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Short Communication

The Orphan C2orf40 Gene is a Neuroimmune Factor in Alzheimer's Disease

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Abstract

Expression of the orphan C2orf40 gene is associated with the aggregation of the neurofibrillary tangle-protein tau in transgenic mice, tumor suppression, the induction of senescence in CNS, and the activation of microglia and peripheral mononuclear leukocytes. This gene also encodes several secreted pro- and anti-inflammatory neuropeptide-like cytokines, suggesting they might be implicated in the inflammatory component(s) of Alzheimer's disease (AD). Accordingly, we evaluated human AD and control brains for expression changes by RT-qPCR, Western blot, and histological changes by immunolabeling. RT-qPCR demonstrated increased cortical gene expression in AD. The molecular form of Ecrg4 detected in cortex was 8-10 kDa, which was shown previously to interact with the innate immunity receptor complex. Immuno cytochemical studies showed intensely stained microglia and intravascular blood-borne monocytes within cerebral cortical white matter of AD patients. Staining was diminished within cortical neurons, except for prominent staining in neurofibrillary tangles. Choroid plexuses showed a decreasing trend. These findings support our hypothesis that c2orf40 participates in the neuroimmune response in AD.

ABBREVIATIONS

AD: Alzheimer's Disease; C2orf4: Human Chromosome 2open Reading Frame 4; CD14: Cluster Of Differentiation 14; CNS: Central Nervous System; CP: Choroid Plexus; DAPI- 4':6-Diamidino 2 Phenylindole; Ecrg4: Esophageal Cancer Related Gene 4, The Precursor Protein Encoded By C2orf40; Ecrg4 (31-148): Amino Acids 31-148 Of Ecrg4; Ecrg4 (71-148): Amino Acids 71-148 of Ecrg4; FTD- Fronto Temporal Dementia; MD2- Lymphocyte Antigen 96; PC- Prohormone Convertase; NFT: Neuro Fibrillary Tangle; RT-qPCR: Reverse Transcription- Quantitative Polymerase Chain Reaction; TLR4: Toll-Like Receptor 4

INTRODUCTION

Despite the early recognition of cerebral amyloid plaques and neurofibrillary tangles in Alzheimer's disease (AD) by Alois Alzheimer in 1906, the causative factors responsible for the pathogenesis of AD remain elusive. The amyloid hypothesis of AD may not account for other age related changes that might be necessary to generate sporadic AD. These include (i) age-related white matter disruption [1], (ii) gliosis and the secretion of neurotoxic pro-inflammatory mediators [2], (iii) vascular impairment [3], (iv) stress on CSF sink action [4-6] and (v) the most common genetic predisposition to AD, Apolipoprotein E haplotype [7,8], is involved in intercellular lipid and cholesterol exchange. As a result of these observations, there is emerging evidence that immunologic, repair and homeostatic responses contributing to glial and neuronal cell dysfunction and death may be required for cognitive decline and progression of AD. This may begin to explain why anti-amyloid therapies (e.g. vaccines, passive antibody therapies, and secretase inhibitors) work in AD transgenic mouse models but have failed to improve cognitive function in humans [9-11].

The identification of new injury responsive genes in AD could provide insight into the etiology of this disease. Therefore, we hypothesized that a recently identified candidate tumor repressor gene called c2orf40 that we associated with the brain injury response [12,13], glioma [14], and innate immunity [15,16] might be differentially regulated in the AD brain. Its homologous mouse gene, 1500015O10Rik, is upregulated over 8-fold in a transgenic

mouse model engineered to develop similar neurofibrillary tangle pathology as human AD patients via neuronal over expression of the protein tau [17]. C2orf40 encodes a pre-protein precursor called esophageal cancer-related gene-4 (Ecrg4 [18]) that has features of a pro-hormone precursor [19]: (i) a hydrophobic leader sequence consistent with sorting for secretion, (ii) highly conserved sequence identity amongst species, and (iii) consensus protease processing sites that can generate up to 8 novel peptides [20,21]. The exact function of Ecrg4 peptides is unknown, but strong evidence points to its epigenetic silencing in various epithelial cancers, [22-27], contrasted by upregulation during senescence by neurons and CNS progenitor cells [28]. A precursor-derived peptide Ecrg4 (31-148) is constitutively secreted and tethered to the cell surface of epithelial cells [13] and circulating leukocytes [15]. It is proteolytically shed from the cell surface generating a shorter peptide [29] immediately following injury [15]. Therefore, peptides may bind to target cells to initiate proliferative, inhibitory, pro- and anti-inflammatory responses depending on its processing [13,15,19-21,23,29-31].

