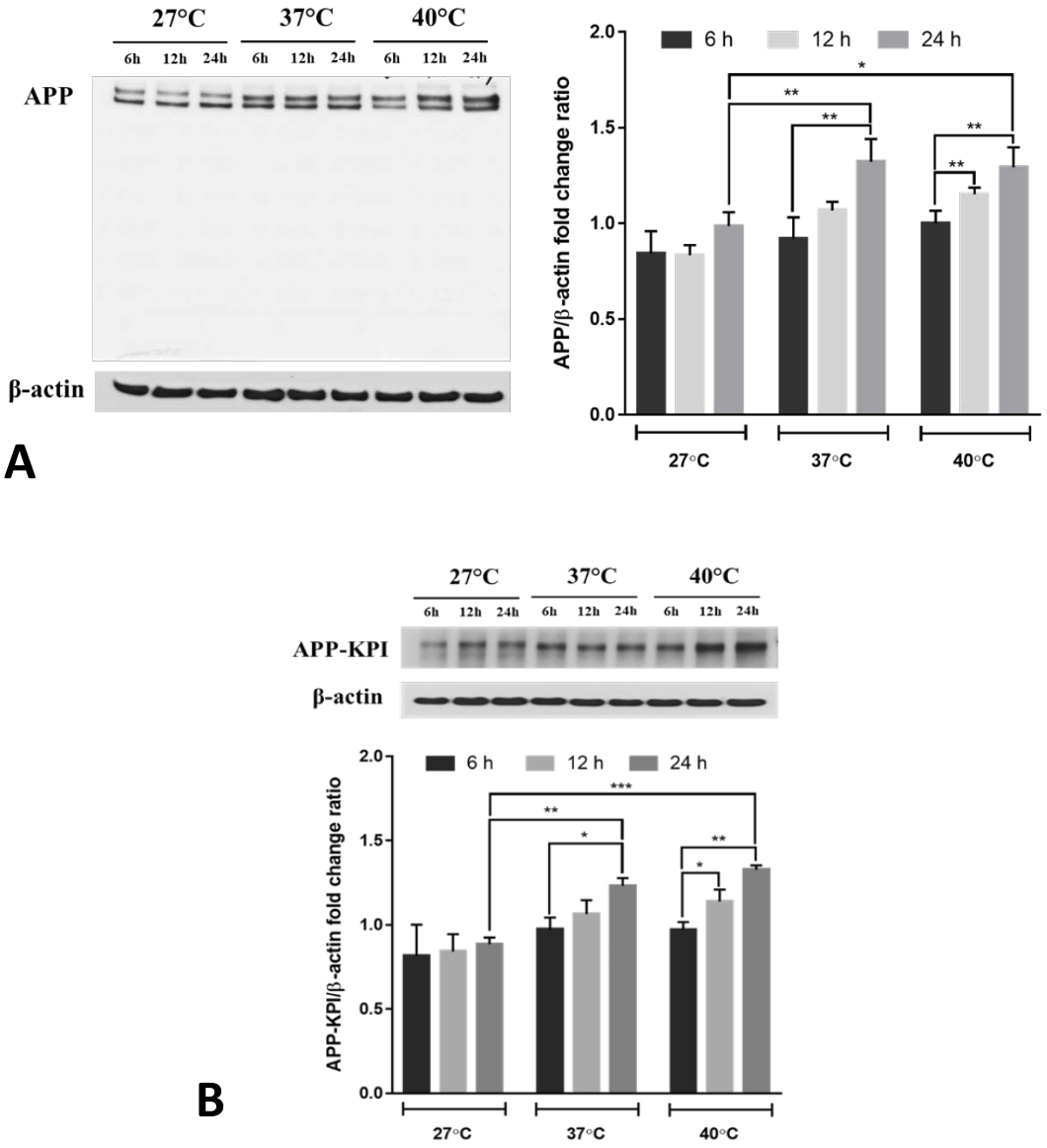


Figure 4: Western blot and relative histograms showing time- and temperature-dependent effects on the expression levels of APP holoprotein detected by Y188 (APP-C terminal antibody) (A) and antibody recognizing the KPI-domain (B) in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

