Epilepsy: neuroinflammation, neurodegeneration, and APOE genotype

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Abstract

Background: Precocious development of Alzheimer-type neuropathological changes in epilepsy patients, especially in APOE ε4 carriers is well known, but not the ways in which other APOE allelic combinations influence this outcome. Frozen and paraffin-embedded tissue samples resected from superior temporal lobes of 92 patients undergoing temporal lobectomies as a treatment for medication-resistant temporal lobe epilepsy were used in this study. To determine if epilepsy-related changes reflect those in another neurological condition, analogous tissue samples harvested from 10 autopsy-verified Alzheimer brains, and from 10 neurologically and neuropathologically normal control patients were analyzed using immunofluorescence histochemistry, western immunoblot, and real-time PCR to determine genotype effects on neuronal number and size, neuronal and glial expressions of amyloid β (Aβ) precursor protein (βAPP), Aβ, apolipoprotein E (ApoE), S100B, interleukin-1α and β, and α and β secretases; and on markers of neuronal stress, including DNA/RNA damage and caspase 3 expression.

Results: Allelic combinations of APOE influenced each epilepsy-related neuronal and glial response measured as well as neuropathological change. APOE ε3,3 conferred greatest neuronal resilience denoted as greatest production of the acute phase proteins and low neuronal stress as assessed by DNA/RNA damage and caspase-3 expression. Among patients having an APOE ε2 allele, none had Aβ plaques; their neuronal sizes, like those with APOE ε3,3 genotype were larger than those with other genotypes. APOE ε4,4 conferred the weakest neuronal resilience in epilepsy as well as in Alzheimer patients, but there were no APOE genotype-dependent differences in these parameters in neurologically normal patients.

Conclusions: Our findings provide evidence that the strength of the neuronal stress response is more related to patient APOE genotype than to either the etiology of the stress or to the age of the patient, suggesting that APOE genotyping may be a useful tool in treatment decisions.

Keywords: Alzheimer’s disease, Apolipoprotein E (ApoE), APOE genotype, Caspase 3, Epilepsy, Neuroinflammation

Background

Epilepsy is the third most common cause of neurological disability worldwide [1] and is associated with precocious development of the neuropathological changes of Alzheimer’s disease (AD) [2-4]. Traumatic brain injury (TBI), which is a major risk factor for the development of epilepsy [5], is also associated with increased risk for later development of AD, and, in both cases, the risk of development of AD is greater with inheritance of apolipoprotein E ε4 alleles (APOE ε4) [3,6]. Exploring possible links between epilepsy-related alterations in neuronal and glial cell responses relative to APOE genotype is potentially important in understanding chronic neurodegenerative sequelae in epilepsy as well as in other forms of brain injury such as TBI [5,7-10].

In both purified rodent neuronal cell cultures and cultures of the human neuroblastoma cell line NT2, excess glutamate induces marked increases in the expression of the neuronal acute phase response protein βAPP, and in the release of its secreted fragment sAPPα [11], which is a powerful inducer of glial activation and increased production and release of the proinflammatory cytokine IL-1β [12]. This sAPP-induced glial activation and cytokine expression and release is differentially modulated in the

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The presence of ApoE3 vs ApoE4 [11], with ApoE3 providing greater protection than ApoE4. In epilepsy, there is overexpression of βAPP and IL-1, as well as the astrocyte-derived, neurtogentic cytokine S100B [13,14]. Furthermore, a comparison between surgical waste tissues from patients undergoing anterior temporal lobectomy surgery for drug-resistant intractable epilepsy showed that APOE ε3,3 and APOE ε4,4 genotypes dramatically alter the expression of βAPP and of IL-1 such that the APOE ε3 allele is more effective with regard to the maintenance of appropriate neuronal acute phase responses that favor neuronal viability than is APOE ε4 [2]. Importantly, several studies have provided evidence that inheritance of an APOE ε4 allele is associated with increased risk for Alzheimer neuropathological changes in epilepsy patients [3]. This is particularly relevant to the possibility that the decrease in the ability of ApoE4 compared with ApoE3 to elevate synthesis of the neuronal acute phase protein βAPP [15] is responsible for less sAPPα release, resulting in diminished neuronal repair and survival [16].