Because of its tie to tau-associated pathology in transgenic mice, senescence, CNS injury and inflammation [13,15,17,28], we hypothesized that c2orf40 expression could be altered in human AD compared to normal aged brain. Accordingly, we examined cerebral cortical and choroid plexus tissue from AD, cognitively normal, and disease control (frontotemporal dementia, FTD) brains obtained at autopsy for any changes in c2orf40 gene expression, Ecrg4 protein fragment(s) and cell localization. Results show that there is a differential expression of c2orf40 in cell types of the AD brain that implicate c2orf40/Ecrg4-derived peptides as neuroimmune factors in AD.

MATERIALS AND METHODS

Samples

Brains from cognitively normal age-matched, AD, and frontotemporal dementia (FTD, used as a disease control) individuals were obtained at autopsy and provided by the Brown University Brain Tissue Resource Center in accordance with protocols approved by the Lifespan/ Rhode Island Hospital Institutional Review Board for human studies and the Brown University Brain Tissue Resource Center (Providence, RI). Demographic and tissue data for samples used in biochemical analysis and immunohistochemical labeling are shown in (Tables 1 and 2). All samples used for immunohistochemical localization studies (Table 2) were fixed in 10% neutral buffered formalin and had post mortem intervals (PMI) of < 22 hr. All AD patients' brain samples were subjected to a formal neuropathological examination from an expert neuropathologist. The diagnosis of AD is based upon NIA Reagan criteria as well as Braak and Braak staging. There were no significant differences in PMI among control and AD groups, as determined by Student's T-test. There was no significant difference in age in samples used for analysis, and there was no significant difference in PMI.

Antibodies

Immunohistochemistry: anti-Ecrg4 IgY antibody generation was contracted to GenWay Biotech, Inc. (San Diego, CA). The c2orf40 ORF encoding Ecrg4 (71-148) was used for immunization. Characterization reveals the epitopes are in this C-terminal region of the precursor and processed product. Commercially available monoclonal mouse anti-human CD68 and polyclonal rabbit anti-tau antibodies were purchased from Cell Marque (Rocklin, CA; Catalog #168M-96) and Dako (Carpinteria, CA; Catalog #A002401-2), respectively.

Western blotting: Rabbit polyclonal antibody was purchased from Sigma Aldrich (St. Louis, MO) catalog #HPA008546. This antibody detects Ecrg4 (31-148), 14 kDa, and Ecrg4 (71-148), 8-10 kDa recombinant proteins, and the same MW proteins from primary cell culture and conditioned medium [13].

Quantitative gene expression analysis: We used the standard curve method of RT-qPCR to quantify c2orf40 gene expression levels relative to β-Actin reference gene in AD patients and control individuals. RNA was purified by Trizol extraction according to manufacturer's protocol (Invitrogen, Carlsbad, CA). One microgram of RNA was used for reverse transcription with the BioRadiScript kit (BioRad, Hercules, CA). C2orf40 and β-actin gene expression levels were quantified on a BioRadi Q5 Real-Time PCR machine, with SYBR green detection (BioRadiQ SYBR Green Supermix). Forward and reverse primers were purchased from Qiagen (Quantitect Primer Assays for SYBR Green, assay Hs_C2orf40_1_SG, and β-actinHs_ACTB_1_SG). Plasmid DNA encoding human c2orf40 and β-Actin was diluted to a logarithmic curve of copy #/µl and used as a standard curve. The copies of gene from each brain sample were calculated by linear regression from this curve. Relative gene expression of c2orf40 was defined as (copy # c2orf40)/(copy #β-Actin) for each sample.

