**APOE4** is Associated with Differential Regional Vulnerability to Bioenergetic Deficits in Aged APOE Mice

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The ε4 allele of apolipoprotein E (APOE) is the dominant genetic risk factor for late-onset Alzheimer’s disease (AD). However, the reason for the association between APOE4 and AD remains unclear. While much of the research has focused on the ability of the apoE4 protein to increase the aggregation and decrease the clearance of Aβ, there is also an abundance of data showing that APOE4 negatively impacts many additional processes in the brain, including bioenergetics. In order to gain a more comprehensive understanding of APOE4’s role in AD pathogenesis, we performed a transcriptomics analysis of APOE4 vs. APOE3 expression in the entorhinal cortex (EC) and primary visual cortex (PVC) of aged APOE mice. This study revealed EC-specific upregulation of genes related to oxidative phosphorylation (OxPhos). Follow-up analysis utilizing the Seahorse platform showed decreased mitochondrial respiration with age in the hippocampus and cortex of APOE4 vs. APOE3 mice, but not in the EC of these mice. Additional studies, as well as the original transcriptomics data, suggest that multiple bioenergetic pathways are differentially regulated by APOE4 expression in the EC of aged APOE mice in order to increase the mitochondrial coupling efficiency in this region. Given the importance of the EC as one of the first regions to be affected by AD pathology in humans, the observation that the EC is susceptible to differential bioenergetic regulation in response to a metabolic stressor such as APOE4 may point to a causative factor in the pathogenesis of AD.

Possession of the ε4 allele of apolipoprotein E (APOE) is the major genetic risk factor for late-onset Alzheimer’s disease (AD). In normal physiology, the apoE protein plays a vital role in the transport of cholesterol and other lipids through the bloodstream, as well as within the brain1,2. Although the three common isoforms of apoE (E2, E3 and E4) differ from each other at only two amino acids—apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158), apoE4 (Arg12, Arg158)—this small change in amino acid sequence has a large effect on the protein structure, resulting in differential affinities towards apoE’s lipid cargo, as well as its receptors [see reviews by1,4].

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Importantly, these isof orm differences also have a major impact on the pathogenesis of late-onset AD. Although the APOE2, APOE3 and APOE4 alleles are normally present at a relative frequency of about 8%, 78% and 14%, respectively, the APOE4 allele is present at a relative frequency of about 37% in AD patients, with individuals who possess one or two APOE4 alleles having an odds ratio for AD of about 3 or 12, respectively. While a number of mechanisms have been proposed to help explain this APOE4-associated susceptibility to AD, the precise cause remains a source of debate. One prominent hypothesis is that this increase in AD risk is due to the capability of apoE4 to increase the aggregation and decrease the clearance of Aβ17–19. However, APOE4 expression has also been shown to have deleterious effects on numerous Aβ-independent pathways, including lipid metabolism, tau pathology, bioenergetics, neuronal development, synaptic plasticity, the neuro-vascularle, and neuro-inflammation [see reviews by14 and15], any number of which could play an important role in the pathogenesis of AD among APOE4 carriers.

In terms of APOE4's effects on bioenergetics, a number of pivotal reports have demonstrated that APOE4 expression leads to widespread dysregulation of the brain's bioenergetic capacity. For example, early reports by Reim aand colleagues demonstrated that both young and old APOE4 carriers display decreased glucose utilization, as measured by fluorodeoxyglucose positron emission tomography (FDG-PET), in brain regions similar to those seen with AD patients16–18. Additional reports have detailed the wide range of bioenergetic insults that APOE4 expression can cause in the brain, including impaired insulin signaling9,20, reduced cerebrovascular blood flow and cognitive function in response to a high fat diet (HFD)21,22, altered genetic expression of glucose-regulating enzymes and transporters23,24, and the generation of a toxic C-terminal fragment of apoE4 that can directly target electron transport chain (ETC) complexes in the mitochondria25–27.

In order to study the diverse effects that APOE4 expression has on the brain, we performed a transcriptomics analysis on the entorhinal cortex (EC) and primary visual cortex (PVC) of 14–15 month-old APOE targeted replacement mice, which express human APOE in place of their mouse Apoe gene and which do not develop overt AD pathology28,29. In addition to other observations30, this transcriptomics analysis revealed the differential regulation of numerous genes related to energy metabolism. Follow up studies showed that, while aged APOE4 mice possess bioenergetic deficits in the hippocampus (Hip) and cortex (Ctx), the EC of these mice appears to possess unique counterbalancing mechanisms that allow it to resist these APOE4-associated decreases in mitochondrial respiration. We hypothesize that this differential regulation of EC bioenergetics may play an important role in the pathogenesis of AD.

Results
Transcriptomics analysis reveals differential expression of energy-related genes in the EC of aged APOE4 mice. In order to investigate the effects of APOE4 expression in an untargeted manner, we performed a transcriptomics analysis on RNA extracted from a brain region that is acutely vulnerable to AD pathology (the EC) vs. a less vulnerable brain region (the PVC) of 14–15 month-old APOE targeted replacement mice (10 APOE3/3 and 19 APOE3/4 males). The raw data generated from this analysis (Supplementary Tables S1 and S2) has already been published as part of a separate study on APOE4's impact on endosomal-lysosomal dysregulation30. However, as is often the case with omics studies, the large amount of data generated from the analysis can inform us about changes in multiple biological pathways that cannot all be investigated in a single study. In this case, in addition to the dysregulation of endosomal-lysosomal genes that were revealed in our pathway analysis, another major KEGG pathway that was observed to be significantly enriched for differentially expressed genes was “Oxidative Phosphorylation” (Fig. 1A). This enrichment was driven by a large number of differentially expressed genes encoding for subunits of ETC complexes I-V, with each of these genes upregulated in the EC of aged male APOE3/4 mice, as compared to aged male APOE3/3 mice (Fig. 1B).