Epilepsy, in particular, exemplifies the intimate relationship between neuronal stress and triggering of glial activation, as such interactions are self-amplifying in epilepsy. For example, glutamate-induced hyperexcitation in purified primary rat neurons results in increases in βAPP, sAPP, and IL-1β, as well as ApoE, and both IL-1β and ApoE induce βAPP expression and sAPP release. Moreover, IL-1β treatment of neurons results in glutamate release [17], promoting a proposed self-perpetuating cycle: glutamate → ApoE → βAPP → sAPP → IL-1β → glutamate. Initially, cycle-engendered early acute phase responses may be beneficial, affording neuronal protection and debris clearance. However, because of the self-perpetuating nature of this cycle and the resultant glutamate release from both glia [18] and neurons [17] chronic neuronal stress ensues, enhancing the probability of neurodegeneration. Furthermore, the potential of such a cycle to be self-regenerative may explain, at least in part, why even those epilepsy patients with the advantage of an APOE ε3,3 genotype may develop Alzheimer-type neuropathological changes.

Evidence of a role for APOE genotype in determining neurodegenerative consequences of epilepsy underscores the need for in-depth analyses of neuronal-glial interactions that may be governed by inheritance of specific APOE allelic combinations. Such analyses provide basic cellular and molecular information regarding pathways involved in neuronal-glial interactions as well as information that may be helpful in clinical decision making.

Results

Comparison between our patient groups – Group 1: patients having one or two alleles of ε2 and no ε4 (APOE ε2,2 (n = 1) and APOE ε2,3 (n = 12)) age range = 14 y-73 y, median age = 40 y, average age = 35 y; Group 2: [APOE ε3,3 patients (n = 53)], age range = 0.25 y-71 y, median age = 32 y, average age = 30.4 y; Group 3: patients having one ε4 allele [APOE ε2,4 (n = 2) APOE ε3,4 (n = 17)], age range = 10 y-48 y, median age = 30.5 y, average = 29.2 y; Group 4: APOE ε4,4 patients (n = 7), age range = 10 y-50 y, median age = 34 y, average age = 32 y. Such grouping allowed for investigation of the degree of influence of each of the 6 combinations of the three APOE alleles and provided the following results.

APOE genotype modulation of glial responses in epilepsy

Glia numbers in a given cross-sectional cortical area

Patients in Group 1 (patients having one or two alleles of ε2 but no ε4) had the lowest number of microglia per neuron: 82% of the neurons had less than two adjacent microglia. Relative to other groups, Group 2 (patients with two APOE ε3 alleles ε3,3) had more IL-1α-immunoreactive microglia adjacent to each neuron: 70% of the neurons had at least two adjacent microglia, with some having as many as 9. In Group 3 (patients having one APOE ε4 allele, ε2,4 and ε3,4), more than 80% of neurons had two or less IL-1α immunoreactive microglia per neuron. In Group 4 (patients having two APOE ε4 alleles, ε4,4), 70% of neurons had one or fewer adjacent microglia (Figure 1).

In a given unit area of cortical layers III and IV of superior temporal gyrus in patients in Group 2 (patients having two APOE ε3 alleles), the total number of microglia counted was greater than that in other groups (Group 2 = 1137 ± 267 vs Group 1 = 460 ± 112; Group 3 = 534 ± 81; Group 4 = 770 ± 187 microglia/mm²; p < 0.001). Although the number of microglia/mm² was influenced by APOE genotype, the relative levels of glial cytokine mRNAs IL-1α and IL-1β were not related to genotype (data not shown).

Astrocyte numbers per unit area were highest in patients with APOE ε3,3 (Group 2) compared to those in patients with other APOE allelic combinations, groups 1, 3, and 4 respectively (175 ± 19 vs 94 ± 38; 96 ± 12; 133 ± 12 astrocyte/mm²; p < 0.001). However, synthesis of the astrocyte-derived cytokine S100B was higher in patients with APOE ε4,4 compared to that in patients carrying other allelic combinations (Figure 2A). Conversely, S100B protein expression was higher in APOE ε3,3 (Group 2) patients (99.7 ± 5.17) than in groups 1, 3, or 4, respectively (95.7 ± 3.55, 92.7 ± 6.21, 92.9 ± 2.87; p < 0.001) (Figure 2B and C).