Western blotting: Choroid plexus and prefrontal cortex tissues were cut from brain blocks and pulverized on dry ice. Protein was extracted in RIPA buffer with protease inhibitor cocktail using standard techniques. Protein was quantified by BCA assay (reagents from ThermoFisher, San Diego, CA, used according to manufacturer's instructions). Protein was size fractionated by SDS-PAGE on 4-12% Bis-Tris Gels in NuPAGE MES-SDS buffer (Life Technologies, Grand Island, NY), and transferred to 0.45 µm nitrocellulose membrane in Tris-Glycine buffer. Membranes were blocked in 10% BSA in TBS/T overnight at 4°C, and then in primary antibody (rabbit anti-c2orf40, SigmaAldrich), at 1:5000 dilution in 1% BSA TBS/T overnight at 4°C. Membranes were washed three times in PBS/T and then incubated with goat anti-rabbit-HRP conjugated secondary antibody for 2 hours at 25°C. Membranes were again PBS washed and then incubated with chemiluminescent substrate reagent (Thermo SuperSignal West Pico Chemiluminescent Substrate according to manufacturer's instructions). Blots were imaged on an IVIS Lumina imaging machine (Waltham, MA).

Immunohistochemistry: Human brain tissue was fixed in neutral-buffered formalin (NBF), and paraffin embedded. After preparing 8 µm sections, tissue was deparaffinized and rehydrated. For antigen retrieval, the tissue was incubated in 10 mM sodium citrate buffer, pH 6 (Ecrg4) or 10 mM TRIS/1 mM EDTA buffer, pH 9 (CD68) at 95°C for 20 minutes and rinsed. Sections were quenched with dual endogenous enzyme-blocking reagent (Dako, Carpinteria, CA, USA) and blocked with 5% normal goat serum and incubated in 0.5 µg/ml chicken anti-Ecrg4IgY (GenWay) overnight at 4°C. Adjacent serial sections were also incubated with either mouse anti-CD68 (diluted 1:600) or rabbit

Table 1: Human brain samples used for biochemical studies.

Tissue	Control		Advanced AD		Frontotemporal Dementia	
Choroid plexus	N	11	N	6	N	2
	Ages	36 to 91	Ages	73-82	Ages	61, 88
	Gender	M - 6	Gender	M - 2	Gender	M - 1
		F - 4		F - 4		F - 2
	Post-mortem Interval	1-27 hr., 1 unknown	Post-mortem Interval	6-17 hr. 3 unknown	Post-mortem Interval	1 and 12 hr.
Cortex	N	4	N	4	None	
	Ages	67 to 91	Ages	80-88		
	Gender	M - 2	Gender	M -1		
		F - 4		F - 3		
	Post-mortem Interval	1-13 hr.	Post-mortem Interval	6-12 hr.		

Table 2: Human brain samples used for immunohistochemical studies.

Case	Age (yr.)	Sex	Post-mortem interval (hr.)	Brain weight (g)	Classification	Braak stage	Clinical diagnosis
1	57	F	15.5	1150	Control	No NT pathology	Respiratory & renal failure
2	68	M	8	1495	Control	No NT pathology	Hypertension; COPD
3	59	M	21	1395	Control	No NT pathology	Papillary renal cell carcinoma; multi-organ failure
4	77	F	13	1075	Advanced AD	Stage VI	Alzheimer's disease; hypoxic-ischemic encephalopathy
5	81	F	13	1033	Advanced AD	Stage V	Alzheimer's disease; cerebrovascular disease
6	61	F	11	1038	Advanced AD	Stage VI	Alzheimer's disease; cerebrovascular disease

anti-tau (diluted 1:600) for 30 min at room temperature. Goat anti-chicken-AlexaFluor594 conjugated IgG (Life Technologies) was used at 1 µg/ml for detection of immunofluorescence staining (Figure 5B). Biotinylated goat anti-chicken secondary antibody (Vector Laboratories, Burlingame, CA) was used at 1 µg/ml (Figures 2-4). For CD68 and tau immunostaining, HRP-labeled polymers conjugated with secondary antibodies (anti-mouse FLEX+; Dako; catalog #K8002 or Dual Link HRP; Dako; catalog #K4061) were applied for 30 min at room temperature, in accordance with the EnVision+ system for immunohistochemical staining. Ecrg4 immunoreactivity was detected using the standard avidin-biotin immunoperoxidase complex method as described elsewhere with reagents from Vector Laboratories [32]. As a control, pre-immune IgY from the same animal was used as the primary antibody for non-specific labeling and processed identically as with anti-Ecrg4 primary antibody. The protocol was optimized such that no signal was detected by pre-immune IgY.

Statistical analysis: Differences in gene expression levels between AD, cognitively normal and FTD controls were assessed by Student's t-test. Differences were determined to be statistically significant if $p < 0.05$

RESULTS AND DISCUSSION

Gene and protein expression changes were observed in AD samples compared to age-matched, cognitively normal controls in several instances, as we hypothesized. Several notable changes were observed in the AD cortex. General cortical gene expression levels (Figure 1A) were increased compared to age-matched, cognitively normal controls by RT-qPCR of prefrontal cortical tissue. Because the prefrontal cortex contains a heterogeneous cell population [33,34], different cortical cell types could contribute this gene expression increase unequally. Further analysis of the cellular distribution of cortical Ecrg4 protein was analyzed by immunohistochemical labeling below. Protein levels assessed by Western blot, however, (Figure 1B) showed no detectable changes in AD compared to control brains, of the 8-10 kDa form of Ecrg4. This peptide form, corresponding to the molecular weight of the carboxy-terminal 71-148 amino acid fragment, has been demonstrated to be a biologically active peptide [16,29].

Data from Ecrg4 immunolabeling studies (Figures 2-4) supports the evidence of overall gene expression differences between AD and controls (Figure 1). Ecrg4 immunohistochemistry showed a strikingly increased expression in cortical tissue affected by the neuroimmune response in AD patients. Strongly Ecrg4-immunoreactive microglial cells with an activated-like spindle morphology were observed in the white matter of the AD entorhinal cortex (Figure 2B, arrow). On the contrary, staining was very faint within control white matter (Figure 2A). The size and morphology of Ecrg4+ microglial cells in AD white matter was similar to CD68+ cells, a marker of microglia [35] (Figure 2C). Anti-Ecrg4 antibody also strongly labeled intravascular monocytes in AD brains (Figure 3B) that are known to respond to

amyloid plaques and contribute to neuroinflammation in AD [36-39]. Staining of blood-borne monocytes was less obvious in the vasculature of age-matched controls (Figure 3A). A substantially decreased presence of intravascular monocytes in control individuals correlates with reports from other groups [40]. Because Ecrg4 was not expressed by quiescent immunosurveilant microglia in the cognitively normal brain, we propose that Ecrg4 is up regulated as part of the inflammatory response in AD. The immunolabeling patterns (Figures 2-4) indicate Ecrg4 up-regulation by immune cells in response to AD.

Neurons of the entorhinal cortex, however, had diminished Ecrg4 immunoreactivity in AD (Figure 4B) compared to controls (Figure 4A). At high magnification, we further observed a pattern of Ecrg4immunolabeling (Figure 4C) consistent with the major neurofibrillary tangle protein tau [41] shows a similar morphology with anti-ECRG4 labeling in cortical neurons (Figure 4D). Taken together, these data suggest that among the types of Ecrg4 immunoreactive cells detected in gray matter of the cortex, the loss of Ecrg4 expression in AD appears to be restricted to non-immune cells suggesting different functions among cell types.

In choroid plexus tissue however, we found a decreasing trend of gene expression in AD compared to cognitively normal controls that was not statistically significant by RT-qPCR ($p = 0.17$) (Figure 5A), and with no significant changes between FTD disease controls and AD. Likewise, protein expression patterns detected by immunofluorescence labeling did not have different features between normal age-matched control (not shown) and AD (Figure 5B) (Figure 5B; Ecrg4 red, DAPI blue). Ecrg4 localized to secretory granules in the cytoplasm that appeared to be more concentrated at the apical surface of the choroid plexus epithelial (CPe) cell layer (Figure 5B arrow), a less so within endothelial cells of the vascular pedicle (Figure 5B, arrowhead). The pattern of staining appeared to be similar to the distribution described previously in normal human and rat CP. We had predicted changes in the choroid plexus because this tissue is known to be impaired and shows histological changes in AD [5,6], and because Ecrg4 levels are altered in a brain injury model [12,13]. The lack of change in our analyses of the CP supports a specific role for Ecrg4 in AD in microglia, peripheral monocytes and neurons (Figures 2-4).

Results indicate that Ecrg4 expression was altered in some but not all cell types in the AD brain. For example, we found specific Ecrg4 positive immune cells in AD, particularly in microglia of the white matter, that have a spindle-like activated morphology, and blood-borne monocytes. These findings are compatible with an up regulation of c2orf40 as part of the "repair-response" neuroimmune response to changes in AD (Figures 2-4). There was little labeling of astrocytes or gray matter microglia where we predicted the presence of inflammatory and repair capable cells expressing Ecrg4 [42,43]. Anti-Ecrg4 did not label oligodendrocytes in control or AD white matter, although protein expression has been reported in pools of oligodendrocyte precursor cells in the adult mouse brain [28]. One can therefore predict that the observed increase in Ecrg4 gene expression (Figure 1) is largely the result of elevated expression levels in activated microglia and/or macrophages that are migrating and proliferating in AD cortex. Therefore, an upregulation of Ecrg4 expression may serve to recruit other microglia or infiltrating monocytes predominantly into cerebral white matter.

The Ecrg4-immunoreactive microglia/macrophages observed in AD patients did not appear to be concentrated around senile plaques in the cerebral cortex (Figures 2 and 4). Such prominent aggregates are notably absent. All AD cases studied were severely demented with high pathological Braak stage (V-VI). Therefore, if Ecrg4+ microglia were localized within or surrounding amyloid-containing plaques, this staining pattern should have been clearly evident within the cerebral cortex. Our findings suggest that neuronal Ecrg4 levels are reduced in cortex and that the majority of Ecrg4 staining was seen in the white matter where it is associated with a diffuse microglial proliferation. In gray matter, Ecrg4 immunostaining patterns appear morphologically similar to NFTs (Figure 4), and not in a pattern indicating concentration around plaques. Although these initial studies are qualitative comparisons between advanced AD patients and cognitively normal individuals, our main finding of Ecrg4-positive cells within AD white matter was striking.

We have also shown that Ecrg4 is a recruitment factor for monocytes and macrophages in other diseases, including glioma [14,15,44]. Several studies indicate brain macrophages in neurodegeneration (under non-irradiated conditions) are leukocyte-derived, although the relative levels of resident compared to recently recruited macrophages are not clear[45,46]. Given that in neurodegenerative and brain injury conditions, many brain microglia are derived from circulating macrophages [43] it would appear plausible that microglial activation in AD may be stimulated by Ecrg4 in a manner similar to these other diseases.

It is still believed that monocytes do not normally cross the blood-brain barrier (BBB) [47,48], and the degree of neuronal dysfunction, and consequent inflammatory activation of microglia, might gauge the recruitment of leukocytes and lymphocytes through the BBB [10,46]. With this in mind, we cannot rule out age-related white matter degradation, or vascular disease as confounding factors to the Ecrg4+ microglia and intravascular monocytes we observed in the white matter of AD patients. Cerebrovascular diseases (e.g. age-related hypertension and Binswanger's 's disease) have been associated with a neuroimmune response particularly in white matter and to a lesser extent gray matter [8,11,49-51]. Cerebral amyloid angiopthy (CAA), which is highly prevalent among AD patients (> 77%, [52,53], is considered a major pathological and etiological feature of the disease [54]. In light of this, it is logical to propose that Ecrg4+ monocytes can marginate through the vasculature into the brain parenchyma, recruited by expression and release by microglia as a potential chemotaxic factor as shown here, and described in glioma models [14], and which has been described for other factors [9,55]. During normal aging, limited recruitment of perivascular monocyte infiltration is seen in cognitively normal individuals [43]. Future studies of interest include histological examination of Ecrg4+ cells in cerebrovascular disease compared with AD patients.

Possible consequences of diminished Ecrg4 immunoreactivity in AD cortical neurons (Figure 4) are less clear. We detected highly Ecrg4-immunoreactive loci (Figure 4C) consistent with a pattern observed for NFTs in the AD gray matter (Figure 4D). While

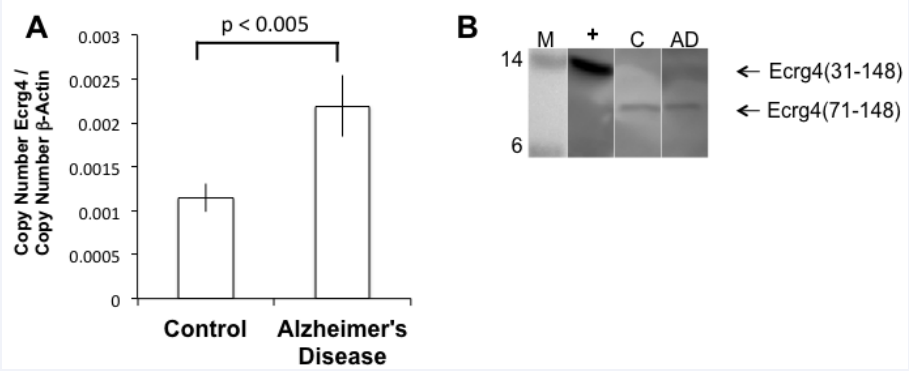

Figure 1 c2orf40 gene and protein expression in cerebral cortex. (A) c2orf40 gene expression is higher in the AD prefrontal cortex (pfc) compared to control. RNA was extracted and used for RT-qPCR from pfc tissue. Gene expression levels were approximately doubled in AD compared to cognitively normal controls with p = 0.0017 determined by two-tailed Student's T-test. Error bars represent mean ± standard deviation with N = 4 cognitively normal, N = 4 AD. (B) The peptide form of Ecrg4 detected in both aged, cognitively normal and AD patients corresponds to the molecular weight of the carboxy-terminal fragment consisting of amino acids 71-148. We were not able to detect changes in overall protein levels between control and AD samples (not shown), although significant changes in gene expression were found (A). However, this is the size of a fragment shown to interact with the TLR4/CD14/MD2 IIR-C (M: molecular weight ladder; +: recombinant protein used as a detection control; C: control, aged matched patient; AD: AD patient).

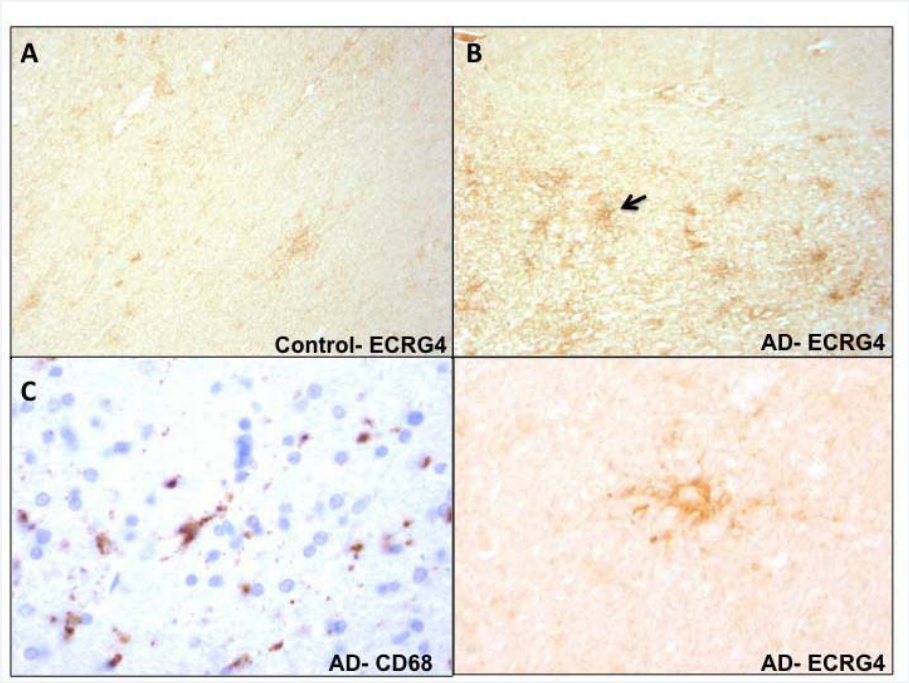

Figure 2 Ecrg4 immunoreactive activated microglia permeate the white matter in AD. White matter from the entorhinal cortex was immunostained for Ecrg4 in cognitively normal control (A) and compared to AD (B). There was a substantial increase of activated microglia into the AD white matter (B, arrow) that have microglial morphology, shown in (C) by CD68 labeling of AD white matter (brown: CD68, blue: counterstain). A higher magnification of an ECRG4+ microglial cell in AD white matter is shown in (D). Only faint background staining was seen in control samples (A). Staining pattern is representative of N = 4 control and N = 4 AD individuals. Magnifications: A: 200x; B: 200x; C: 400x; D: 400x).

neuronal protein expression of Ecrg4 was decreased throughout the cell soma and axon, Ecrg4 protein may be redistributed to NFTs in AD, but was more evenly distributed throughout the neuron in age-matched controls (Figure 4A). Western blot analysis of groups of cognitively normal control compared to AD brains did not indicate a considerable difference in protein levels of a C-terminal derived fragment (Ecrg4 (71-148), not shown).