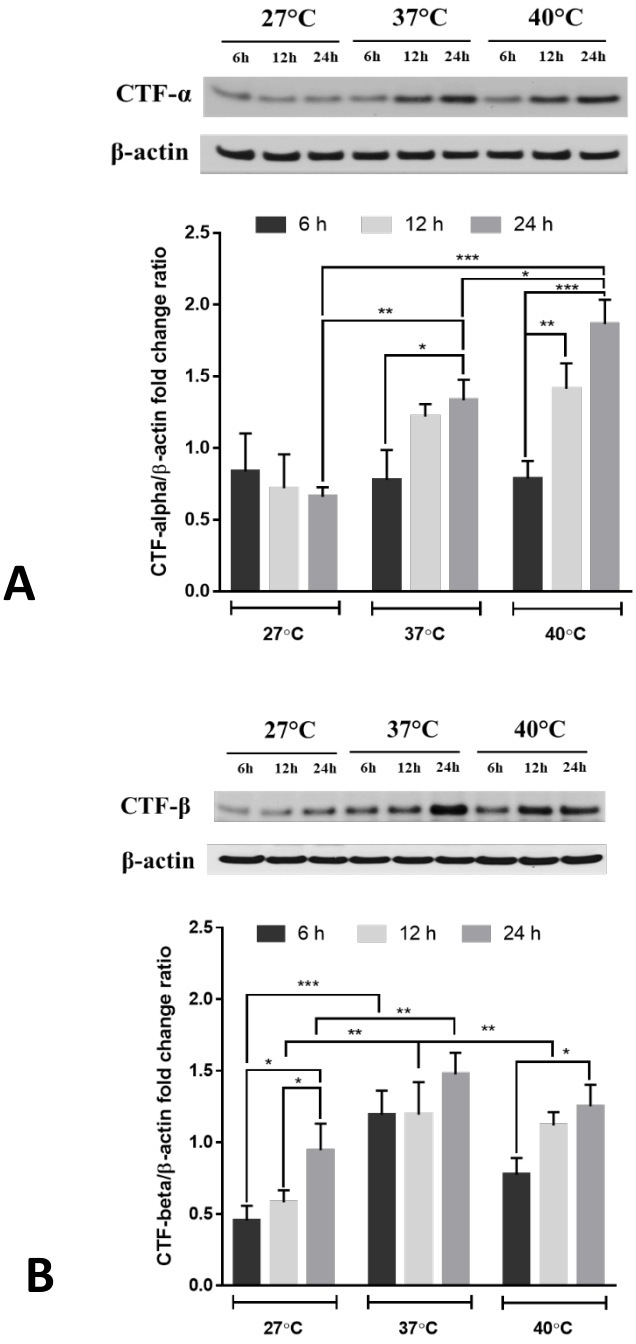


Figure 5: Western blot and equivalent histograms showing time- and temperature-dependent effects on the expression levels of the nonamyloidogenic C-terminal fragment (α-CTF) (A) and the amyloidogenic Aβ-containing C-terminal fragment (β-CTF) (B) in the cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

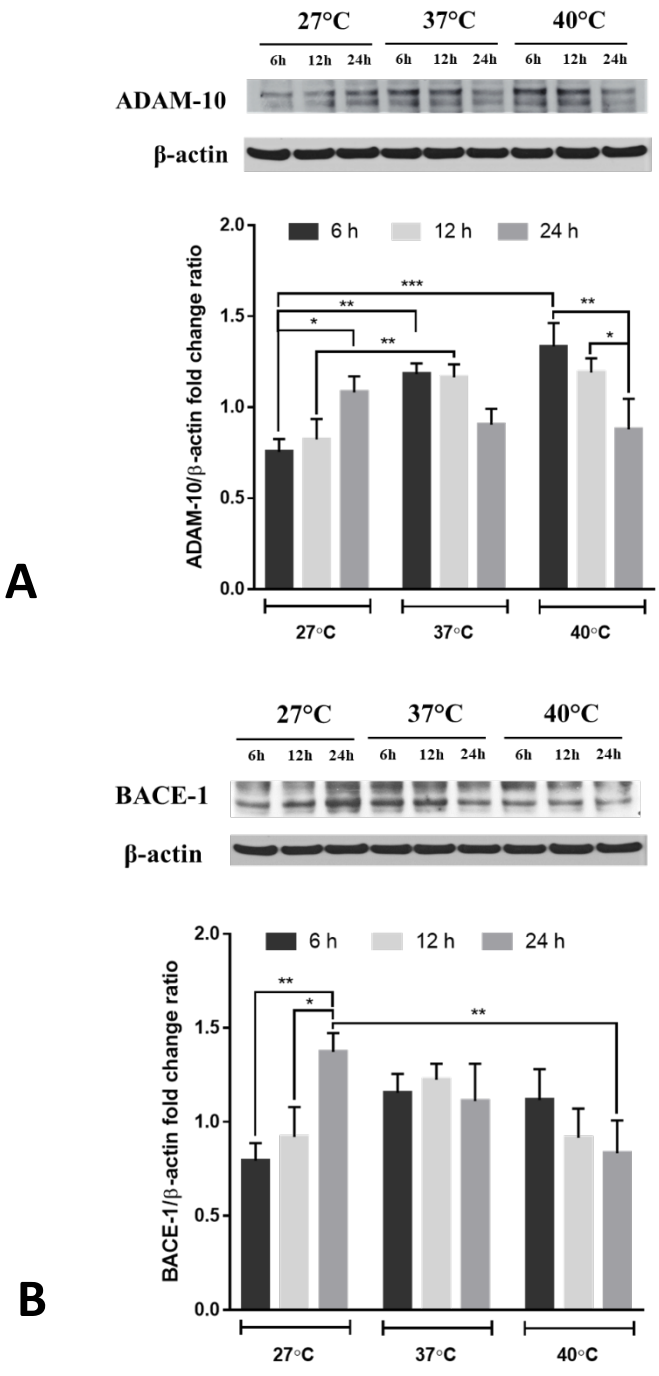


Figure 6: Western blot and analogous histograms showing time- and temperature-dependent effects on the levels of the expression of ADAM10 (A), an α-secretase nonamyloidogenic pathway protein and BACE1 (B), a β-secretase protein in cultured astrocytes. All Western blots were re-probed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