APOE genotype modulation of neuronal responses in epilepsy compared to that in Alzheimer patients

Neuronal numbers in a given cortical area and neuronal cross-sectional area in epilepsy

As previously reported [2], we observed no differences in neuronal numbers between epilepsy patients with either an APOE ε3,3 or APOE ε4,4 genotype. However, the cross-sectional area of neurons from patients in Group 1
as well as Group 2 (those having one or two APOE ε2 alleles, but no APOE ε4 allele, or those having APOE ε3,3 genotype, respectively) was greater (439 ± 32 μm² vs 389 ± 29 μm²). APOE ε4,4 neurons (Group 4) had the smallest neuronal area (213 ± 17 μm²) among the Groups, p < 0.001 (Figure 3).

Neuronal numbers in a given cortical area and neuronal cross-sectional area in Alzheimer patients vs control patients
In tissue from patients with Alzheimer’s disease, the sizes of APOE ε3,3 neurons were larger than those of APOE ε4,4 neurons (286 ± 23 μm² vs 227 ± 25 μm², p < 0.01)
In contrast, the size of neurons in analogous tissue from neurologically normal controls did not differ with APOE genotype (APOE ε3,3 neurons, 293 ± 32 μm² and APOE ε4,4 neurons, 282 ± 21 μm²) (Figure 4D-F). The cross-sectional area of APOE ε4,4 neurons was less than that of APOE ε3,3 neurons in both epilepsy and Alzheimer patients, perhaps suggesting that APOE ε4,4 neurons are not able to follow the same response to neuronal stress as do neurons of other genotypes. No APOE genotype-related changes in neuronal cell numbers were detected in our epilepsy patient Groups 1, 2, 3, and 4 (302 ± 39, 277 ± 25, 280 ± 32, 272 ± 21 per mm², respectively).

**Acute phase responses in epilepsy relative to APOE genotype**

To assess the neuronal response to the hyperexcitation stress in epilepsy, we measured the relative tissue levels of the messenger RNAs for both βAPP and ApoE in tissue samples from patients (n = 92) with each of the different APOE allelic combinations, using real time PCR analysis. βAPP mRNA expression did not show significant differences with regard to APOE genotype (data not shown). However, ApoE mRNA levels were higher in Group 4 (the APOE ε4,4 group) than in other groups (Figure 7A). Despite the lack of increased expression of βAPP mRNA among our patients, there was a dramatic increase in the levels of βAPP protein in those with APOE ε3,3 (Group 2) compared to those levels in APOE ε4,4 (Group 4), suggesting that the elevation of βAPP in this group was due more to translation than transcription. Conversely, ApoE protein levels, measured by western blot analysis showed that APOE ε4,4 patients (Group 4) had lower levels than did other APOE genotypes (18.2 ± 8.4 vs 61.8 ± 15.3, 55.4 ± 23.3, 26.9 ± 7.1; Group 4 vs 1, 2, and 3, respectively, p < 0.01) (Figure 7B,C), suggesting that even the increase in ApoE mRNA in tissues samples from Group 4 patients was not sufficient to raise ApoE protein levels to those noted in other groups. Moreover, Group 4 (APOE ε4,4) patients had lower tissue levels of actin than did patients in Groups 1, 2, and 3, respectively (57.0 ± 13.0 vs 83.8 ± 13.0, 114.3 ± 39.5, 106.1 ± 19.0, p < 0.01). This may explain, at least in part, the differences noted in neuronal size. For example, without regard to cell type, size may influence actin levels. Alternatively, actin levels may influence cell...
size, as actin has been used to estimate cell size in some studies [20].