Correlating with our finding of elevated c2orf40 gene expression in AD patients (Figure 1A), Woo *et al* [17] reported Ecrg4 gene expression was up regulated 8.35 fold in brains of human Tau 23 over expressing mice, a model for NFT-like pathology. Further studies, including quantitative co-localization analysis of Ecrg4 within NFTs would confirm in humans correlation between Ecrg4 and NFTs first suggested by Woo et al. The consequences

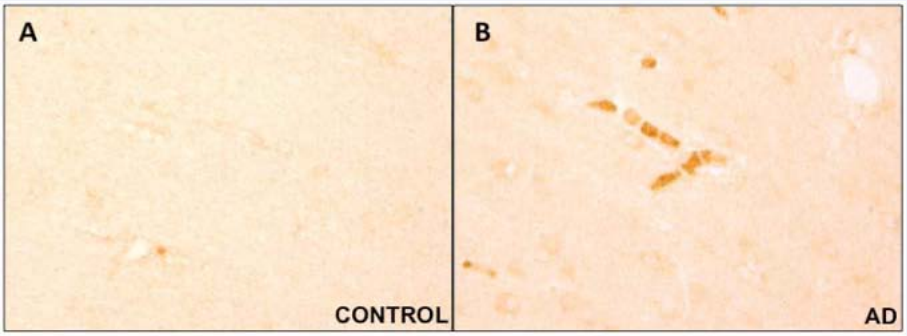

Figure 3 Ecrg4 immunoreactive intravascular monocytes are seen in AD cerebral cortical white matter (B) and none are seen in cognitively normal white matter (A). Circulating monocytes are recruited into the AD brain parenchyma to participate in the neuroimmune response. Staining pattern is representative of N = 4 AD patients, while no intravascular monocytes were seen in N = 4 control individuals. (Magnifications: A: 400x; B: 400x).

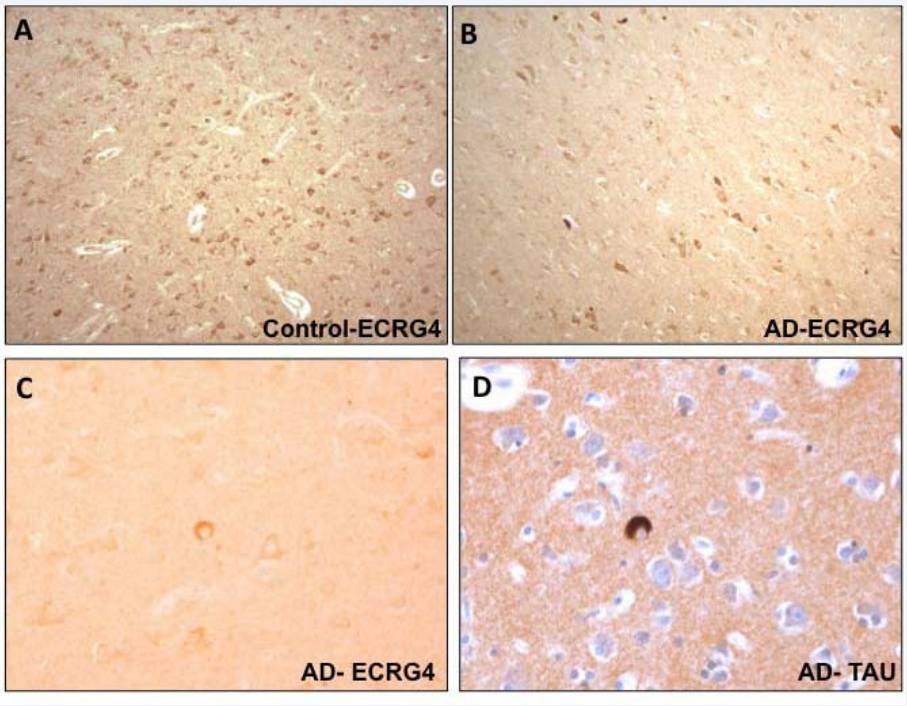

Figure 4 Ecrg4 immunoreactivity is decreased in AD cortical neurons. Anti-Ecrg4 antibody strongly labeled cortical neurons in control cortex (A), but staining was diminished in AD (B). At a higher magnification of AD neurons (C), ECRG4+ regions of neurons have the morphology consistent with neurofibrillary tangles. Tau labeling of AD neurons in the AD brain (D) confirms this morphology (brown: Tau, blue: counterstain). This labeling pattern was consistent among N = 4 control and N = 4 AD individuals. Magnifications: A: 100x; B: 100x; C: 400x; D: 400x.

of this subcellular distribution are unclear at this point.

We surprisingly found a decreasing trend, but no statistically significant difference in expression level of c2orf40 between AD and controls in the CP. The finding was contrary to our hypothesis that levels would be decreased because c2orf40 expression in the CPe is tied to brain homeostatic responses [12,13]. Comparing expression in the CP of larger sample groups and between earlier and later stages of AD may show differences of c2orf40/Ecrg4 and are of interest to future studies. Likewise, because of our recent findings that a C-terminal derived fragment of Ecrg4 is a chemotaxic factor for recruitment of macrophages and microglia in the brain [14,15], future studies would include a potential correlation of early and later stages of AD, expression levels of c2orf40/Ecrg4 and recruitment of peripheral monocytes through the blood-CSF barrier [40,56,57]

CONCLUSION

These are the first experiments that ask whether changes in c2orf40 and the Ecrg4protein it encodes are found in AD patients. The major finding of these studies is the striking observation of highly Ecrg4-immunoreactive microglia and intravascular monocytes within the white matter regions of AD patients' brains. These results warrant further investigation into the role of Ecrg4 in AD.

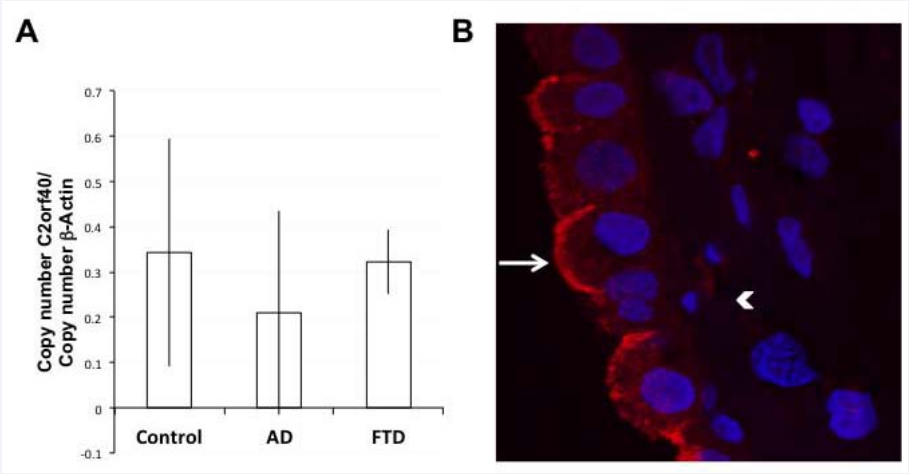

Figure 5 c2orf40/Ecrg4 expression is unchanged in the AD choroid plexus compared to cognitively normal and frontotemporal dementia (FTD). (A) Gene expression assessed by RT-qPCR using the standard curve method showed no significant difference between the groups (p = 0.17 AD compared to normal controls), with N = 11 cognitively normal, N = 2 FTD and N = 6 AD. Similarly, no significant differences were found between control and AD with FTD. Error bars represent mean ± standard deviation. (B) Immunofluorescence labeling of the CP in AD. No distinguishable difference in the pattern of Ecrg4 immunolabeling (red, blue: DAPI) was seen between AD and normal aged controls (not shown). Anti-Ecrg4 localized primarily to CP epithelial cells in a granular, secretory vesicle-like pattern, with increasing intensity towards the apical surface (arrow). Some trace, faint staining can be seen in the CP endothelial layer below (arrowhead). The image in panel B was collected with a 60x PlanApo lens and scan zoom of 3x.

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