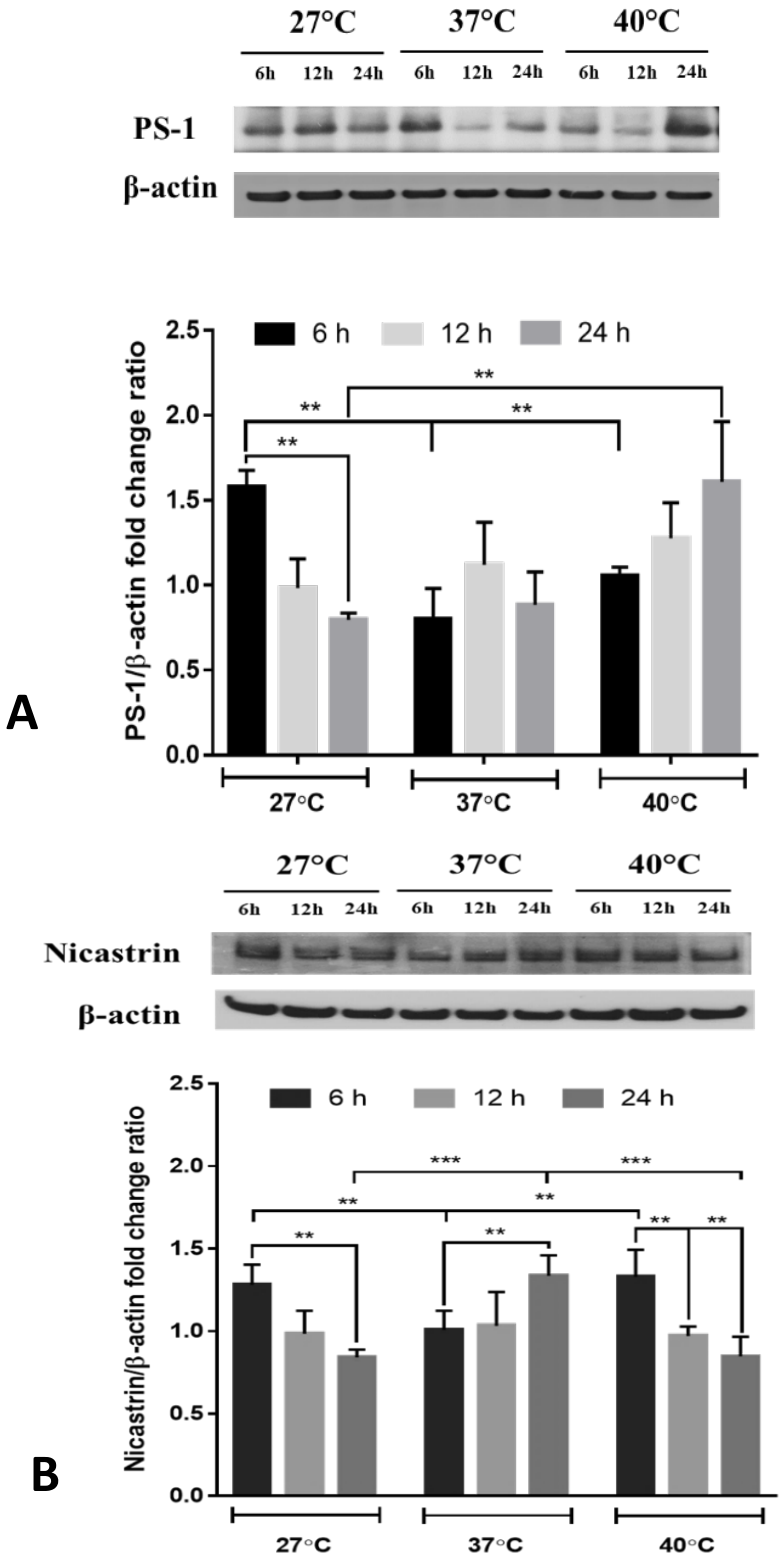


Figure 7: Western blot and correlated histograms showing time- and temperature-dependent effects on the levels of the expression of PS1 (A) and nicastrin (B), cofactors of γ-secretase protein in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. **p<0.01, ***p<0.001