Seahorse analysis reveals decreased mitochondrial respiration with aging in the hippocampus and cortex of APOE4 vs. APOE3 mice, but not in the EC. In order to validate and expand upon the differential expression of oxidative phosphorylation (OxPhos) genes that we observed in our transcriptomics analysis, we conducted a series of Seahorse experiments on mitochondria isolated from the EC and other important brain regions of APOE4/4 vs. APOE3/3 mice. This APOE4/4 vs. APOE3/3 comparison was utilized in order to maximize the APOE genotype-associated differences observed with our original transcriptomics analysis on APOE3/4 vs. APOE3/3 mice. Utilizing the Seahorse XF24 platform, we first measured the oxygen consumption rate (OCR) as a measure of mitochondrial respiration from the EC and PVC of 15-month-old APOE mice (4 APOE3/3 and 4 APOE4/4 males; samples pooled within each genotype and region). As shown in Supplementary Fig. S1, we observed a significant reduction in both the Complex I-mediated and Complex II-mediated respiratory activity in mitochondria from the EC of APOE mice, with a more global decrease in mitochondrial function within the aged APOE4 brain, we performed additional experiments measuring the OCR of mitochondria isolated from the EC, Hip and Ctx of 6-month-old APOE mice (3 APOE3/3 and 3 APOE4/4 males; samples pooled within each genotype and region) and 20-month-old APOE mice (2 APOE3/3 and 2 APOE4/4 males; samples pooled within each genotype and region). As shown in Fig. 2, in the presence of pyruvate-malate (Complex I-mediated activity), we observed a reduction in State 3 respiration of mitochondria from the Ctx of 6-month-old male APOE4/4 vs. APOE3/3 mice, and very slight reductions in State 3 respiration of mitochondria from the Hip and EC of these mice. On the other hand, as shown in Fig. 3, there were significant reductions in Complex I- and Complex II-mediated respiration of mitochondria from both the Hip and the Ctx of 20-month-old male APOE4/4 vs. APOE3/3 mice. Intriguingly, however, in mitochondria from the EC of these 20-month-old APOE4/4 vs. APOE3/3 mice, we did not observe any significant differences in basal mitochondrial respiration in Complex I- or Complex II-mediated respiration, and we actually observed significant increases...
in State 3 respiration in Complex I-mediated respiration in mitochondria from the EC (Fig. 3B). Furthermore, using the data generated from this Seahorse analysis, we determined the respiratory control ratio (RCR) (state3u/state4o), which is a measure of the coupling efficiency between OxPhos and ATP production31,32 (Fig. 3C). This
Figure 2. Seahorse analysis reveals minimal differences in mitochondrial respiration in the cortex, hippocampus, and EC of young male APOE4 mice. Seahorse analysis was performed in order to analyze the effects of differential APOE isoform expression on mitochondrial respiration in mitochondria that were isolated from the cortex (Ctx), hippocampus (Hip) and EC of 6-month-old APOE mice (3 APOE4/4 males, tissues pooled vs. 3 APOE3/3 males, tissues pooled). (A) The oxygen consumption rate (OCR) from each region shows minor reductions in Complex I-mediated mitochondrial respiration in the Ctx, but not in Complex II-mediated mitochondrial respiration in the Ctx or in Complex I- or Complex II-mediated mitochondrial respiration in the Hip and EC of the young APOE4/4 mice. (B,C) Bar graphs showing the average OCR from (B) State 3 and for (C) the Respiration Control Ratio (RCR; state 3u/state 4o) in each region of the APOE4/4 mice, as a percentage of the APOE3/3 OCR from the equivalent tissues. The dotted blue line represents the normalized levels in the APOE3/3 tissues. (*denotes p < 0.05; **denotes p < 0.01; ***denotes p < 0.001; ****denotes p < 0.0001).
Figure 3. Seahorse analysis reveals decreased mitochondrial respiration in the cortex and hippocampus, but not in the EC, of aged male APOE4 mice. Seahorse analysis was performed in order to analyze the effects of differential APOE isoform expression on mitochondrial respiration in mitochondria that were isolated from the cortex (Ctx), hippocampus (Hip) and EC of 20-month-old APOE mice (2 APOE4/4 males, tissues pooled vs. 2 APOE3/3 males, tissues pooled). (A) The oxygen consumption rate (OCR) from each region shows decreased mitochondrial respiration in the Ctx and Hip, but not the EC of the aged APOE4/4 mice. (B, C) Bar graphs showing the average OCR from (B) State 3 and for (C) the Respiration Control Ratio (RCR; state 3/4) in each region of the APOE4/4 mice, as a percentage of the APOE3/3 OCR from the equivalent tissues. The dotted blue line represents the normalized levels in the APOE3/3 tissues. (** denotes p < 0.01; *** denotes p < 0.001; **** denotes p < 0.0001).
analysis indicates that EC mitochondria from the 20-month-old APOE4/4 mice are highly coupled, resulting in increased production of ATP per unit of oxygen.

These observations suggest that, while APOE4 expression leads to an overall reduction in mitochondrial function in the brains of aged APOE4 mice, which may be related to other APOE4-associated bioenergetic deficits observed using alternative approaches\textsuperscript{19–24}, mitochondria in the EC seem to undergo differential bioenergetic regulation, characterized by increased coupling efficiency. Importantly, these region-specific differences in the effects of APOE4 expression on mitochondrial respiration did not appear to be the result of changes in mitochondrial mass, as the levels of Tom20 protein, as well as the levels of PGCL RNA and the ratio of Mitochondrial Nuclear DNA, were unchanged between APOE genotypes in the EC, Hip, and Ctx of 21-month-old male APOE mice (Supplementary Fig. S2A,C,D). Differential APOE isoform expression also did not result in changes in the levels of specific ETC complex proteins in the EC, Hip or Ctx (Supplementary Fig. S2B).

In order to investigate whether there is a gender-effect associated with these region-specific differences in bioenergetic regulation, we also measured the OCR of mitochondria isolated from the EC and PVC of 14–15 month-old male APOE4/4 vs. APOE3/3 mice. As shown in Supplementary Fig. S3, the aged female APOE4/4 mice also showed a reduction in State 3 respiration in Complex I-mediated respiration in mitochondria from the Ctx, but not the EC. However, the Complex I-mediated respiration deficits in the Ctx were not nearly as robust in the female mice as they were in the males. Interestingly, in mitochondria from both the Ctx and the EC of these aged female APOE4/4 mice, we also observed a significant increase in Complex II-mediated (but not Complex I-mediated) State 3 respiration, suggesting that increased Complex II-mediated mitochondrial respiration (most likely from increased β-oxidation of fatty acids) might prevent these aged female APOE4/4 mice from developing the levels of reduced Complex I-mediated mitochondrial respiration observed in the aged male APOE4/4 mice.

Computational analysis of untargeted metabolomics data reveals differential expression of ketones in the EC of aged APOE4 mice. In order to investigate the source of these differential bioenergetic effects in the EC, we conducted a series of additional analyses on the EC of aged male APOE4/4 vs APOE3/3 mice. We have already published on an untargeted metabolomics study that we performed on metabolites extracted from the EC and PVC of 14–15 month-old male APOE4/4 vs. APOE3/3 mice, which is part of a larger study elucidating the effects APOE4 expression on neuronal hyperactivity\textsuperscript{25}. Intriguingly, this metabolomics study uncovered increases in numerous energy-related metabolites, including several TCA cycle metabolites (malate, citrate and isocitrate), as well as fructose-6-phosphate, carnitine, and ATP, each of which had higher levels in the EC of the aged APOE4/4 vs. APOE3/3 mice (Fig. 4A). Furthermore, the metabolomics data indicates a higher ATP:ADP ratio in the EC of these aged APOE4/4 vs. APOE3/3 mice (Fig. 4B), which, in light of our transcriptomics data, suggests an increase in the rate of oxidative metabolism in this region. Furthermore, our metabolomics data also revealed an APOE4-associated decrease in the levels of numerous free fatty acids in aged male APOE4/4 vs. APOE3/3 mice (Fig. 4C), which, unlike each of the previously mentioned energy-related metabolites (with the exception of carnitine), were differentially altered in both the EC and the PVC. These changes in fatty acid and carnitine levels are suggestive of differences in β-oxidation activity in both the EC and the PVC of these aged male APOE4/4 mice.

In addition to these and other differentially expressed metabolites that were identified during the course of this untargeted metabolomics study (Tables 1 and 2), there were also hundreds of additional differentially expressed metabolites that we were unable to identify using the tools that were available to us at the time that this study was conducted. Therefore, in order to extract more information out of this untargeted metabolomics dataset, we utilized the PIUMet (Prize-collecting Steiner forest algorithm for Integrative Analysis of Untargeted Metabolomics) method\textsuperscript{34} to predict the identities of these differentially expressed metabolites from the EC of aged APOE4/4 vs. APOE3/3 mice. PIUMet uses network optimization to analyze the data in the context of a vast database of protein-protein and protein-metabolite interactions, revealing both known and uncharacterized pathways that contain the putative metabolites.

We used PIUMet to analyze the 304 untargeted metabolite features whose levels were differentially altered in the EC of 14–15 month-old male APOE4/4 vs. APOE3/3 mice (corrected p-value < 0.05). Among these features, 124 had matches in PIUMet's underlying database of metabolites (Supplementary Table S5). PIUMet was able to infer the identity of 32 metabolite features and revealed a network of protein-protein and protein-metabolite interactions associated with their dysregulation. The resulting network is enriched in 18 putative GO biological processes (Table 3) and 124 total metabolites (Supplementary Fig. S4). Among these, the most intriguing GO biological processes were fatty acid metabolic process, inosine 5’-monophosphate (IMP) metabolic process, ketone catabolic process, steroid metabolic process, and vitamin metabolic process, while the most interesting putative metabolite identifications were acetone, adenylosuccinate, alpha-tocotrienol, and oleamide. In regards to this study, the identification of acetone and the ketone catabolic process GO term are particularly interesting. Ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) are primarily generated in the liver from fatty acid breakdown via β-oxidation, and are used as a supplemental energy source when glucose levels are low. These ketone bodies readily cross the blood brain barrier and can act as a potent fuel for the brain.\textsuperscript{35–37} Alternatively, there is evidence that astrocytes also have the ability to produce ketones\textsuperscript{38–40}, both through the breakdown of fatty acids, as well as through the breakdown of ketogenic amino acids such as leucine\textsuperscript{41,42}. Thus, the putative identification of acetone and the ketone catabolic process GO term in this analysis may point to an upregulation of ketone metabolism in the EC of these aged APOE4 mice.