**The influence of APOE genotypes on epilepsy-induced neuropathological changes**

None of the 15 patients having at least one APOE ε2 allele had Aβ plaques. This is in contrast to the fact that Aβ plaques were present in carriers of all other genotypes [13 of the 53 patients with APOE ε3,3, 24.5%; 3 of 17 patients with APOE ε3,4, 17.6%; and in 1 of the 6 patients with APOE ε4,4, 16.7% (one of the seven APOE ε4,4 patients was not examined immunohistochemically because of a limited amount of tissue)] (Figure 8A-C). These findings raise the possibility of a protective role of APOE ε2 alleles against plaque formation in epilepsy. The elevated plaque density in those with APOE ε3,3 and APOE ε3,4 compared

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**Figure 4** Cross sectional neuronal area in Alzheimer patients and in neurologically normal controls, relative to APOE genotype. Superior temporal gyrus tissue sections from Alzheimer patients (A-C) and neurologically normal patients (D-F) immunoreacted with Pan neuronal marker (green) in Alzheimer patients having APOE ε3,3 (A) appeared to be larger than those from Alzheimer patients with APOE ε4,4 (B). Cross-sectional area measurements of neurons (μm²) confirm this difference (286 ± 23, vs 227 ± 25; p = 0.009) (C). Cross-sectional area of neurons in control patients with APOE ε3,3 (D) was not different from that in control patients with APOE ε4,4 (E) (250 ± 16, vs 244 ± 28; p = 0.67) (F).

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**Figure 5** Neuronal stress as estimated by heat shock-related damage associated with expression of 8-OH guanosine. Numbers of neurons expressing RNA/DNA oxidative damage was similar in the different APOE genotype groups (A-C). However, the extent of damage per neuron, as assessed by Stress Marq (8-OH guanosine) fluorescence intensity, was greater in patients with APOE ε4,4 than APOE ε2,2 or ε3,3 genotypes (80 ± 3 vs 55 ± 6 and 55 ± 6 arbitrary units, p = 0.001) (D).
Figure 6 APOE genotype influences cell cycle-related DNA damage. Illustration of a western blot representative of 8 gels from patients (n = 92) with different APOE genotypes, showing actin bands (green) and pro-caspase 3 bands (red) (A). Wilcoxon distribution scores for pro-caspase 3 fluorescence intensity for all the 92 patients: APOE ε3,3 (Group 2) had lower levels of pro-caspase than other genotypes (B) with p values for comparisons with Groups 1,3,4 equal to < 0.002, < 0.001, and < 0.02, respectively.

Figure 7 ApoE transcription and translation relative to APOE genotype. Wilcoxon distribution scores for ApoE mRNA levels were higher in patients carrying at least one APOE ε4 allele (Group 3 and Group 4) than in those carrying other APOE genotypes (Groups 1 and 2); the difference was significant only between Group 2 and Group 3 (p < 0.01) (A). ApoE protein levels, illustrated in one of eight western blots of different epilepsy samples (n = 92) with actin (red) in the upper row and ApoE (green) in the lower row with the middle sample being recombinant ApoE protein used as a positive control (Std) (B). ApoE protein levels for all the patient samples (n = 92), quantified by western blot analysis, showed that APOE ε4,4 patients (Group 4) had lower levels of ApoE than did other APOE genotypes (18.2 ± 8.4 vs 61.8 ± 15.3, 55.4 ± 23.3, 26.9 ± 7.1 arbitrary units) for groups 1, 2, and 3, respectively, p < 0.01 (C).
to other genotypes is consistent with the findings in these patients of elevated ApoE and S100B; both induce elevation of βAPP expression, which is then available for Aβ cleavage and deposition. Although APOE genotype did not influence expression of the mRNAs for the β-secretases (BACE1 and 2), α-secretase mRNA expression was elevated in those with APOE ε4,4 genotype (Group 4) compared to other genotypes (Figure 8D). The APOE ε4,4-related increase in α-secretase mRNA expression might be viewed as an attempt at compensation for other deficiencies as α-secretase obviates the production of Aβ and at the same time increases secretion of sAPPα, which may provide a neuron-sparing action [16]. It is interesting to note that in epilepsy with the early appearance of Aβ plaques and glial activation, there is little or no evidence of neurofibrillary tangle formation [21].