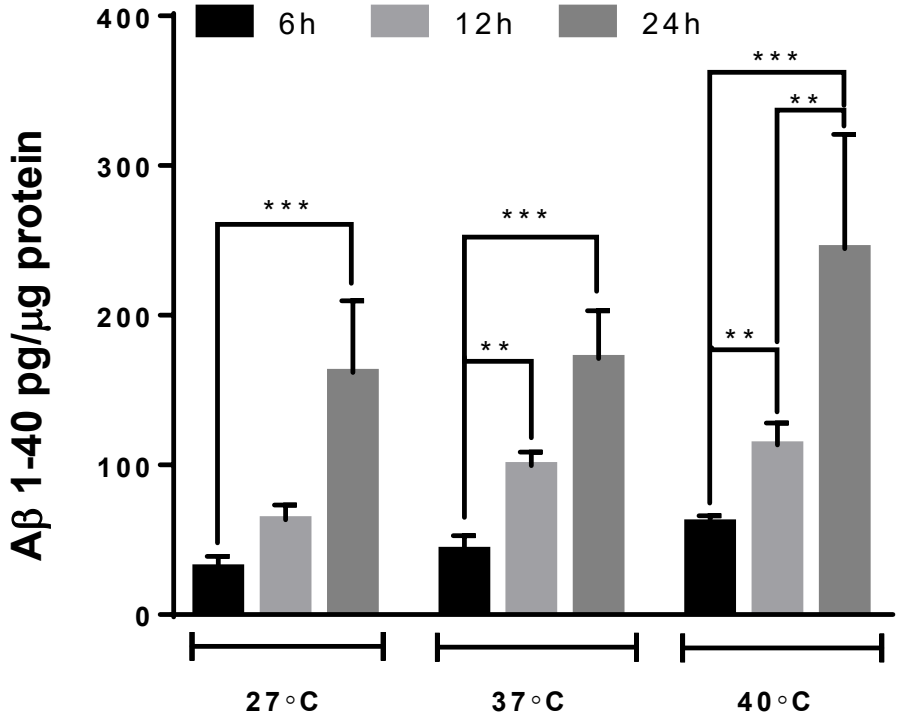


Figure 8: Histograms showing time-and temperature-dependent effects on the levels of the secreted $A\beta_{1-40}$ in cultured astrocytes. Data were normalized as pg of $A\beta_{1-40}$ per μg of protein in concentrated media. Data represent means ± SEM from 3 independent experiments. **p<0.01, ***p<0.001.

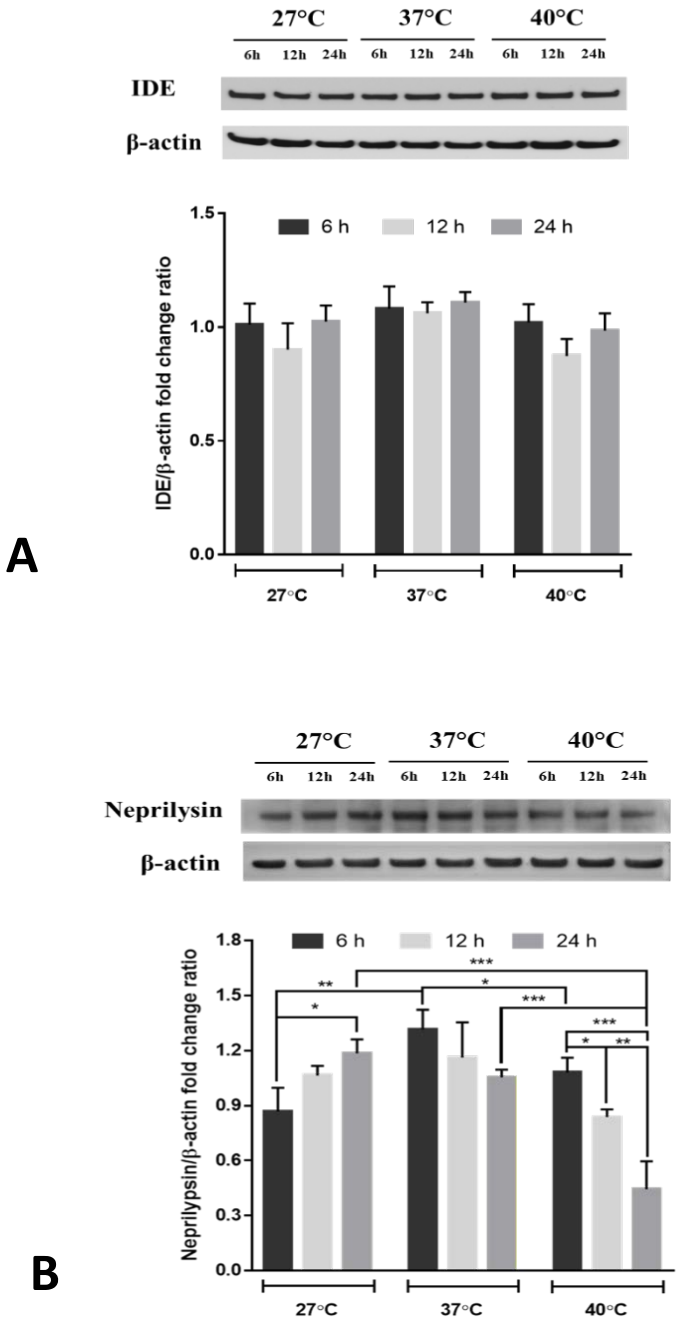
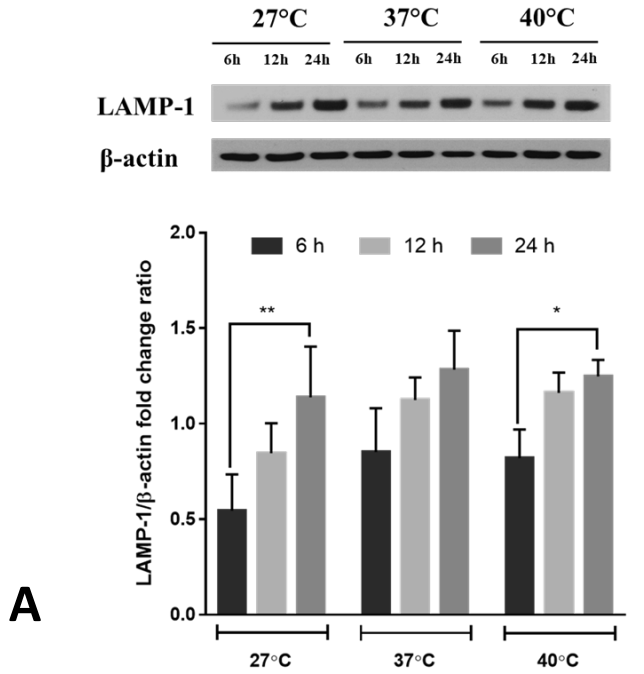


Figure 9: Western blot and equivalent histograms showing time-and temperature-dependent effects on the levels of the expression of the IDE (A) and neprilysin (B) in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.