Proton nuclear magnetic resonance spectroscopy analysis reveals differential expression of phosphocreatine, glutamate and GABA in the EC of anesthetized aged APOE4 mice. 1H NMR spectroscopy provides simultaneous quantification of the concentration of several highly abundant metabolites
in the brain, including neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA), as well as other biological metabolites such as creatine, glutathione, and N-acetylaspartate (NAA). Since metabolomics and 1H NMR spectroscopy have been described as complementary techniques, with advantages and disadvantages
| Metabolite                                      | Regulation in E4/4 | Fold Change | p-value   | FDR       | Detection Mode | CAS Number |
|------------------------------------------------|--------------------|-------------|-----------|-----------|----------------|------------|
| **Fatty Acids**                                |                    |             |           |           |                |            |
| gamma-Linolenic Acid                           | down               | 1.31        | 1.19E-03  | 0.093     | positive       | 506-26-3  |
| Myristic Acid                                  | down               | 3.92        | 0.004     | 0.118     | positive       | 544-63-8  |
| Docosahexaenoic Acid                           | up                 | 1.36        | 0.011     | 0.154     | positive       | 6217-54-5 |
| Stearic Acid                                   | down               | 1.69        | 0.011     | 0.154     | positive       | 57-11-4   |
| 12-Hydroxydocosanoic Acid                     | down               | 1.36        | 0.021     | 0.213     | positive       | 505-95-3  |
| Arachidonic Acid                               | down               | 1.40        | 0.037     | 0.230     | negative       | 506-30-9  |
| Palmitic Acid                                  | down               | 1.32        | 0.049     | 0.243     | negative       | 57-10-3   |
| **Oligosaccharides**                           |                    |             |           |           |                |            |
| trisaccharide                                  | up                 | 1.99        | 0.001     | 0.049     | negative       | 512-69-6  |
| tetrasaccharide                                | up                 | 2.24        | 0.015     | 0.169     | negative       | 10094-58-3|
| disaccharide                                   | up                 | 2.20        | 0.015     | 0.169     | negative       | 63-42-3   |
| **Vitamin and Vitamin Derivatives**            |                    |             |           |           |                |            |
| Phylloquinone                                  | up                 | 1.47        | 0.003     | 0.102     | positive       | 84-80-0   |
| Tocopherol                                     | up                 | 2.74        | 0.005     | 0.123     | negative       | 59-02-9   |
| Dehydroascorbic acid                           | up                 | 1.76        | 0.008     | 0.134     | positive       | 490-83-5  |
| **Energy-Related Metabolites**                 |                    |             |           |           |                |            |
| Inosine 5′-monophosphate (IMP)                 | up                 | 2.26        | 0.003     | 0.0720    | negative       | 131-99-7  |
| D-Fructose 6-phosphate                         | up                 | 1.10        | 0.011     | 0.169     | negative       | 643-13-0  |
| Succinoadenosine                               | up                 | 1.47        | 0.011     | 0.169     | negative       | 4542-23-8 |
| Carnitine                                      | up                 | 1.27        | 0.021     | 0.213     | positive       | 541-15-1  |
| Citric Acid/Isocitric Acid                     | up                 | 1.24        | 0.028     | 0.230     | negative       | 77-92-9/1637-73-6 |
| Malic Acid                                     | up                 | 1.16        | 0.037     | 0.230     | negative       | 97-67-6   |
| ATP                                            | up                 | 1.24        | 0.049     | 0.243     | negative       | 56-65-5   |
| **Cholesterol Metabolites**                    |                    |             |           |           |                |            |
| Lanosterol                                     | up                 | 2.27        | 0.015     | 0.169     | negative       | 79-63-0   |
| Cholesterolyl Acetate                          | up                 | 2.29        | 0.015     | 0.169     | negative       | 604-35-3  |
| **Amino Acids**                                |                    |             |           |           |                |            |
| Leucine                                        | up                 | 1.26        | 0.015     | 0.169     | negative       | 61-90-5   |
| Proline                                        | up                 | 1.12        | 0.037     | 0.230     | negative       | 147-85-3  |
| Glycine                                        | up                 | 1.10        | 0.037     | 0.230     | negative       | 56-40-6   |
| **Tryptophan Metabolites**                     |                    |             |           |           |                |            |
| Quinolactic Acid                               | up                 | 1.68        | 0.001     | 0.049     | negative       | 93-10-7   |
| 5-Hydroxyindole-3-carboxylic acid              | up                 | 2.49        | 0.015     | 0.169     | negative       | 2922-83-0 |
| 5-Hydroxytryptophylacetic acid                 | up                 | 1.32        | 0.049     | 0.243     | negative       | 492-27-3  |
| **Cysteine and Methionine Metabolites**        |                    |             |           |           |                |            |
| 5-Adenosylothyminecysteine                     | up                 | 1.15        | 0.021     | 0.213     | positive       | 979-92-0  |
| **Histidine Metabolism**                       |                    |             |           |           |                |            |
| Carnosine                                      | up                 | 1.31        | 0.037     | 0.230     | negative       | 305-84-0  |
| **Arginine and Proline Metabolites**           |                    |             |           |           |                |            |
| 4-Oxoproline                                   | up                 | 1.30        | 0.003     | 0.102     | positive       | 4347-18-6 |
| **Tyrosine Metabolites**                       |                    |             |           |           |                |            |
| Vanillylglycol (MHPG)                          | up                 | 1.64        | 0.011     | 0.169     | negative       | 67423-45-4|
| Tyramine                                       | up                 | 1.06        | 0.028     | 0.216     | positive       | 51-67-2   |
| Thymidine                                      | down               | 1.49        | 0.028     | 0.216     | positive       | 50-89-5   |
| Uracil                                         | down               | 1.38        | 0.049     | 0.243     | negative       | 66-22-8   |
| **Purine Metabolism**                          |                    |             |           |           |                |            |
| GMP                                            | up                 | 1.14        | 0.028     | 0.230     | negative       | 85-32-5   |
| **Miscellaneous**                              |                    |             |           |           |                |            |
| Hydroxybutyric acid                            | up                 | 1.35        | 0.002     | 0.055     | negative       | 5094-24-6 |
| Methylglutarylcarbine                          | up                 | 2.42        | 0.005     | 0.134     | positive       | 102673-95-0|
| Trimethylamine N-oxide                         | up                 | 2.65        | 0.021     | 0.213     | positive       | 1184-78-7 |
| 2-Hydroxypryridine                             | up                 | 1.36        | 0.037     | 0.275     | positive       | 142-08-5  |
| N-Acetylneuraminic Acid                        | up                 | 1.23        | 0.037     | 0.230     | negative       | 131-48-6  |