Discussion

Our results show that, compared to other APOE allelic combinations, neuronal resilience and glial activation were greatest in epilepsy patients with APOE ε3,3 genotype. Neuronal resilience correlated with the highest expression of acute phase response proteins βAPP and ApoE, greatest neuronal size, and least indications of DNA fragmentation, oxidation, and potential cell cycling. Similarly, compared to patients with other allelic combinations, patients with APOE ε3,3 genotype had more IL-1α-immunoreactive microglia in a given cross-sectional cortical area, more microglia adjacent to neurons, and more astrocytes per given cross-sectional area. In addition, the tissue levels of the astrocyte-derived, neuron-sparing neurtotrophic cytokine S100B were highest in patients with APOE ε3,3 genotype. These findings are consistent with the idea that APOE genotype influences neural responses to the neuronal stress engendered by the hyperexcitation of epilepsy. Several neural stresses elicit elevation of neuronal acute phase proteins βAPP and ApoE, which, in turn, promote microglial and astrocytic activation with increased expression of glial cytokines such as IL-1β and S100B. These cytokines are known to regulate the expression of each other and both are known to induce the expression of βAPP and ApoE for promotion of neuronal survival and maintenance [15,17,22-24]. Together, our findings are consistent with the idea that hyperexcitation first elicits compensatory responses that include over-expression of βAPP, release of sAPP, and glial activation with induction of IL-1β and S100B. The fact that carriers of one or two APOE ε3 allele(s) were shown here to be more adept than carriers of other APOE genotypes at eliciting specific neuronal and glial responses that have been associated with neuronal repair and survival [16,25] suggests that neuronal resilience is, at least in part, dependent on which specific ApoE variant is present.

Our findings that inheritance of two APOE ε4 alleles is associated with smaller neurons in both epilepsy and Alzheimer’s disease, but not in neurologically normal controls, together with the dramatic elevation of markers of stress in APOE ε4,4 carriers, suggests that such
Aβ plaques and hippocampal atrophy in temporal lobectomy tissues from epilepsy patients have been observed in other studies [4,26]. Our finding that the incidence of Aβ plaques is dependent on specific APOE allelic subtypes, namely, that having even one APOE ε2 allele is associated with an absence of Aβ plaques even in our oldest patient (71y), may in some way be related to a reported decreased risk for AD [27] and a protective effect of inheritance of APOE ε2 against Alzheimer-like neuropathological changes [28]. The Aβ plaques, which were noted in our 10-year old APOE ε4,4 patient, appeared to be mature dense core neuritic plaques, while those in carriers of other allelic combinations appeared as diffuse neuritic plaques. These findings support previous studies showing a relationship between the presence of an APOE ε4 allele and precocious development of AD in epilepsy patients [3,29], and suggests the need for further investigation into the role of APOE ε4 alleles in Aβ plaque maturation. These findings may have relevance to the fact that plaque maturity is associated with both formation of Aβ dense cores as well as increases in ApoE immunoreactivity as Aβ plaques mature [30].

The fact that most of the Aβ plaques present in our patients were found in APOE ε3,3 carriers may, in principle, be mostly related to increases in βAPP expression, especially in view of concomitant increases in proteins, cytokines, and neurotransmitters that are known to induce increases in neuronal [APP, viz., ApoE [15], IL-1 [17], S100B [23], and glutamate [17], perhaps identifying mechanisms by which the advantage of having APOE ε3 alleles may be accompanied by the disadvantage of fostering Aβ deposition. Interestingly, with regard to APOE genotype and Alzheimer neuropathological change, carrying even one APOE ε2 allele appears to have a protective effect against the formation of Aβ plaques, regardless of age and sex. These interpretations of our results are predicated on the idea that Aβ plaques are the consequence of hyperexcitation-induced, neuronal stress-related cycles that are important in the neuropathological progression observed in epilepsy and which occur as a consequence of disease severity and duration, perhaps regardless of genotype, age, and sex. Our observation regarding greater plaque maturity among those with APOE ε4,4 relative to other APOE genotypes is consistent with observations in Alzheimer patients [31,32] and in mice [33], which show that ApoE binding to Aβ disrupts Aβ clearance across the blood-brain barrier in an isoform-specific manner, with ApoE4 having a greater disruptive effect than ApoE3 or ApoE2 [33].

The lower tissue levels of actin in patient carriers of APOE ε4,4 compared to those with other APOE allelic combinations may explain, at least in part, the APOE ε4,4-related smaller neuronal cell size [20]. Variation in actin expression according to APOE genotype, as we show here, suggests that actin should not be used as a tool for normalizing the relative expression of proteins in situations in which actin varies with specific parameters.

Conclusions
Our finding that even one APOE ε2 allele is protective against Aβ plaque deposition is consistent with previous reports associating this genotype with protection against Alzheimer’s disease [34]. The robust neuronal-glial response to the neuronal stress of epilepsy in APOE ε3,3 carriers suggests that overall they have an advantage over other genotypes as indicated by an ability to increase neuronal acute phase protein expression, a greater neuronal size, and increased resilience, as indicated by lower levels of markers for RNA and DNA damage, and lower susceptibility to inappropriate cell cycling and death pathways. However, the fact that the percentage of patients with Aβ plaques was highest in APOE ε3,3 carriers suggests that these beneficial effects occur at the expense of an increase in the possibility of cleavage of excess βAPP and formation of Aβ, as well as increasing deposition of ApoE in such plaques and in this way perhaps favoring plaque formation. APOE ε4,4 carriers in our study were disadvantaged compared to other genotypes, having the smallest neurons among the genotypes, lowest acute phase responses, and highest markers of stress. Taken together, our findings suggest that APOE genotype may be important in decisions regarding timing of surgical intervention for intractable epilepsy, as well as in decisions regarding exposure of individuals to activities with high risk for TBI.

Methods
Patients and specimens
Epilepsy tissue samples
Resected temporal lobe tissues were obtained from 92 epilepsy patients (58 males and 34 females; 1 APOE ε2,2, 12 APOE ε2,3, 53 APOE ε3,3, 2 APOE ε2,4, 17 APOE ε3,4 and 7 APOE ε4,4) with an age at surgery ranging from 0.25 to 73 years (median age = 32 y, average age = 31.7 y). All patients underwent anterior temporal lobectomy for treatment of medication-resistant intractable epilepsy. For more information on patients regarding neuropathological evaluation please see [35]. Surgical waste obtained from the anterior portion of the superior temporal gyrus, an area some distance from sclerotic areas and epileptogenic foci, was dissected at 4 mm intervals, and alternate
sections were preserved by flash freezing for molecular analyses and by formalin fixation for histological evaluation. For uniformity, immunohistochemical examination was restricted to cortical layers III and IV of superior temporal gyrus.

**Autopsy tissue samples**

Temporal lobe tissues analogous to that collected from epilepsy surgical waste was collected from autopsied brain tissue of neuropathologically diagnosed Alzheimer patients [n = 6 males and n = 4 females; 5 *APOE* ε3,3 (M-60 y; M-73 y; F-76 y; F-80 y; F-88 y) and 5 *APOE* ε4,4 (F-72 y; M-74 y; M-74 y; M-82 y; M-86 y)]. Analogous tissue samples were also obtained from neurologically and neuropathologically normal individuals [n = 8 males and n = 2 females; 6 *APOE* ε3,3 (M-69 y; M-78 y; M-81 y; M-83 y; F-87 y) and 4 *APOE* ε4,4 (M-70 y; M-78 y; F-79 y; M-85 y)]. As with the processing of the tissue samples from our epilepsy patients, autopsy tissue samples collected less than eight-hours postmortem from Alzheimer and control patients were identical, i.e., analogous samples to be used for molecular analyses were snap frozen in liquid nitrogen and for immunohistochemical analyses sections of formalin fixed brains were used.

Surgical waste and autopsy tissue are both exempt from IRB review under 46.1015(b), and this study was approved as an exempt study by the University of Arkansas Institutional Review Board.

For further analysis of data obtained from epilepsy patients, we categorized genotypes into four groups. Group 1: patients having one or two alleles of ε2 and no ε4 [*APOE* ε2,2 (n = 1) and *APOE* ε2,3 (n = 12)]; Group 2: [*APOE* ε3,3 patients (n = 53)]; Group 3: patients having one ε4 allele [*APOE* ε2,4 (n = 2) *APOE* ε3,4 (n = 17)]; Group 4: [*APOE* ε4,4 patients (n = 7)]. This grouping allowed for investigation of the degree of influence of each of the three *APOE* alleles on observed results.

**Immunofluorescence immunohistochemistry**

Reagents: Antibodies: rabbit anti-human IL-1α (Peprotech 4:1000); goat anti-human ApoE (Invitrogen 1:50); mouse anti-human Aβ/βAPP (Covance 1:1000); rabbit anti-human Pan Neuronal Marker (PNM – Millipore ABN 1:500); mouse anti-human DNA/RNA oxidative damage antibody (8-OH-guanosine – Stress Marq Biosciences 5 μg/ml); rabbit anti-phosphorylated tau (AT8 – Abcam 1:3000); rabbit anti-actin (Santa Cruz 1:1000) were diluted in antibody diluent (DAKO). Secondary antibodies were Alexa Fluor® 488: donkey anti-rabbit or goat anti-rabbit; Alexa Fluor® 594: donkey anti-goat or mouse. Mounting media containing Prolong Gold anti-fade reagent with DAPI (Invitrogen) was used to stain nuclei.

**Procedures**

Paraffin-embedded tissue was sectioned at 7 μm and processed as previously described [2]. Sections destined for IL-1α, PNM, Stress Marq, and Aβ immunoreaction were pretreated by placing them in boiling sodium citrate buffer (0.01 M, pH 6.0) for 20 minutes; sections for ApoE immunoreaction were placed in trypsin solution for 10 min at 37°C, and all were blocked using protein block

| Gene analyzed | Human sequences | Annealing temp. (Co) | Cycle no. |
|---------------|-----------------|----------------------|-----------|
| ApoE          | F: CCCAGGTCAACCCAGGAACCT | 60                  | 40        |
|               | R: AGITCCGATTTGTAGCCCTCA |                      |           |
| βAPP          | F: AACACCGTGAGGCTCTTT  | 60                  | 40        |
|               | R: ATGCCACGCTGAGAC  |                      |           |
| α-secretase (ADAM 10) | F: AGTGTACGTGTCAGACTTC | 60                  | 40        |
|               | R: TITGGACGGTAGTGGTCG |                      |           |
| BACE 1        | F: CAGTCAAATCCATAAGGCAG | 60                  | 40        |
|               | R: GTTGTAACCTCAACCATTAGG |                      |           |
| BACE 2        | F: AGAGTATAACGGACAGCAAGGC | 60                  | 40        |
|               | R: CCAGTCCAGAAACACCACAG |                      |           |
| IL-1 α        | F: GGAGACGCTTGTTGATGAGCAA | 60                  | 40        |
|               | R: TGGCTAATAACCTACAAGCTCTT |                      |           |
| IL-1 β        | F: GCACGATTCGACCTGACGAT | 60                  | 40        |
|               | R: CACCAAGCTTTTTTGCTGTGAGT |                      |           |
| S100B         | F: CCCCAGGGACTCTGGTAAACAG | 60                  | 40        |
|               | R: CACGGTGCGACCTTATCAC |                      |           |

Table 1 Human gene sequences for ApoE, βAPP, α-secretase, BACE 1, BACE 2, IL-1α, IL-1β, and S100B, with PCR annealing temperatures and number of amplification cycles
(DAKO), and immunoreacted by overnight incubation at room temperature. Appropriate Alexa Fluor-tagged secondary antibodies were diluted in antibody diluent 1:200, and sections were incubated for 60 minutes, washed three times for 5 minutes each in distilled water, and covered-slipped with prolong Gold with DAPI.