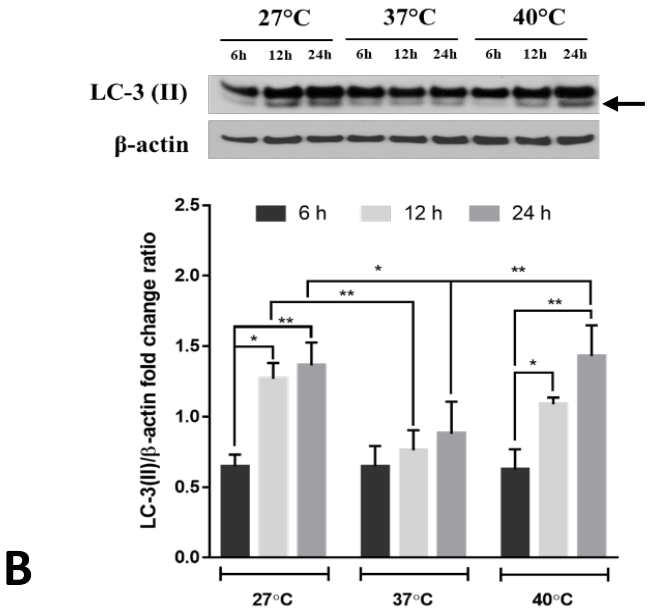


Figure 10: Western blot and equivalent histograms showing time-and temperature-dependent effects on the levels of the expression of the lysosomal marker LAMP1 (A) and the autophagy marker LC3-II (B) in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. *p<0.05, **p<0.01.

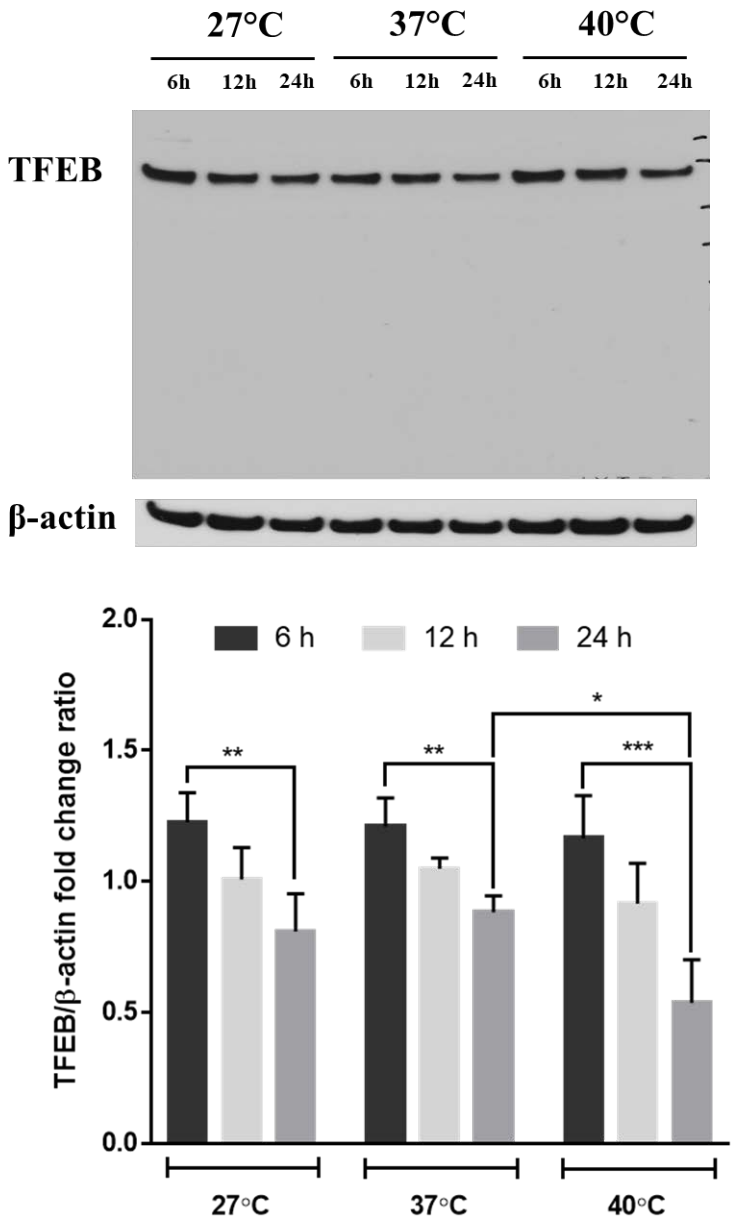


Figure 11: Western blot and equivalent histograms showing time-and temperature-dependent effects on the levels of the expression of the TFEB in cultured astrocytes. All Western blots were reprobed with β-actin antibody to evaluate protein loading in each lane. Data represent means ± SEM from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