Table 1. Differentially expressed targeted metabolites from the EC of aged male APOE4/4 vs. APOE3/3 mice.
| Metabolite                        | Regulation in E4/4 | Fold Change | p-value | FDR   | Detection Mode | CAS Number |
|----------------------------------|--------------------|-------------|---------|-------|----------------|------------|
| **Fatty Acids**                  |                    |             |         |       |                |            |
| gamma-Linolenic Acid             | down               | 1.19        | 0.003   | 0.068 | positive       | 506-26-3   |
| Palmitic Acid                    | down               | 1.32        | 0.021   | 0.212 | negative       | 57-10-3    |
| 10-Hydroxydecanoate              | up                 | 2.22        | 0.015   | 0.195 | positive       | 1679-53-4  |
| Docosahexaenoic Acid             | down               | 2.03        | 0.021   | 0.212 | negative       | 6217-54-5  |
| Arachidonic acid                 | down               | 1.45        | 0.021   | 0.212 | negative       | 506-32-1   |
| **Oligosaccharides**             |                    |             |         |       |                |            |
| Trisaccharide                    | up                 | 1.82        | 0.015   | 0.212 | negative       |            |
| Tetrasaccharide                  | up                 | 2.70        | 0.021   | 0.212 | negative       |            |
| **Vitamin and Vitamin Derivatives** |                |             |         |       |                |            |
| Dehydroascorbic acid             | up                 | 2.03        | 0.001   | 0.062 | positive       | 490-83-5   |
| Phyloquinone                     | up                 | 1.90        | 0.001   | 0.062 | positive       | 84-80-0    |
| Ascorbic acid                    | up                 | 105.84      | 0.005   | 0.192 | negative       | 50-81-7    |
| Tocopherol                       | up                 | 2.28        | 0.008   | 0.192 | negative       | 59-02-9    |
| **Energy-Related Metabolites**   |                    |             |         |       |                |            |
| Acetylcarnitine                  | up                 | 1.40        | 0.003   | 0.068 | positive       | 3040-38-8  |
| Carnitine                        | up                 | 1.31        | 0.011   | 0.154 | positive       | 541-15-1   |
| Coenzyme A (CoA)                 | up                 | 2.82        | 0.028   | 0.282 | positive       | 85-61-0    |
| Pyruvate                         | up                 | 1.43        | 0.028   | 0.238 | negative       | 127-17-3   |
| Acetoacetic Acid                 | up                 | 1.40        | 0.028   | 0.238 | negative       | 541-50-4   |
| **Cholesterol Metabolites**      |                    |             |         |       |                |            |
| Taurodeoxycholate                | up                 | 1.94        | 0.003   | 0.068 | positive       | 516-50-7   |
| Cholesteryl Acetate              | up                 | 2.24        | 0.008   | 0.192 | negative       | 604-35-3   |
| Lanosterol                       | up                 | 2.50        | 0.011   | 0.192 | negative       | 79-63-0    |
| **Amino Acids**                  |                    |             |         |       |                |            |
| Tyrosine                         | up                 | 1.25        | 0.037   | 0.270 | negative       | 60-18-4    |
| Serine                           | down               | 1.18        | 0.049   | 0.270 | negative       | 56-45-1    |
| Glutamate                        | down               | 1.08        | 0.049   | 0.282 | positive       | 56-86-0    |
| **Cysteine and Methionine Metabolites** |    |             |         |       |                |            |
| Pterin                           | down               | 3.91        | 0.002   | 0.192 | negative       | 2236-60-4  |
| **Histidine Metabolites**        |                    |             |         |       |                |            |
| Urocanic acid                    | down               | 1.34        | 0.021   | 0.212 | negative       | 104-98-3   |
| Carnosine                        | up                 | 1.32        | 0.028   | 0.282 | positive       | 305-84-0   |
| **Arginine and Proline Metabolites** |                |             |         |       |                |            |
| Phosphocreatine                  | up                 | 1.62        | 0.037   | 0.282 | positive       | 67-07-2    |
| 4-Guanodinobutyric Acid          | down               | 1.09        | 0.049   | 0.270 | negative       | 463-00-3   |
| **Threonine Metabolites**        |                    |             |         |       |                |            |
| 2-Ketobutyric Acid               | up                 | 1.43        | 0.021   | 0.212 | negative       | 600-18-0   |
| **Tyrosine Metabolites**         |                    |             |         |       |                |            |
| Homovanillic Acid                | down               | 1.30        | 0.049   | 0.270 | negative       | 306-08-1   |
| **Riboflavin Metabolites**       |                    |             |         |       |                |            |
| 2, 4-Dihydroxypteridine (Lumazine) | up             | 2.07        | 0.008   | 0.154 | positive       | 487-21-8   |
| Flavin adenine dinucleotide (FAD) | up               | 1.19        | 0.021   | 0.212 | negative       | 146-14-5   |
| Lumichrome                       | down               | 1.71        | 0.049   | 0.282 | positive       | 1086-80-2  |
| **Pyrimidine Metabolites**       |                    |             |         |       |                |            |
| 2-Aminoisobutyric acid           | up                 | 1.38        | 0.011   | 0.154 | positive       | 62-57-7    |
| UMP                              | up                 | 1.22        | 0.049   | 0.270 | negative       | 58-97-9    |
| CMP                              | up                 | 1.29        | 0.049   | 0.282 | positive       | 63-37-6    |
| **Miscellaneous**                |                    |             |         |       |                |            |
| Lipoic Acid                      | up                 | 3.76        | 0.007   | 0.192 | negative       | 1077-28-7  |
| Thiourea                         | down               | 1.45        | 0.008   | 0.192 | negative       | 62-56-6    |
| Butyrylcarnitine                 | up                 | 1.24        | 0.011   | 0.154 | positive       | 25576-40-6 |
| Hydroxybutyric Acid              | up                 | 1.49        | 0.011   | 0.192 | negative       |            |
| Indoxyl Sulfate                  | down               | 2.12        | 0.021   | 0.212 | negative       | 2642-37-7  |
| 3-Hydroxymethylglutaric Acid     | up                 | 1.27        | 0.028   | 0.238 | negative       | 503-49-1   |
| 2-Hydroxypropridine              | up                 | 1.45        | 0.037   | 0.282 | positive       | 142-08-5   |

Continued
to both [see review by43], we chose to perform 1H NMR spectroscopy on the EC of 18–19 month-old APOE mice (7 APOE3/3 and 7 APOE4/4 males) in order to gain additional information about the metabolic changes occurring in the EC of aged APOE mice. While the EC’s close proximity to sinus spaces makes the NMR analysis of this brain region challenging, we were able to achieve full width half maximums of 8–15 Hz using a customized second and third order shim coil. Figure 5A shows the position of the volume of interest (VOI) that was used to measure metabolite levels in the EC. A representative spectrum from this region is shown in Fig. 5B, along with the spectral position of some of the major metabolites. To our knowledge, this is the first in vivo 1H NMR study of the EC performed in mice.

As shown in Fig. 5C and Table 4, this 1H NMR study revealed significant differences in the levels of multiple metabolites in the EC of aged male APOE4/4 vs. APOE3/3 mice, including decreased levels of creatine (Cre), glutamate (Glu), GABA, and taurine (Tau). Perhaps the most interesting observation, however, was the large increases in the level of phosphocreatine (PCr) and the ratio of PCr:Cr observed in the APOE4 EC. Creatine is mainly phosphorylated in mitochondria, where creatine kinase uses the newly synthesized ATP to produce PCr and ADP44. This PCr is then shuttled to the cytosol where it acts as a rapidly mobilized reserve of phosphate groups that can be used to replenish ATP levels, whereas the increased levels of ADP in the mitochondria will stimulate oxygen uptake and oxidative metabolism, especially during enhanced neuronal activity45. Thus, the observation that PCr levels are increased in aged APOE4/4 mice supports and expands upon the bioenergetics observations from our transcriptomics, metabolomics and Seahorse analyses. In addition to these changes in PCr levels, the observation that glutamate and GABA are both decreased in the EC of APOE4/4 mice may also be related to these bioenergetic differences between APOE genotypes and brain regions. Although glutamate and GABA are mostly known for their roles as neurotransmitters in excitatory and inhibitory neurons, they can also be utilized as energy metabolites46–49.

Taken together, our data suggest that, in contrast to other brain region such as the hippocampus and cortex, the EC of aged APOE4 mice exhibits an increased rate of oxidative metabolism and ATP turnover, as compared to aged APOE3 mice. We hypothesize that this differential regional mitochondrial respiration that we observe here is downstream of the decreased glucose utilization observed in APOE4 carriers40–42 and may be related to the neuronal hyperactivity that we observed in the EC of aged APOE4 mice33. In addition, we hypothesize that the unique bioenergetic compensatory mechanisms that we observe in the EC may lead to an increased rate of

| Metabolite                                      | Regulation in E4/4 | Fold Change | p-value | FDR  | Detection Mode | CAS Number |
|------------------------------------------------|--------------------|-------------|---------|------|----------------|------------|
| N-Acetylspermidine Acid                        | up                 | 1.92        | 0.037   | 0.270| negative       | 110-16-7   |
| Maleic acid                                    | up                 | 1.17        | 0.037   | 0.282| positive       | 131-48-6   |
| N-Acetylneuraminic Acid                       | down               | 1.19        | 0.049   | 0.282| positive       | 35989-16-3 |
| 2-Aminoadipic Acid                             | down               | 1.17        | 0.049   | 0.270| negative       | 542-32-5   |
| Cytidine diphosphate choline (CDP-choline)     | down               | 1.11        | 0.049   | 0.282| positive       | 987-78-0   |
| Glutathione                                    | up                 | 1.49        | 0.049   | 0.282| positive       | 70-18-8    |

Table 2. Differentially expressed targeted metabolites from the PVC of aged male APOE4/4 vs. APOE3/3 mice.

| GO Term                                             | Enrichment | p-value | FDR  |
|-----------------------------------------------------|------------|---------|------|
| carboxylic acid transport                           | 12.35      | 9.80E-20| 1.43E-16|
| carboxylic acid transmembrane transport             | 22.34      | 1.36E-16| 1.04E-13|
| long-chain fatty acid metabolic process             | 18.08      | 2.77E-13| 1.50E-10|
| fatty acid metabolic process                        | 7.8        | 1.45E-11| 5.40E-09|
| indololylamine metabolic process                    | 44.68      | 2.48E-09| 6.68E-07|
| disaccharide metabolic process                      | 63.29      | 6.92E-09| 1.77E-06|
| IMP metabolic process                               | 48.69      | 3.47E-08| 7.43E-06|
| steroid metabolic process                           | 6.87       | 1.78E-07| 3.24E-05|
| maltose metabolic process                           | 126.58     | 4.81E-07| 8.06E-05|
| kynurenine metabolic process                        | 50.63      | 7.51E-07| 1.18E-04|
| IMP biosynthetic process                            | 50.63      | 7.51E-07| 1.19E-04|
| tryptophan catabolic process                        | 50.63      | 7.51E-07| 1.20E-04|
| de novo IMP biosynthetic process                    | 63.29      | 9.46E-06| 1.13E-03|
| ketone catabolic process                            | 3.90E-05   | 3.92E-03|
| dopamine metabolic process                          | 20.25      | 4.12E-05| 4.06E-03|
| neurotransmitter catabolic process                  | 37.97      | 5.54E-05| 5.25E-03|
| keratan sulfate catabolic process                   | 31.65      | 1.00E-04| 8.37E-03|
| vitamin metabolic process                           | 6.38       | 3.60E-04| 2.35E-02|

Table 3. GO biological processes observed in the PIUMet analysis of differentially expressed untargeted metabolites from the EC of aged male APOE4/4 vs. APOE3/3 mice.
reactive oxygen species (ROS) generation in this region, which may play a causative role in the pathogenesis of AD among APOE4 carriers.

**Discussion**

Possession of the APOE4 allele greatly increases an individual’s risk of developing AD. Although numerous theories have been proposed, the precise cause of this association remains unknown. In order to further investigate the effects of APOE4 gene expression in AD-relevant brain regions, we initially chose to perform a transcriptomics analysis on RNA extracted from an AD-vulnerable brain region (the EC) vs. a less vulnerable brain...
EC of these mice. This decrease in mitochondrial respiration occurs downstream of glycolysis, whereas this decreased aerobic respiration does not appear to occur in the APOE4 mice.

APOE3/3 mice are primarily related to decreased aerobic respiration in Complex I-mediated respiration compared to Complex II-mediated respiration (Fig. 3 and Supplementary Fig. S3). This observation is consistent with previous reports showing that APOE4 expression has numerous deleterious effects on the brain's bioenergetic capabilities19–24, although it is important to note that the decline in mitochondrial functionality that we observed in the cortex and hippocampus, while significant, is likely below the pathological threshold50. Intriguingly, however, this APOE4-associated decrease in mitochondrial respiration with age was not observed in the EC. Additional studies, including proton nuclear magnetic resonance (1H NMR) spectroscopy and a bioinformatics analysis of our untargeted metabolomics data provide further support and elucidation of this region-specific bioenergetic regulation by APOE4, including the observations of increased PCr levels and decreased glutamate and GABA levels (Fig. 5), as well as an upregulation of the ketone pathway (Table 3), in the EC of aged APOE4 mice.

Mitochondria are part of a multifaceted network of metabolic pathways that are modulated by the cell to compensate for any reductions in respiratory activity and ATP production31,32. Mitochondria have been described as having a "reserve respiratory capacity" that allows them to increase their oxidative capacity and ATP production depending on the cell's energy demands31,34. Furthermore, mitochondrial adaptability is tissue and cell-type specific, depending on the different compensatory mechanisms and the availability of substrates30,35. Perhaps the most intriguing finding from our study, however, is that, in addition to these tissue and cell-type specific differences in mitochondrial adaptability, it appears that within the brain, mitochondria also have regional differences in their response to metabolic stressors such as APOE4 expression.

As shown in previous studies, the brains of APOE4 carriers display decreased glucose utilization in numerous AD-relevant brain regions, as observed by FDG-PET16–18. We hypothesize that the differential regional bioenergetic characteristics observed in the current study may represent region-specific responses to the decreased glucose utilization caused by APOE4 expression. For example, in both the EC and the Ctx as a whole, it appears that there are attempts to use alternative energy sources, such as the β-oxidation of fatty acids, to compensate for APOE4’s effects on glucose utilization. This is represented by the decreased fatty acid levels and increased carnitine levels observed in the metabolomics data from both the EC and PVC of the aged male APOE4/4 vs. APOE3/3 mice. In addition, the increased Complex II-mediated mitochondrial respiration observed in both the EC and the Ctx of aged female APOE4/4 vs. APOE3/3 mice suggests that aged female APOE4 mice may utilize β-oxidation of fatty acids to an even greater extent than the aged male APOE4 mice. However, the data from our Seahorse and 1H NMR spectroscopy studies, as well as further mining of our transcriptomics and metabolomics data, suggests that the mitochondria in the EC of aged APOE4 mice also utilize additional mechanisms to counteract any APOE4-associated reductions in glucose utilization, with the net effect being an increased coupling efficiency between oxygen consumption and ATP production.

This additional response in the EC is most clearly observed in our Seahorse data, where mitochondria in the EC of aged male APOE4 mice show increased ADP-stimulated oxygen consumption and increased maximal respiration rate, without changes in proton leak (Fig. 3) or mitochondrial mass (Supplementary Fig. S2). In addition, we also observed that the RCR is increased in the EC of aged male APOE4/4 vs. APOE3/3 mice (Fig. 3C), further confirming that ADP-driven mitochondrial respiration in the aged APOE4 EC is better coupled to ATP production than in the Hip, the PVC, and the Ctx. Interestingly, the reduction in mitochondrial respiration observed in the Ctx of both aged male and female APOE4/4 vs. APOE3/3 mice by Seahorse analysis appears to be more robust in Complex I-mediated respiration compared to Complex II-mediated respiration (Fig. 3 and Supplementary Fig. S3), whereas there is no decreased respiration in the EC of aged female APOE4/4 mice (Supplementary Fig. S3), and in the EC of aged male APOE4/4 mice, this Complex I-mediated respiration actually begins to outpace the Complex I-mediated respiration observed in the aged male APOE3/3 mice (Fig. 3). Since Complex I-mediated mitochondrial respiration is driven by pyruvate, this suggests that the APOE4-associated bioenergetic deficits observed in the Hip and Ctx of aged APOE4 mice are primarily related to decreased aerobic respiration that occurs downstream of glycolysis, whereas this decreased aerobic respiration does not appear to occur in the EC of these mice.

| Metabolite       | APOE3/3 concentration (mM) | APOE4/4 concentration (mM) | p-value     |
|------------------|---------------------------|-----------------------------|-------------|
| Creatine (Cr)    | 3.73 +/- 0.25             | 3.09 +/- 0.21               | 3.04E-04    |
| Phosphocreatine (PCr) | 0.84 +/- 0.27             | 2.55 +/- 0.18               | 1.00E-05    |
| Gamma-aminobutyrate (GABA) | 2.14 +/- 0.12             | 1.98 +/- 0.13               | 0.036       |
| Glutamate (Glu)  | 7.65 +/- 0.15             | 6.58 +/- 0.17               | 5.30E-05    |
| Glycerophosphocholine (GPC) | 1.25 +/- 0.12             | 0.67 +/- 0.07               | 2.90E-05    |
| Taurine (Tau)    | 6.16 +/- 0.14             | 5.31 +/- 0.15               | 1.59E-05    |
| Cr + PCr         | 5.58 +/- 0.11             | 5.85 +/- 0.12               | 8.90E-04    |
| Glu + Gln        | 10.43 +/- 0.21            | 9.22 +/- 0.23               | 2.30E-05    |
| NAA + NAAG       | 6.19 +/- 0.14             | 5.81 +/- 0.12               | 8.90E-04    |

Table 4. Metabolite concentrations in the EC of aged male APOE3/3 and APOE4/4 mice, as observed by 1H NMR spectroscopy.
While the reason for the increased mitochondrial coupling in the EC of aged APOE4 mice requires further investigation, we note that our transcriptomics data reveals an increased expression of some mitochondrial transporter genes in this brain region (Supplementary Tables S1 and S2). Mitochondrial transporters link biochemical pathways in the cytosol and mitochondria in order to ensure a sufficient rate of solute flux into mitochondria to fuel metabolic pathways. Regulation of these transporters is an essential part of the overall regulation of cellular metabolism, and their expression is highly variable depending on the tissues and the metabolic conditions. Intriguingly, the expression of the gene for adenine nucleotide translocase 1 (ANT1; Slc25a4) was significantly increased in the EC of aged male APOE3/4 vs. APOE3/3 mice. ANT1 participates in ATP-for-ADP exchange through the inner mitochondrial membrane, which supplies the cytoplasm with newly synthesized ATP from OxPhos. It can also interact with creatine kinase (CK), gaining preferential access to ATP, which is needed to synthesize PCr in cells with high energetic needs. Similarly, expression of the gene for the voltage dependent-anion channel 1 (VDAC1; Vdac1), one of the main gatekeepers for the crosstalk between mitochondria and cytosol, is also increased in the EC of these aged APOE3/4 mice, as is the gene for the mitochondrial phosphate transport protein (PTP; Slc25a3), which transports inorganic phosphate (P) to the mitochondrial matrix in order to supply the P, required for ADP phosphorylation into ATP. Another important mitochondrial transporter system that ensures the balanced cooperation between OxPhos and glycolysis (and to a lesser extent the pentose phosphate pathway) in order to maintain ATP levels is the malate-aspartate shuttle. In order to transfer the two NADH molecules generated during glycolysis into mitochondria, the enzymes malate dehydrogenase (MDH1) and glutamate oxaloacetate transaminase 1 (GOT1) mediate a series of metabolic conversions involving malate, oxaloacetate, aspartate and glutamate. Interestingly, our transcriptomics data reveals that the expression of both Slc25a22 (GOT1) and Slc25a2 (MDH1) is upregulated in the EC of aged male APOE3/4 vs. APOE3/3, suggesting that the activity of the malate-aspartate shuttle.

A summary of all these bioenergetics-related changes that we observe in the EC of aged male APOE4 mice is depicted in Fig. 6. Taken together, the observed differences on mitochondrial functionality among the brain regions studied suggest that the EC possesses a different subset of compensatory mechanisms in order to improve OxPhos efficiency under metabolic stress. While this apparent metabolic compensation in the EC may be regarded as a positive attribute, it may have negative sequelae in the long run. For example, it has been shown that over time, elevated mitochondrial respiratory activity and increased coupling efficiency result in an excess of free radical production and resulting oxidative damage within cells. Mild mitochondrial uncoupling, and the resulting increase in proton leak and decrease in mitochondrial membrane potential, has been previously suggested as a neuroprotective mechanism that cells utilize to prevent ROS formation by blocking the accumulation of electrons at early steps of the transport chain.

The fact that we observed increased Complex II-mediated mitochondrial respiration in the Ctx and EC of aged female APOE4 mice (Supplementary Fig. S3) adds further complexity to this story, but it also adds further evidence of increased ROS production in the brains of these aged APOE4 mice. When glucose supply is not high enough to provide a cell with sufficient ATP to meet its energy demands, the cell may resort to the ß-oxidation of fatty acids in order to help fuel the electron transport chain. During this ß-oxidation process, these fatty acids are metabolized into acetyl-CoA molecules, which feed into the TCA cycle, a system that includes the conversion of succinate to fumarate by Complex II. However, unlike glucose metabolism, which generates a high ratio of NADH:FADH2 (about 5:1), the ß-oxidation of fatty acids generates a much lower NADH:FADH2 ratio (about 2:1 for palmitate, for example). This increased EADH2 ratio can increase the production of ROS, both by maintaining the electron transport chain in a highly reduced state due to limited NADH supply, as well as by increasing the activity of flavoprotein-ubiquinone oxidoreductase, which is a potent source of superoxide production. Thus, although the EC from the aged female APOE4 mice did not require as much compensatory activity in its Complex I-mediated respiration as we observed in the aged male APOE4 mice, it is possible that the presence of a compensatory effect in Complex I-mediated respiration in the EC of aged female APOE4 mice, in combination with an overall increase in ROS production throughout the brain of these mice resulting from the increased Complex II-mediated respiration, may lead to even greater pathogenic effects in the EC of aged female vs. aged male APOE4 mice.

More work will of course be necessary in order to further elucidate this phenomenon, including an understanding of the specific contribution of different cell-types, as well as determining whether these same EC-specific compensatory mechanisms that we observed in the aged APOE4 mice exist in the EC of human APOE4 carriers. Given the limitations of human imaging studies and the lack of published omics data on human EC tissues from APOE4 vs. APOE3 carriers, it is difficult to directly compare our results to published human data at the present moment. It has been reported, however, that AD patients who possess at least one APOE4 allele show increased relative tau accumulation and increased brain atrophy in the EC compared to APOE4 non-carriers. Importantly, there is also evidence of EC-specific changes in human APOE4 carriers long before any symptoms of AD appear. For example, decreased entorhinal thickness has been observed in both young and middle-aged APOE4 carriers as compared to non-carriers, and young adult APOE4 carriers exhibit reduced EC-related grid-cell-like representations than non-carriers. Furthermore, middle-aged APOE4 carriers have been shown to possess increased incorporation of docosahexaenoic acid (DHA) specifically in the EC region compared to non-carriers, a finding that may be related to results from our metabolomics study, where DHA was the only fatty-acid observed to be upregulated in the EC of aged APOE4/d vs. APOE3/3 mice (Fig. 2B). Although the reason for this APOE4-associated upregulation of DHA incorporation in the EC is unknown, it is interesting to note that DHA concentrations have been found to be positively correlated to the rates of energy metabolism in cells and tissues.

It will also be important to investigate whether the other phenotypes that we have observed in the EC of these aged APOE4 mice, such as endosomal-lysosomal dysregulation and neuronal hyperactivity, are related to the bioenergetic observations described here. For example, Zhao et al. reported that APOE4 expression causes...
insulin receptors to be inefficiently recycled via the endosomal-lysosomal system, potentially causing the insulin resistance that the authors observed in the brains of aged APOE4 mice. As the endosomal-lysosomal dysregulation that we observed in the brains of aged APOE4 mice was not restricted to the EC, this may play a role in the APOE4-associated bioenergetic deficits that we observed in the Hip and Ctx of these mice. On the other hand, the neuronal hyperactivity that we observed appears to be primarily localized to the EC within the hippocampal formation. Intriguingly, neuronal activity and glucose utilization have previously been shown to exist in a near 1:1 stoichiometric relationship, suggesting that the bioenergetic counterbalancing that we observe in the EC of aged APOE4 mice may be directly correlated to the observed neuronal hyperactivity in these mice.

Finally, it will be important to determine whether these bioenergetic differences in the EC are involved in the increased risk of AD among APOE4 carriers. The EC is a unique brain region in many ways. It is vital for learning and memory (especially spatial memory), acting as a bridge between the hippocampus and the neocortex. As such, it is known to have higher energy demands and to be more metabolically active than many other brain regions. During AD pathogenesis, the EC is one of the first regions to develop tauopathy, which eventually develops in the hippocampus and other cortical regions as the disease progresses. It is possible, therefore, that due to its unique bioenergetic needs, the EC may possess compensatory mechanisms for increasing its bioenergetic capacity under conditions of metabolic stress such as APOE4 expression. Alternatively, these compensatory mechanisms may be initiated in order to fuel the greater energy demands that come from neuronal hyperactivity in the EC prior to AD onset. Regardless of the cause, it is possible that, in the process of inducing these compensatory bioenergetic mechanisms, other negative consequences may result, such as increased ROS production and increased tau hyperphosphorylation/aggregation, such that it results in an accelerated pathogenesis of AD.

Methods

Mice. Human APOE targeted replacement mice were first developed by Sullivan et al. and were acquired from Dr. Sullivan, or from Taconic Biosciences. All mice used in this study were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Columbia University Medical Center and the Nathan Kline Institute's Institutional Animal Care and Use Committee (IACUC).

RNA-sequencing. Transcriptomics analysis of aged APOE mice was performed as previously described. Briefly, male mice expressing human APOE3/3 (10 mice) or APOE3/4 (19 mice) were aged to 14–15 months, at which point they were sacrificed by cervical dislocation, and brain tissues containing the EC and PVC were dissected and snap frozen on dry-ice. Brain tissues were stored in RNase-free eppendorf tubes at −80°C prior to extraction. Total RNA was extracted from frozen tissues by homogenizing each tissue sample using a battery-operated pestle mixer (Argos Technologies, Vernon Hills, IL) in 1 ml of TRIzol reagent according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). RNA concentration was measured using a nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA), and RNA integrity (RIN) was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples possessed a RIN of 8 or higher. RNA was
stored at −80 °C prior to use. Starting with 2 μg of total RNA per sample, Poly(A) + mRNA was purified, fragmented and then converted into cDNA using the TruSeq RNA Sample Prep Kit v2 (Illumina cat# RS-122–2001) as per the manufacturer’s protocol (Illumina, San Diego, CA). For RNA-Sequencing of the cDNA, we hybridized 5 μM of each library to a flow cell, with a single lane for each sample, and we used an Illumina cluster station for cluster generation. We then generated 149 bp single end sequences using an Illumina HiSeq 2000 sequencer. For analysis, we used the standard Illumina pipeline with default options to analyze the images and extract base calls in order to generate fastq files. We then aligned the fastq files to the mm9 mouse reference genome using TopHat (v2.0.6) and Bowtie (v2.0.2.0). In order to annotate and quantify the reads to specific genes, we used the Python module HT-SEQ with a modified NCBIM37.61 (containing only protein coding genes) gtf to provide reference boundaries. We used the R/Bioconductor package DESeq 2 (v1.10.1) for comparison of aligned reads across the samples. We conducted a variance stabilizing transformation on the aligned and aggregated counts, and then the Poisson distributions of normalized counts for each transcript were compared across APOE3/4 vs. APOE3/3 groups using a negative binomial test. We corrected for multiple testing using the Benjamini-Hochberg procedure and selected all genes that possessed a corrected p-value of less than 0.05. Finally, a heat map based on sample distance and a volcano plot based on fold change and adjusted p-values were generated using the R/Bioconductor package ggplot2 (v2.0.0). For pathway analysis, enriched KEGG pathways were identified using the ClueGo application (version 2.1.7) in Cytoscape (version 3.2.1). Briefly, all differentially expressed EC genes from the RNA-Seq analysis were entered into the application and searched for significantly enriched KEGG pathways possessing a Benjamini-Hochberg adjusted p-value of less than 0.05.

Seahorse analysis. In order to investigate the effect of differential APOE isoform expression on electron transport chain activity, mitochondria were isolated from tissues collected from 15-month-old male mice expressing APOE3/3 (4 mice) or APOE4/4 (4 mice) (experiment 1), from 6-month-old male mice expressing APOE3/3 (3 mice) or APOE4/4 (3 mice) (experiment 2), from 20-month-old male mice expressing APOE3/3 (2 mice) and APOE4/4 (2 mice) (experiment 3), and from 20-month-old female mice expressing APOE3/3 (2 mice) and APOE4/4 (2 mice) (experiment 4). Tissue samples were pooled by region and genotype, and the oxygen consumption rates (OCR) were measured in a Complex I or Complex II-mediated fashion, as previously described. Briefly, each mouse was sacrificed by cervical dislocation, and the different brain areas from each brain hemisphere were immediately removed and placed into ice-cold PBS containing protease inhibitors. Following tissue collection, samples were homogenized in ~10 volumes of isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA and 0.5% fatty acid-free BSA, pH 7.2) and rinsed 3 times. Tissues were then homogenized using a Teflon glass homogenizer with 2–3 strokes. Homogenized samples were then centrifuged at 800 × g for 10 min at 4 °C. Following centrifugation, fat/lipid was aspirated off, and the remaining supernatant was decanted through two layers of cheesecloth into a separate tube and centrifuged at 8000 × g for 10 min at 4 °C. After removal of the supernatant, the pellet was resuspended in mitochondrial isolation buffer, and the centrifugation was repeated. The resulting pellet was resuspended in mitochondrial isolation buffer, and total protein concentrations were determined using Bradford Assay reagent (Bio-Rad). For Complex I experiments, 8 μg of protein was added to each well and for Complex II analysis 6 μg per well. To prepare samples for OCR measurements, samples were prepared in mitochondrial assay buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA and 0.2% fatty acid-free BSA, pH 7.2) plus substrate (pyruvate and malate for the Complex I-mediated ETC activity assay, or succinate and rotenone for the Complex II-mediated ETC activity assay).

Respirometry was performed using the XF24e Extracellular Flux Analyzer (Seahorse Bioscience). Oxygen consumption was measured in basal conditions (Seahorse media with 25 mM glucose and 2 mM pyruvate) and after the sequential addition of 1 μM oligomycin (Complex V inhibitor), 0.75μM FCCP (uncoupler) and 1 μM rotenone/1 μM antimycin A (Complex I and Complex III inhibitors, respectively). All results are averages of five or more biological replicates. Every biological replicate consisted of three technical replicates. OCR data was generated by the Seahorse XF24 1.5.0.69 software package and displayed in point-to-point mode. Final calculations of total OCR were performed by normalizing APOE4/4 values to APOE3/3 for each of the three experiments and then combining the data in Prism.

Metabolomic analysis. Metabolomics analysis of aged APOE mice was performed as previously described. Briefly, male mice expressing human APOE3/3 (8 mice), APOE3/4 (9 mice), or APOE4/4 (7 mice) were aged to 14–15 months, at which point they were sacrificed by cervical dislocation to maintain the brain environment, and individual brain regions were immediately removed and snap-frozen on dry ice. Tissues were stored at −80 °C for prior to extraction. Metabolite extraction was performed using a methyl tert-butyl ether (MTBE)/methanol extraction protocol modified from previous reports. Briefly, individual EC or PVC tissues were homogenized in 400 μl of ice-cold methanol using a bead mill homogenizer (Tissuelyser II, Qiagen) at 25 beads/sec, 2x for 45 sec each. Following homogenization, samples were incubated in 1200 μl of MTBE for 1 hr at room temperature to separate organic-soluble lipids from aqueous-soluble lipids and other small molecules. Finally, 360 μl of ultrapure water was added (for a final ratio of 3:1:0.9 MTBE:methanol:water) to resolve the two liquid phases, and each sample was centrifuged at 10,000 × g for 10 min. The lower aqueous phase and the upper organic phase were collected from each sample and stored in separate tubes, and the remaining protein pellets were resuspended in 25 mM ammonium bicarbonate, pH 8, with 2.5% SDS. A BCA protein assay was performed on each protein fraction, and both the aqueous phase and organic phase were normalized to their protein concentration equivalent with 50% and 100% methanol, respectively. All samples were then stored at −80 °C prior to analysis. Metabolite profiling was performed using an Agilent Model 1200 liquid chromatography (LC) system coupled to an Agilent Model 6430 time-of-flight (TOF) mass analyzer as described previously. Metabolite separation was accomplished using aqueous neutral phase (ANP) gradient chromatography on a Diamond Hydride column.
PIUMet analysis. We used the previously published PIUMet algorithm34 to analyze the untargeted metabolomics data from the EC of aged APOE4/4 vs. APOE3/3 mice. PIUMet discovers dysregulated molecular pathways and components associated with changes in untargeted metabolomics data by analyzing a network built from curated protein-protein and protein-metabolite interactions (PPMI). PIUMet represents each metabolite peak as a node in this network and connects it to metabolites with masses that correspond to the m/z values of the peak. Using the prize-collecting Steiner tree algorithm, it searches the PPMI interactome to find an optimum subnetwork that connects the input metabolite peaks via their putative identities and other metabolites and proteins that were not detected in the experiments. The Gene Ontology (GO) analysis of the resulting network further reveals molecular pathways associated with the APOE4 genotype. We selected 304 untargeted metabolite peaks or features that were significantly altered between the aged APOE4/4 and APOE3/3 mice (corrected p-value < 0.05). We then assigned a prize to each input data point to show the significance of their alterations, as –log (P values) of the significance of changes between two phenotypic conditions. PIUMet accepts several parameters that regulate the size of the resulting networks, including w and beta. While w tunes the number of trees in the resulting network, beta tunes the number of input nodes that are included in the output. Another parameter, mu, controls the bias toward high degree nodes. Higher values of mu result in a lower number of high degree nodes in the resulting networks, and thus less bias to highly studied molecules or ubiquitous interactions such as those with ions. We determined the optimum parameters (w = 6.0, beta = 0.5, and mu = 0.0005) based on their effects on the size of the resulting networks. We further generated 100 networks by adding random noise to the underlying database, and calculated robustness scores for each node. Nodes with robustness scores less than 60% were removed from the results. Additionally, for each node we calculated a specificity score, based on the number of their presence in the 100 random networks obtained from a set of mock metabolite peaks randomly selected from the input feature list that mimic real input. A score of 100 indicates that the node did not show in any of the randomly generated networks, while a zero score shows the node appears in all the randomly generated networks. 79% of the resulting nodes have specificity scores of over 60%.

1H NMR spectroscopy. All animal procedures for in vivo proton MR spectroscopy were performed as described previously31,42, and in accordance with the National Institutes of Health guidelines with approval from the Institutional Animal Care and Use Committee at the Nathan S. Kline Institute for Psychiatric Research. All animals were anesthetized using an isoflurane vaporizer set at the following percentages: 3% for induction, 2% during pilot scanning and 1.5% during data acquisition. An animal monitoring unit (model 1025, SA Instruments, Stony Brook, NY, USA) was used to record respiration and rectal temperature. Respiration was measured with a pressure transducer placed under the abdomen just below the ribcage. Body temperature was maintained using forced warm air, controlled by a feedback circuit between the heater and thermistor. After induction, the animals were placed on a holder and restrained using a bite bar and ear bars placed half way into the auditory canal. Oxygen was used as the carrier gas delivered to a cone positioned before the bite bar, where gases mixed with air and passed over the rodent’s nose. All animals were maintained at 37.0 ± 0.2 °C. All data were obtained on a 7.0T Agilent (Santa Clara, CA, USA) 40 cm bore system. The gradient coil insert had an internal diameter of 12 cm with a maximum gradient strength of 600 mT/m and minimum rise time of 200 µs, with customized second and third order shim coils. A Rapid (Rimpac, Germany) volume transmit coil (72 mm ID) and a two-channel receive-only surface coil was used for RF transmission and reception, respectively.

The shim settings for the selected volume of interest (VOI) were automatically adjusted using FASTMAP4 (Fast, Automatic Shimming Technique by Mapping Along Projections), a high order shim method, which samples the magnetic field along a group of radial columns which focus on the center of a localized voxel. It is a method for optimizing the field homogeneity in a cubical local region. The water signal was suppressed using variable power RF pulses with optimized relaxation delays (VAPOR)2. The spectral acquisition consisted of a short echo time Point Resolved Spectroscopy (PRESS) sequence with the following parameters: repetition time = 4 s, echo time = 7.5 ms, number of averages = 512 acquired in blocks of 128, number of points = 2048 and bandwidth...
of acquisition = 5 kHz, total acquisition time = 34 minutes. Outer volume suppression was also used to minimize signal contamination by extra cranial muscle and lipids. The VOI size was 3.3 μl (2.0 × 1.3 × 1.3 mm³) placed in the EC. The target VOI is depicted in Fig. 5 overlaid on a schematic of the coronal brain slice at approximately 2.92 mm relative to Bregma. An anatomical T₂-weighted pilot scan was used to position the VOI (coronal). These scans were acquired with a fast spin echo sequence with the following parameters: field of view = 20 mm with 256 × 256 matrix size, slice thickness = 0.5 mm, number of slices = 11, repetition time = 4 s, echo train length = 8, echo spacing = 15 ms, effective echo time = 60 ms, number of averages = 8, total acquisition time = 8 minutes 40 s. All data were processed using the LCModel software developed by Provencher1. This software calculates the best fit to the acquired data of a linear combination of model spectra acquired from in vitro solutions of all the brain metabolites of interest. This basis set of the model spectra has the same echo time, sequence acquisition and field strength as the acquired data of the study. The LCModel software outputs the estimated concentration along with estimated standard deviations (Cramer-Rao lower bounds) expressed in percent of the estimated concentration, which can be used as a quantitative measure of reliability. To improve statistical significance, Miller et al. recently demonstrated the use of weighted averaging in NMR spectroscopic studies83. In this current study, we also used weighted averaging to calculate the standard unequal variance t-test (Welch’s t-test) as outlined by Miller83 and in the reference manual of the LCModel software84. All data used in the final analysis had Cramer-Rao lower bounds of 20% or less.

The metabolites measured were: alanine (Ala), aspartate (Asp), creatine (Cr), phosphocreatine (PCr), γ-Aminobutyric Acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glycerophosphocholine (GPC), phosphocholine (PC), glutathione (GSH), myo-inositol (Ins), lactate (Lac), N-Acetylaspartate (NAA), N-Acetylaspartateglutamate (NAAG) and taurine (Tau). It is often quite difficult to resolve Glu from Gln, NAA from NAAG and PC from GPC, particularly if the spectral quality is poor due to an inadequate shim. So, in addition to the principal metabolites, the total of Glu and Gln, NAA and NAAG, and PC and GPC are also reported. These total concentrations are thought to be a more reliable metric. An unsuppressed water signal was also used for absolute concentration calculation and eddy current correction. This internal reference method assumes known values of water concentrations of gray and white matter2–7.

There are several limitations to 1H NMR spectroscopy which can make reliable measures particularly challenging. One such limitation is that is that 1H NMR metabolite measures are inherently low in signal to noise. The metabolite signals measured are approximately 10,000-fold less than the proton signal used in imaging. The EC is also a small structure and thus requires a small VOI which reduces the measured signal strength. However, at 7T with shim values in the range 8–15 Hz and a short echo time of 8 ms, the spectral peaks were reasonably separated, allowing for direct and reliable measures of metabolite concentrations. For metabolites which have significant spectral overlap, total values (e.g. Glu and Gln) are also reported. Another error source is chemical shift displacement. The bandwidth of the 180° RF pulse is 5 kHz, which would give a displacement error of 0.3 mm in the vertical and horizontal directions over the range of 0.2 to 4.2 ppm. However, this displacement was relatively small and still allowed for sufficient specificity. Despite the challenges of in vivo mouse brain 1H NMR, we were able to achieve high quality spectra in this difficult region with high quantification accuracy.

**Data availability**

The transcriptomics datasets are available in the NCBI Gene Expression Omnibus (GEO) repository: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102334. The metabolomics datasets are available in the MetaboLights repository: http://www.ebi.ac.uk/metabolights/MTABLS530.

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**References**

1. Mahley, R. W. & Rall, S. C. Jr. Apolipoprotein E: far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet*. 1, 507–537 (2000).
2. Han, X. The role of apolipoprotein E in lipid metabolism in the central nervous system. *Cell Mol. Life Sci.* 61, 1896–1906 (2004).
3. Holtzman, D. M., Herz, J. & Bu, G. Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2, a006312 (2012).
4. