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# PRKAG2 Gene Expression Is Elevated and its Protein Levels Are Associated with Increased Amyloid- $\beta$ Accumulation in the Alzheimer's Disease Brain

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**Abstract.** Increased amyloid- $\beta$  (A $\beta$ ) accumulation associated with abnormal autophagy-lysosomal activity and nutrient kinase dysregulation are common features in Alzheimer's disease (AD) brain. Recent studies have identified PRKAG2 and TIPRL genes that control nutrient kinase regulated autophagy, and here we determined if their expression is altered in postmortem AD brains. Gene and protein expression of TIPRL were unchanged. However, gene expression of PRKAG2 was increased 3-fold and its protein levels positively correlated with A $\beta$  accumulation in the AD brain. In summary, our findings suggest that increased PRKAG2 is an important contributing factor to A $\beta$  accumulation in the AD brain.

**Keywords:** Alzheimer's disease, amyloid- $\beta$ , autophagy, PRKAG2, TIPRL

## INTRODUCTION

Autophagy is an important clearance pathway for misfolded and aggregate proteins and its dysfunction has been implicated in the impaired clearance of aggregate proteins in neurodegenerative diseases including Alzheimer's disease (AD), frontotemporal dementia (FTD), and Lewy body dementia (LBD). The autophagy pathway is impaired at different steps in various types of dementias and mostly associated with increased autophagy initiation and reduced

autophagosome degradation [1]. AD brain shows a marked upregulation of lysosomal activity, including extensive involvement of autophagosomes and acid hydrolases such as cathepsin D with amyloid- $\beta$  (A $\beta$ ) protein deposits [1, 2]. Whereas, LBD is characterized by a decrease in Atg7 expression which is predicted to result in deficient initiation of the autophagic process [3]. A high proportion of FTD cases have a hereditary component and several pathogenic gene mutations impact pathways associated with later steps of autophagy including the CHMP2B (charged multivesicular body protein 2B) and VCP (valosin containing protein) genes, which lead to impaired autophagosome maturation [4].

Autophagy is closely regulated by nutrient signaling kinases [mammalian (or mechanistic) target

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of rapamycin complex 1] (mTORC1) and AMP-activated kinase (AMPK). mTORC1 and AMPK act as the main upstream sensors, while Unc-51 like autophagy activating kinase 1 (ULK1) complex integrates the upstream signals and transduces them to the downstream autophagy pathway [5]. Signaling from mTORC1 and AMPK pathways is essential for regulating anabolic and catabolic pathways and growing evidence suggests that these nutrient sensing kinases are dysregulated in AD and other neurodegenerative diseases [6]. Studies have identified regulatory genes PRKAG2 (Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 2) and TIPRL (TOR Signaling Pathway Regulator) that modulate the ability of AMPK and mTORC1 to control autophagy during stress. AMPK is a heterotrimeric protein complex composed of 3 subunits including a non-catalytic regulatory gamma subunit PRKAG2. AMPK is an evolutionarily conserved master switch in the cellular energy balance activated during metabolic stress [7] [8]. PRKAG2 homolog, SNF4A $\gamma$  is required for regulation of developmental and stress-induced autophagy in a drosophila model [9]. TIPRL facilitates amino-acid regulated mTORC1 signaling through its association with protein phosphatase 2A (PP2Ac). TIPRL promotes H2AX (histone H2A variant) phosphorylation during DNA damage [10] and also functions as a negative regulator of autophagy [11]. The above studies show an important role for PRKAG2 and TIPRL in regulating nutrient kinase signaling and autophagy during stress. But whether their expression levels are altered in the AD brain and if they are associated with impaired autophagy and accumulation of A $\beta$  is unknown.

The main aim of this study was to determine the gene and protein expression levels of autophagosome marker microtubule-associated protein 1A/1B-light chain 3B (LC3), lysosomal hydrolase Cathpesin D, autophagy regulators PRKAG2 and TIPRL in frontal cortex and hippocampal regions of postmortem brain tissues of patients with AD, FTD, LBD, and in healthy controls. We also assessed whether A $\beta$  accumulation correlated with autophagy related proteins LC3BI, LC3BII, Cathpesin D, PRKAG2, and TIPRL. Our findings show that gene expression of PRKAG2 was increased 3-fold in AD-hippocampus and AD-frontal cortex and its protein levels positively correlated with A $\beta$  accumulation in the brain. Whereas, gene and protein expression of TIPRL were unchanged. Our findings suggest that increased PRKAG2 may be an important contributing factor to A $\beta$  accumulation in AD brain.

## MATERIAL AND METHODS

### *Postmortem brains*

All experiments were conducted in frontal cortex (F) and hippocampus (H) regions of postmortem brain tissues of patients with AD ( $n=15$ ), FTD ( $n=5$ ), LBD ( $n=5$ ), and healthy controls ( $n=5$ ) obtained from the Sydney Brain Bank (SBB), approved by Human Research Ethics Committee of Edith Cowan University (approval number 14820).

### *cDNA synthesis and gene expression analysis in brain homogenates*

Frozen brain tissues (100 mg) were suspended in 300  $\mu$ l Tris-buffered saline (TBS), pH 7.4 with ceramic beads and homogenized at 4000 RPM for 3 cycles of 45 s using a MoBio Powerlyzer<sup>TM</sup> at 4°C. The brain homogenates were aliquoted into 100  $\mu$ l lots and stored at -80°C. RNA and cDNA preparation were prepared from the brain homogenates using RNeasy lipid tissue mini extraction kit and Qiagen reverse transcription kit. For the quantification of the products (RNA and cDNA), Qubit<sup>TM</sup> dsDNA HS Assay kits (ThermoFisher) were used. Gene expression of autophagosome marker LC3B (Hs00797944\_s1), lysosomal hydrolase Cathpesin D (CTSD, Hs00157205\_m1), PRKAG2 (Hs00211903\_m1), TIPRL (Hs00295580\_m1) and endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs03929097\_g1) was conducted on cDNA synthesized from brain homogenates using Taqman assays on the QuantStudio 12K system. Fold change in gene transcripts was measured using comparative Ct method using the QuantStudio(TM) Real-Time PCR Systems software platform, with GAPDH as endogenous control and frontal cortex from healthy controls (Cont-F) as the reference biological group.

### *Westerns quantification for brain homogenates*

Brain homogenates in TBS were used for western blotting analysis and protein quantification of A $\beta$ , LC3B isoforms LC3BI and LC3BII, Cathpesin D, PRKAG2, TIPRL and control GAPDH. All samples were loaded in duplicate using 4–12% BisTris gels, including a pooled brain homogenate sample as an internal control in each gel. An average of all the internal controls (mean internal control) for each antibody

and an internal control factor for each gel was calculated. Average of the duplicate of every sample was calculated and normalized using the internal control factor for the respective gel. A $\beta$  antibody WO2 (3.5 kDa) was a generous gift from Prof. Colin Masters, Melbourne. Other antibodies including GAPDH (37 kDa), LC3B (14 and 16 kDa), mature Cathepsin D (30 kDa), PRKAG2 (60 and 75 kDa isoforms), and TIPRL (32 kDa) were purchased from Cell Signaling or Abcam.

### Statistical analysis

To determine statistical significance for comparisons of gene and protein markers measured in brain tissue samples as Mean  $\pm$  Standard Deviation (SD), 1-way ANOVA was used, while Pearson's correlation was used to assess correlation. Statistical significance was considered post-Tukey correction. All the statistical analyses were conducted on Graphpad Prism software.

## RESULTS

### Increased PRKAG2 gene expression in AD brain

Increased accumulation of A $\beta$  and elevated lysosomal activity are pathological hallmarks of AD, so we initially assessed expression levels of these markers in frontal cortex (F) and hippocampal (H) regions of postmortem brain tissues of patients with AD, FTD, LBD, and in healthy controls. We first assessed A $\beta$  and GAPDH levels in postmortem brain tissues by western immunoblotting (Fig. 2, Supplementary Figures 1 and 2). A $\beta$  was mostly detected as a monomer at 4 kDa; however, its length was not known. A $\beta$  levels were significantly higher in AD-F as compared to Cont-F. A $\beta$  levels was also high in AD-H as compared to Cont-H, but was not significant. LBD-F also showed increased A $\beta$  levels, as compared to Cont-F indicative of the AD like neuropathology routinely observed in LBD brains [12]. Although our findings show increased A $\beta$  levels in AD-H compared to Cont-H, it was not significant. It is now widely accepted that A $\beta$  accumulation in the brain does not always correlate with neurodegeneration and there are individuals who have low brain amyloid with neurodegeneration (A-N+) and ones who have high amyloid without neurodegeneration (A+N-) [13]. We therefore postulate that this maybe a contributing factor to the low level of A $\beta$  detected in the hippocampi of AD subjects.

To address this issue in more details, we assessed A $\beta$  protein levels quantified by western immunoblotting (6E10 antibody) in individual samples of frontal cortex and hippocampal samples of AD and control (Supplementary Figure 2). Low A $\beta$  levels (as compared to Fig. 2) were observed in AD frontal cortex samples 13 and AD hippocampus samples 2, 7, and 11. High A $\beta$  levels (as compared to Fig. 2) was observed in control hippocampus sample 3. Overall, this data supports the growing literature suggesting that A $\beta$  accumulation does not always correlate with neurodegeneration.

Next we assessed gene and protein expression of autophagosome marker LC3B and lysosomal hydrolyase Cathepsin D in postmortem brain tissues. Fold change in gene transcription was measured using comparative Ct method with GAPDH as endogenous control and Cont-F as the reference biological group. No significant changes were observed in the expression of LC3B and Cathepsin D between the different groups. Also, no significant change in the protein levels of LC3BI (16 kDa) and LC3BII (14 kDa) was observed between the different groups. However, levels of mature Cathepsin D (30 kDa) was increased in AD-F and LBD-F regions as compared to Cont-F ( $*p < 0.05$ ). We observed increased levels of mature cathepsin D protein in Cont-H, as compared to Cont-F; however, this was not significant. Overall, our findings above were similar to previous studies of postmortem AD brains [1] showing A $\beta$  accumulation associated with increased Cathepsin D, indicative of elevated lysosomal activity in AD, particularly in the frontal cortex region.

We next assessed gene and protein expression of autophagy regulators PRKAG2 and TIPRL (Fig. 2). No significant changes were observed in the gene or protein expression of TIPRL between different groups. A small decrease in TIPRL protein was observed in AD-F and AD-H compared to healthy control, but was not significant. Notably, a 3-fold increase in PRKAG2 gene expression was observed in AD-F as compared to FTD-F, LBD-F, and Cont-F ( $*p < 0.05$ ). Despite a marked upregulation of PRKAG2 gene expression in AD-F, no significant change in levels of PRKAG2 protein (60 and 75 kDa isoforms) were observed between different groups. We observed increased expression of PRKAG2 protein in frontal cortex compared to hippocampus in LBD, FTD, and healthy controls; however, this was not significant. Overall, these findings showing elevated PRKAG2 gene expression combined with increased mature Cathepsin D protein is in agreement