The present study clearly shows that ambient temperature can influence cell viability and APP metabolism in rat primary cultured astrocytes. This is supported by the sfollowing lines of evidence; i) a hyperthermic condition enhances viability of cultured astrocytes without any alteration in GFAP levels, ii) levels of APP and its cleaved products (i.e., APP-CTFα and APP-CTFβ) are also markedly enhanced in a hyperthermic condition compared to normal and hypothermic conditions, iii) the steady state levels of APP secretases (ADAM10, BACE1 and components of the γ-secrease complex such as PS1 and nicastrin) as well as Aβ degrading enzymes (IDE and neprilysin) are differentially altered in cultured astrocyes in various temperature conditions, iv) the secretory levels of $A\beta_{1-40}$ are markedly increased with time in hypothermic, normal and hyperthermic conditions as well as across the temperatures and v) the markers of the autophagic-lysosomal system, but not TFEB levels, are found to be increased with time in cultured astrocytes. These results, taken together, suggest that hyperthermic conditions may have a role in enhancing APP metabolism, leading to increased secretion of Aβ-related peptides from astrocytes.

Body temperature plays a fundamental role in regulating various biochemical reactions including reaction rates, equilibrium amounts, viscosity and molecular arrangements. It is partly influenced by the circadian clock and is highest during active phases and lowest during inactive phases. Although the mammalian body can adapt physiologically to some variations in hot and cold environments, long-term changes may affect normal physiological functions especially in pathological settings (Fox et al., 1973; Holtzman and Simon 2000; Rikke and Johnson 2007; Blatteis 2012; Almeida et al., 2018). Several studies have shown that the core body temperature of healthy humans over 60 years of age is approximately 0.4^{o}C lower, than that of healthy adults 20-60 years of age. Moreover,

older individuals are found to maintain their core temperature less efficiently than younger adults during prolonged exposure to cold ambient temperature. These changes may be due to an age-related reduction of peripheral vasoconstriction, loss of body fat, decreased muscle mass and lower basal metabolism, leading to reduced metabolic heat production. Several other factors such as commonly prescribed medications, malnutrition, alcohol intake and anesthesia can also increase hypothermia in the elderly (Fox et al., 1973; Holtzman and Simon 2000; Kenny and Munce 2003; Kelly 2006; Lu et al., 2010; Blatteis 2012; Waalen and Buxbaum 2011; Almeida et al., 2018). Since aging is one of the important risk factors for AD, a better understanding of the contributions of age-associated thermoregulatory deficits and lower body temperature will provide critical information on AD pathogenesis and the potential treatment strategy.

Hypothermia was first proposed as a risk factor for AD by Holtzman and Simon in 2000 based on their observation that elderly people have a tendency to display cold intolerance and the evidence from their own studies that Down syndrome patients who invariably develop AD exhibit a statistically significant decrease in body temperature compared to individuals without Down syndrome (Holtzman and Simon 2000). Interestingly, 3xTgAD mice, a transgenic model of AD, were also reported to exhibit a lower body temperature at 12-14 months of age compared to age-matched control mice (Vandal et al. 2016). Over the years, multiple lines of experimental approaches suggest an impact of temperatures on the hallmarks (i.e., tau-positive neurofibrillary tangles and Aβ-containing neuritic plaques) of AD as well as cognitive impairments. The effect of cold on the phosphorylation of tau protein has been reported both in *in vitro* and *in vivo* models with different thermic conditions varying between 3°C - 10°C below 37°C, which is considered the normothermic condition for most homeothermic animals. This suggests a link between age-dependent deficits in

temperature homeostasis and tau phosphorylation leading to aggregation in AD and other dementias (Feng et al., 2005; Planel et al., 2007a; Bretteville et al., 2012; Maurin et al., 2014; de Paula et al., 2016).

Evidence further suggests that if the phospho-tau/temperature relationship is linear, a decrease of 0.4°C body temperature in an aged healthy individual may lead to a ~30% increase in phospho-tau levels compared young adults (Carrettiero et al., 2015; Almeida et al., 2018). Interestingly, cold-induced tau phosphorylation has also been reported in hibernating animals and following the use of intravenous (chloral hydrate and sodium pentobarbital) and inhalation (isoflurane) anesthetics – both of which are reversible after restoration to the normal temperature (Ancelin et al., 2001; Planel et al., 2007a; Stieler et al., 2011; Carrettiero et al., 2015). Increased tau phosphorylation has been ascribed to a dysfunction of tau kinases and/or phosphatases along with a failure in the degradation of tau *via* the proteasomal pathway (Planel et al., 2007a; Whittington et al., 2010; Bretteville et al., 2012; Julien et al., 2012; Maurin et al., 2014; Almeida et al., 2018). At present, however, no information is available on whether the temperature-dependent phosphorylation of tau protein is restricted only to neurons but also evident in astrocytes. Nevertheless, hypothermia has been shown to promote memory disruption and impairment of cognitive function (Ancelin et al., 2001; Eckenhoff and Planel 2012; Carrettiero et al., 2015), raising the possibility that an age-associated decrease in core body temperature, as a risk factor, can influence not only tau phosphorylation but also progressive memory impairment associated with AD pathology.

In contrast to tau phosphorylation, relatively little is known about the influence of temperature on APP levels/processing leading to generation of Aβ-related peptides. Some earlier *in vitro* studies

indicated that higher temperatures are able to enhance the expression of APP and the rate of Aβ oligomerization as well as fibril formation, implying a negative effect of elevated temperatures on the development/progression of AD pathology (Ciallella et al., 1994; Kusumoto et al., 1998; Gursky and Aleshkov, 2000; LeVine 2004). On the other hand, Vandal et al. (2016), reported that exposure of 3xTgAD and Non-Tg control mice to cold (i.e., 4°C) for 24hr increased full-length APP levels in the cortex along with an increase in the $A\beta_{1-42}/A\beta_{1-40}$ ratio – which is considered to be an important factor in the development of AD pathology. However, these changes were evident only in female, but not in male, 3xTgAD mice. Conversely, exposing 3xTgAD mice to a warm environment set at 28°C for 7 days led to a reduced insoluble $A\beta_{1-42}$ level in the parietal cortex without any alteration in the area of Aβ-containing neuritic plaques in the hippocampal region. The levels of APP holoprotein, however, did not differ between 3xTgAD and Non-Tg control mice. Interestingly, the steady-state levels of the Aβ degrading enzymes IDE and neprilysin were also not altered, but the level of low-density lipoprotein receptor-related protein 1 (LRP1), which is involved in the efflux of Aβ through the blood-brain barrier, was found to be significantly increased after exposure to the warm environment for 7days. Most importantly, exposing mice to a warmer environment for 7 days improved their cognitive performance as detected using an object recognition memory test – thus indicating that increased ambient temperature may have some beneficial effects on AD-related pathology (Vandal et al. 2016). Various studies have also shown that anesthetics, which can reduce body temperatures, can promote not only Aβ oligomerization and toxicity but also can increase Aβ production in cultured cells (Eckenhoff et al., 2004; Xie et al., 2006; Carnini et al., 2007; Zhang et al., 2008). Additionally, administration of volatile anesthetics (e.g. 0.8-1% halothane) for 120min/day for 5 days has been shown to enhance plaque deposition in the female Tg2576-Tg mouse model of AD (Bianchi et al. 2008). However, some other studies using injected anesthetics did not

report any alteration of endogenous APP or Aβ peptides in non-Tg control mice or rats (Palotas et al., 2005; Planel et al., 2007a). Whether the variation of these results is due to injected vs inhaled anesthetics or wild-type animals vs animals with pre-existing Aβ pathology remains to be determined. Nevertheless, a recent prospective clinical study of 2300 middle age Finish men followed over 20 years reported that repeated heat exposure through sauna bathing is inversely associated with dementia and AD pathology. The frequency of sauna bathing is also found to be proportional to lowering the risk of developing dementia and AD. This effect could be mediated by a number of factors, including better brain vascular functions and/or higher body temperature (Laukkanen et al., 2017).

Until now most of the preclinical studies have been carried out using cultured neurons/cell lines or animal models of AD to measure the effects of temperatures on APP levels/processing. No information is available on how temperature can influence APP metabolism in astrocytes either under *in vitro* or *in vivo* paradigms. The present study, using the MTT assay, clearly shows that a hyperthermic condition can enhance viability of cultured astrocytes without altering the expression level of GFAP – an intermediate filament protein that is altered in response to injury or disease pathology such as AD. Since the MTT assay measures mitochondrial activity which may be influenced by temperature-regulated cellular metabolism, it is important to validate our results in future studies using markers of cell survival/death following exposure to hypo-/hyperthermic conditions. A recent study using cultured astrocytes from the spinal cord, in keeping with our results, showed that a mild hypothermic condition (i.e., 33^{o}C) can also influence viability of astrocytes and their growth (Kang et al., 2016).

It is of interest to note that APP holoprotein levels are markedly higher at 37°C as well as at 40°C compared to hypothermic 27°C as reported in an earlier study (Ciallella et al., 1994). This is accompanied by enhanced APP-CTFα and APP-CTFβ levels, thus raising the possibility that an increased production or decreased clearance mechanism may underlie the cause of the observed changes. Since APP-CTFα and APP-CTFβ are products of non-amyloidogenic α-secreatse and amyloidogenic β-secretase pathways respectively, we subsequently evaluated the levels of the α-secretase ADAM10 and β-secretase BACE1 which are found to be differentially altered across three temperature conditions. Given the evidence that steady-state levels of ADAM10 and BACE1 often do not correspond with the activity of the enzymes, it will be interest to determine how the activity of the enzymes is influenced following exposure to hypothermic, normal and hyperthermic conditions. The increased secretion of $A\beta_{1-40}$ at 37°C and 40°C compared to 27°C may be the consequence of increased production leading to release of the peptide. This notion can be validated only after analysing the activity of the γ-secretase enzyme complex as the steady-state levels of PS1 and nicastrin showed variations following exposure to three temperature conditions. It is, however, of interest to note that the levels of Aβ-degrading enzyme neprilysin, but not IDE, are substantially decreased at hyperthermic conditions, thus providing indirect support for the increased release of $A\beta_{1-40}$ following its accumulation in cultured astrocytes.