Image analysis
As previously described [2,15], a quantitative approach was used to assess numbers of glia and neurons. Three images per slide (40× magnification) were captured at identical exposure settings using a Nikon Eclipse E600 microscope equipped with a Coolsnap monochrome camera. Each of the three images, spanning 37,638.6 μm², was acquired, analyzed, and thresholded using NIS-Elements BR3 software (Nikon.com). Results regarding neuronal and glial numbers are presented as numbers/mm². Data were analyzed by ANOVA to assess differences among groups. Significance was provided by p ≤ 0.05.

Real time (RT) polymerase chain reaction (PCR) amplification
Total RNA was extracted from brain tissue using TriReagent™ RNA (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s instructions. Real-time RT-PCR was performed as previously described [15]. Briefly, for comparisons of mRNA levels among different RNA samples, RT reactions were performed simultaneously using reagents from Life Technologies (Grand Island, NY). RT-PCR was performed using reagents from SyberGreen Master Mix from Life Technologies (Grand Island, NY). The sequences of primers for ApoE, βAPP, α-secretase (ADAM 10), BACE 1, BACE 2, IL-1α, IL-1β, and S100B are given in Table 1. Equal amounts of RT-PCR from each sample were pooled to use for standard curve reaction with each primer set to verify linearity and a suitable slope. All given mRNA tissue levels are relative to 18s.

Western immunoblot assay
Proteins were extracted from brain tissue in a lysis buffer comprised of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 1 mM EGTA, 1 mM EDTA, and 1% sodium deoxycholate; lysate protein was quantified using a Micro BCA assay reagent kit (Pierce, Rockford IL) as described previously [2]. Aliquots (30 μg each for ApoE and 50 μg for caspase 3) were loaded onto a 4-12% Criterion™ precast Gel Bis-Tris from Bio-Rad (Hercules, CA), subjected to electrophoresis at 80V for 3 h, and transferred to PVDF 0.45 μm Immobilon-FL (Millipore). Blots were blocked in 1-Block Buffer (Applied Biosystem Inc., Bedford, MA) for 60 minutes, then incubated overnight at 4°C with either mouse monoclonal anti-ApoE (1:500) (Santa Cruz sc-58242) primary antibody, rabbit anti-human Caspase3\Cleaved Caspase 3 (1:500) (Cell Signaling # 9661-2), or rabbit anti-actin (Santa Cruz 1:1000), and incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibody (1:1000). For protein detection, we used ProteinSimple Multifluor Western Blotting Kit (Santa Clara, CA), and for image capture we used CellBioscence FluorChem Q digital imager (Santa Clara, CA). Autoradiographs were digitized and analyzed using NIH Image software, version 1.60.

Statistical analysis
Data were analyzed using an unpaired t-test and Wilcoxon distribution score, and results are expressed as mean ± SD. In the Wilcoxon distribution plot, the length of the box represents the interquartile range (the distance between the 25th and 75th percentiles), the symbol in the box interior represents the group mean, the horizontal line in the box interior represents the group median, the vertical lines (the whiskers) issuing from the box extend to the group minimum and maximum values. Values were considered significantly different when the p-value was ≤ 0.05.

Abbreviations
Aβ: Amyloid β; AD: Alzheimer’s disease; ApoE: Apolipoprotein E; AU: Arbitrary units; βAPP: Amyloid β precursor protein; Stress Marq: 8-OH guanosine; IL-1: Interleukin-1.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
OA conducted and helped design all experiments and analyses and interpretation of the data, and writing the manuscript. REM conducted neuropathological evaluations and contributed to interpretation of results and writing of the manuscript. FAB was the neurosurgeon who provided the tissue and reviewed the manuscript. WSTG helped design the study with OA, verified and helped with interpretation of the data, and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors particularly appreciate the irreplaceable contribution made by the patients and the technical expertise and advice provided by Dr. Ling Liu and Professor Steven Barger, and the technical help and advice provided by Mr. Richard A. Jones and Ms. JoAnn Biedermann, and Dr. Songthip T. Ounpraseuth for his help in statistical analyses. This work was supported in part by a grant from the National Institute on Aging AG12411, The Alexa and William T. Dillard Foundation, and the Windgate Foundation.

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Published: 29 July 2013
Received: 3 May 2013 Accepted: 28 June 2013