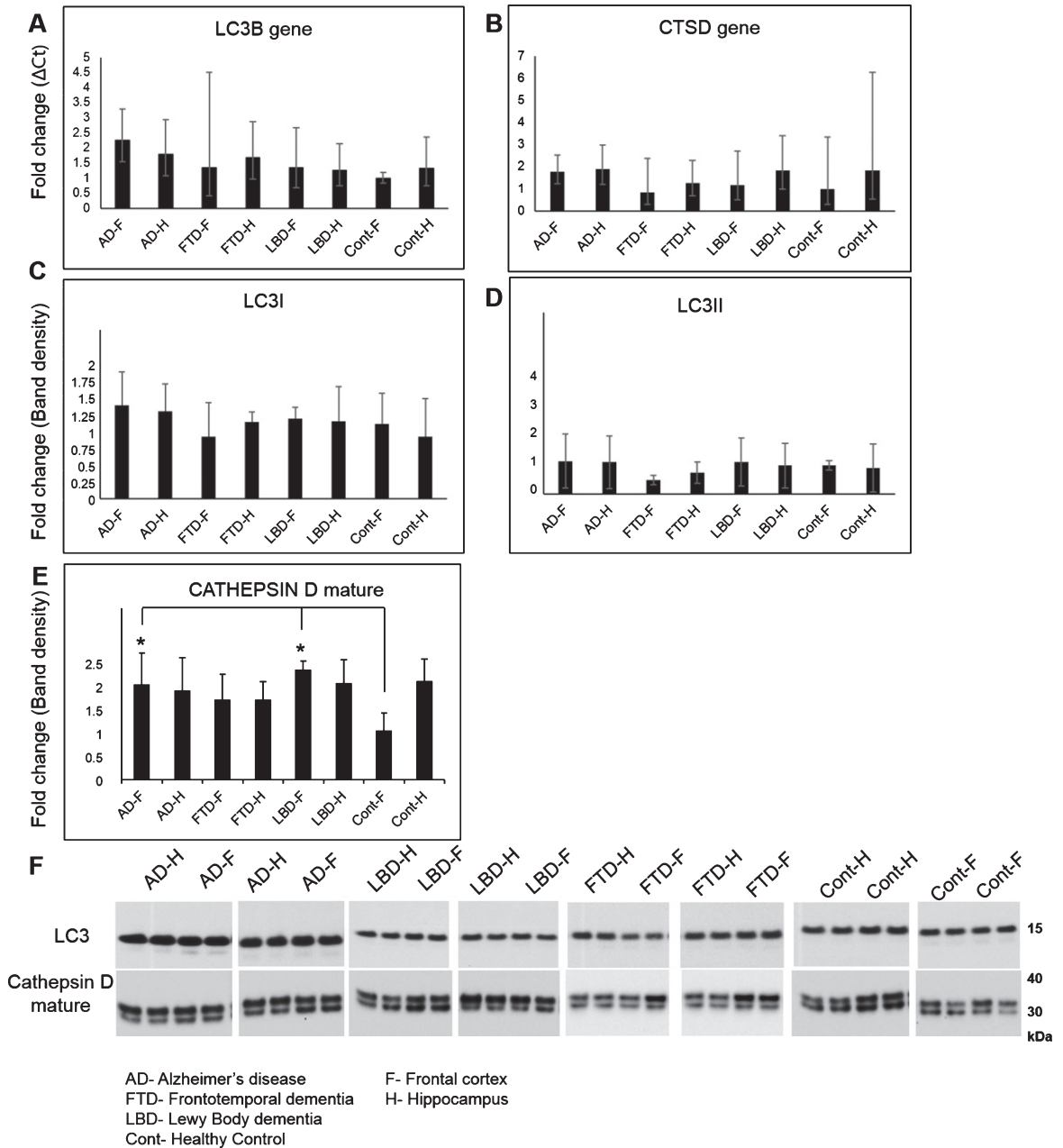


Fig. 1. Gene and protein expression of autophagy markers LC3B and Cathepsin D in AD, FTD, LBD, and control brains. Gene expression of autophagosome marker LC3B (Hs00797944\_s1), lysosomal hydrolase Cathepsin D (CTSD, Hs00157205\_m1) and endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs03929097\_g1) was conducted on cDNA synthesized from homogenates of frontal cortex (F) and hippocampus (H) regions of postmortem brain tissues from AD, LBD, FTD, and healthy controls (Cont) using Taqman assays on the QuantStudio 12K system. Fold change in gene transcripts of LC3B (A) and CTSD (B) was measured using comparative Ct method with GAPDH as endogenous control and Cont-F as the reference biological group. No significant changes were observed in the expression of LCB and Cathepsin D between the different groups. Protein levels of LC3B isoforms LC3I (C) and LC3II (D) and mature isoform of Cathepsin D (E) was also measured using western blotting analysis of the brain homogenates. A representative image of the western blot is shown here (F). No significant change in levels of LC3I (16 kDa) and LC3II (14 kDa) were observed between the different groups. Levels of mature Cathepsin D (30 kDa) was increased in AD-F and LBD-F regions as compared to Cont-F (Mean $\pm$ SD, \* $p$ <0.05).

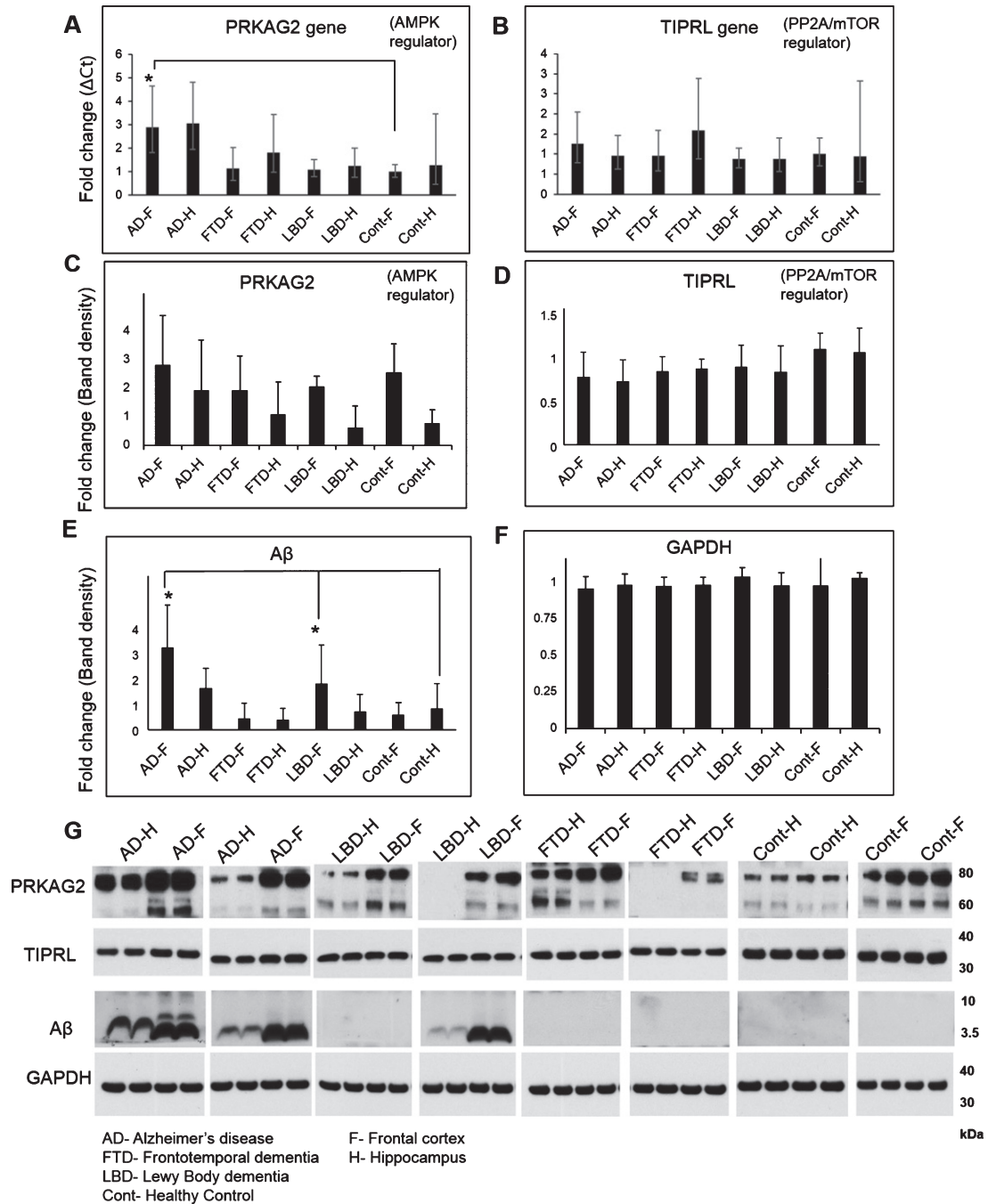


Fig. 2. Gene and protein expression of autophagy regulators PRKAG2, TIPRL, and Aβ in AD, FTD, LBD, and control brains. Gene expression of autophagy regulators PRKAG2 (Hs00211903.m1), TIPRL (Hs00295580.m1), and endogenous control GAPDH (Hs03929097.g1) was conducted on cDNA synthesized from homogenates of frontal cortex (F) and hippocampus (H) regions of postmortem brain tissues from AD, LBD, FTD, and healthy controls (Cont) using Taqman assays on the QuantStudio 12K system. Fold change in gene transcripts of PRKAG2 (A) and TIPRL (B) was measured using comparative Ct method with GAPDH as endogenous control and Cont-F as the reference biological group. A 3-fold increase in PRKAG2 transcripts was observed in AD-F as compared to FTD-F, LBD-F and Cont-F (Mean±SD, \**p*<0.05). No significant changes were observed in the expression of TIPRL between the different groups. Protein levels of PRKAG2 (C), TIPRL (D), Aβ (E), and GAPDH (F) was also measured using western blotting analysis of the brain homogenates. A representative image of the western blot is shown here (G). No significant change in levels of PRKAG2 (60 and 75 kDa isoforms) and TIPRL (32 kDa) were observed between the different groups. Aβ (4 kDa) was significantly higher in AD-F and LBD-F as compared to Cont-F (Mean±SD, \**p*<0.05).

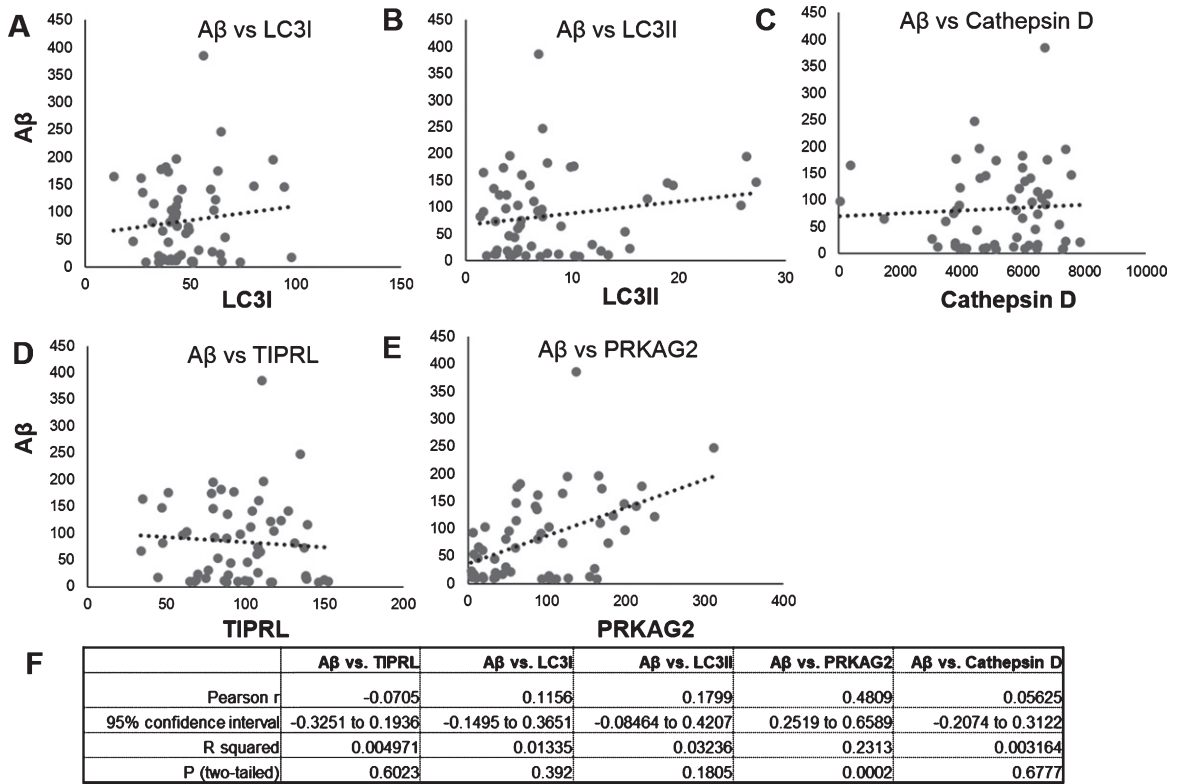


Fig. 3. Correlation of Aβ and autophagy proteins LC3, Cathepsin D, PRKAG2, and TIPRL mRNA and protein levels. Correlation of Aβ protein levels (Fig. 2) to protein levels of LC3B isoforms, LC3I (Fig. 2C), LC3II (Fig. 2D), mature Cathepsin D (Fig. 2E), PRKAG2 (Fig. 3C), and TIPRL (Fig. 3D) was done using Pearson's correlation analysis. Aβ levels positively correlated to PRKAG2 levels ( $r=0.4809$ ,  $p<0.0005$ ).

with previous studies showing increased autophagy induction in AD brain.

*PRKAG2 protein levels positively correlate with increased Aβ accumulation in brain*

Our findings above show increased lysosomal activity and expression of autophagy regulator PRKAG2 in the AD brain. To determine if Aβ accumulation is related to changes in PRKAG2 and autophagy-lysosome pathway in the brain, we performed a Pearson correlation analysis of Aβ levels to levels of PRKAG2, TIPRL and autophagy markers LC3B and Cathepsin D. No significant correlation was observed between Aβ and LC3B, Cathepsin D or TIPRL. However, a significant positive correlation was observed between Aβ and PRKAG2 ( $r=0.4809$ ,  $p=0.0002$ ). The variability observed in the correlation is possibly due to the significantly higher levels of Aβ in AD and LBD brains as compared to FTD and control brains, as shown in Fig. 2. Although PRKAG2

protein was not significantly increased in AD, we observed that increased Aβ accumulation was associated with increased PRKAG2 protein expression. PRKAG2 is a regulator of stress-induced autophagy and overall the data suggests that increased PRKAG2 gene expression maybe a response to increased Aβ accumulation and proteotoxic stress in the brain. Although there was increased transcript levels of PRKAG2 in AD, we did not observe its translation into protein. Growing evidence suggest that the spatial and temporal variations of mRNAs, as well as the local availability of resources for protein biosynthesis, strongly influence the relationship between protein levels and their coding transcripts and that transcript levels are not sufficient to predict protein levels in many scenarios, especially during cell dysfunction and disease [14]. It is therefore possible that the lack of translation of increased PRKAG2 gene expression to protein levels in AD postmortem brains is possibly due to impaired protein synthesis as a result of brain atrophy and neurodegeneration.

## DISCUSSION

Autophagy is the primary catabolic pathway activated by nutrient signaling kinases AMPK and mTORC1 in response to cellular stressors including nutrient and energy starvation. Increased induction of autophagy is relatively frequent in neurodegenerative diseases, but whether this greater induction rate signifies disease development is yet to be established. Our findings showing increased gene expression of PRKAG2, a positive regulator of autophagy suggest that it may be a contributing factor to autophagy induction in AD. The autophagy pathway is impaired at different steps in neurodegenerative diseases and mostly associated with increased autophagy initiation and reduced autophagosome degradation [1, 15]. Our findings showing increased PRKAG2 gene expression in AD combined with a positive association with A $\beta$  levels suggest that PRKAG2 dysfunction is specific to AD and related to A $\beta$  accumulation, a pathological hallmark of AD.

Mutations in PRKAG2 are associated with heart muscle disease [16, 17] and single nucleotide polymorphisms (SNPs) in PRKAG2 have shown to be associated with cognitive impairment and metabolic dysfunction in old age [18]. Whether these PRKAG2 mutations and SNPs alter PRKAG2 expression in the brain leading to autophagy dysfunction and accumulation of protein aggregates like A $\beta$  is unknown. Transcription factor EB (TFEB) is the master regulator of lysosome biogenesis and autophagy, including PRKAG2 transcription [19]. The role of TFEB in AD pathogenesis remains contradictory. Increased TFEB expression and its targets have been observed in AD patient derived fibroblasts [20] and in the hippocampus of AD subjects [21]. Similar upregulation of TFEB and its targets have been reported in transgenic AD mice [22, 23]. These findings strongly support TFEB gain-of-function in AD may contribute to increased PRKAG2 expression observed in our findings (Fig. 2). However, in contrast, AD patient lymphocytes and monocytes has markedly decreased TFEB expression [24]. In agreement, subcellular fractionation of AD patient brain samples documented a selective loss of nuclear TFEB [25]. These opposing results demonstrate the need for a thorough dissection of TFEB dysfunction in AD and its potential role in regulating PRKAG2 activation in AD brain.

Our findings suggest that increased PRKAG2 gene expression could be a response to increased A $\beta$  accumulation and proteotoxic stress in the brain. Increased

autophagy induction has been previously observed in experimental models in which a disease-related protein such as  $\alpha$ -synuclein or SOD1 is overexpressed [26, 27]. It is possible that autophagy induction in response to mutant or damaged proteins and aggregates could be compensatory and neuroprotective at early stages of disease, but may be counterproductive if lysosomal clearance becomes compromised. Impaired lysosomal clearance maybe induced by protein aggregates like A $\beta$  which in turn could also secondarily elevate autophagy induction, promoting further damage to the neuronal processes. The molecular mechanisms that link defective autophagy and A $\beta$  accumulation in AD are unclear and our findings suggest that the combination of increased autophagy induction via PRKAG2 and defective clearance of autophagosomes can create conditions favorable for A $\beta$  accumulation in AD. Further investigation of PRKAG2 gene and variants in modulating neuronal autophagy and A $\beta$  accumulation in disease models and patients will be critical to establish its pathogenic role in AD.

## DISCLOSURE STATEMENT

Authors' disclosures available online (<https://www.j-alz.com/manuscript-disclosures/19-0948r2>).

## SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <https://dx.doi.org/10.3233/jad-190948>.

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