The autophagy-lysosomal system plays a critical role in the regulation of APP and Aβ metabolism. Several lines of experimental evidence suggest that the autophagic pathway as well as the lysosomal system are impaired in AD pathology and contribute to the generation as well as accumulation of Aβ peptides. Biogenesis and function of lysosomes are usually controlled by a ubiquitously expressed master regulator, TFEB – a member of the MiT family of transcription factors (Koh et al.,

2008; Hage et al., 2010; Kegel et al., 2010; Zhang et al., 2011) Under normal conditions, TFEB is phosphorylated at Ser^{211}/Ser^{142} residues by the regulator of the autophagic pathway, i.e., mammalian target of rapamycin complex 1 (mTORC1) and is retained in the cytoplasm through its interaction with members of the 14-3-3 protein family. However, when not phosphorylated due to inactivation of mTORC1 or dephosphorylated by serine-threonine phosphatase calcineurin, TFEB is translocated to the nucleus to promote transcription of the coordinated lysosomal expression and regulation (CLEAR) network genes such as lysosomal hydrolases, membrane proteins, proton pumps and others that increase the number of lysosomes and promote the degradation of lysosomal substrates (Deane et al., 2004; Chong et al., 2006; Sagare et al., 2007; Miners et al., 2011; Weller et al., 2011; Ha et al., 2012). Lysosomal biogenesis has been shown to protect cells against toxicity in *in vitro* and *in vivo* paradigms (Farris et al., 2003; Weller et al., 2011; Nalivaeva et al., 2012; Wang et al., 2012). The significance of the astrocytic lysosomal pathway in AD pathology is highlighted by the fact that enhancing astrocytic lysosomal biogenesis has been shown to attenuate Aβ levels/deposition in an animal model of AD (Xiao et al., 2014). Our results revealed that steady-state levels of the lysosomal marker LAMP1 and autophagy marker LC3-II are increased with time in all three temperature conditions, but the changes were more apparent at 27°C and 40°C than at 37°C. Although no significant alteration in LAMP1 levels was noted across the temperatures, LC3-II levels were found to be increased in both hypo-/hyperthermic conditions compared to the normal condition (37°C). Given the evidence that TFEB levels decreased time-dependently but showed very little variation across the three temperature conditions, it is likely that increased levels of LAMP1 and LC3-II may represent accumulation of lysosomal and autophagic vacuoles in cultured astrocytes due to impaired clearance rather than increased lysosomal biogenesis.

Normal astrocytes, unlike neurons, generate very little Aβ due to low expression of APP and BACE1 (Zhao et al., 1996; Calhoun et al., 1999; Bordji et al., 2010; Zhao. et al., 2011; Thal 2012). Following activation as a consequence of insults or pathological conditions such as AD, astrocytes are known to display higher levels of APP or its processing enzymes (Thal et al., 2000; Rossner et al., 2001; Hartlage-Rubsamen et al., 2003; Nagele et al., 2003; Heneka et al., 2005). Increased Aβ production and secretion at an early stage of disease pathology/insults possibly triggers an inflammatory reaction leading to enhanced levels of proinflammatory cytokines such as TNFα and IFNγ in astrocytes that can activate APP processing *via* an amyloidogenic pathway (Seifert et al., 2006; Li et al., 2011; Zhao et al., 2011). Thus, a vicious feed-forward cycle may occur, which in turn promotes increased synthesis/accumulation as well as release of astrocytic Aβ peptides. This is partly substantiated by the evidence that reducing astrocyte activation by targeting their inflammatory pathway can attenuate neurologic changes in an animal model of AD (Furman et al., 2012). There is also evidence that increasing lysosomal biogenesis in astrocytes can attenuate Aβ levels/deposition in an animal model of AD (Xiao et al., 2014). Activated astrocytes, which are closely associated with neuritic plaques in AD brains, may also contribute to the pathology *via* other mechanisms including i) impairing metabolic support and glutamate recycling, thereby increasing neuronal vulnerability to toxicity (Steele and Robinson, 2012) , ii) generating reactive oxygen and nitrogen species that may influence death of neurons (Farfara et al., 2008), iii) exacerbating Aβ-induced toxicity as demonstrated in cultured conditions (Domenici et al., 2002; Garwood et al., 2011) and iv) regulating efficient clearance of Aβ from the brain (Saido and Leissring 2012). These results, together with the present evidence that ambient temperature is able to influence APP/Aβ metabolism in astrocytes, raise the possibility of a role not only for the ambient temperature but also for activated astrocytes in the development/progression of AD pathology.

Table-2: Changes in various markers compared to 37°C

Marker proteins	27°C			40°C		
	6h	12h	24h	6h	12h	24h
GFAP	NA	NA	NA	NA	NA	NA
APP	NA	NA	+	NA	NA	NA
APP-KPI	NA	NA	-	NA	NA	NA
CTF-α	NA	NA	-	NA	NA	+
CTF-β	-	-	-	NA	+	NA
ADAM10	-	-	NA	NA	NA	NA
BACE1	NA	NA	NA	NA	NA	NA
PS1	+	NA	NA	+	NA	NA
NICASTRIN	+	NA	-	+	NA	-
IDE	NA	NA	NA	NA	NA	NA
NEPRILYSIN	-	NA	NA	-	NA	-
LAMP1	NA	NA	NA	NA	NA	NA
LC3-II	NA	+	+	NA	NA	+
TFEB	NA	NA	NA	NA	NA	-

(+) indicates significant increase; (-) indicates significant decrease; (NA) indicates no significant alteration

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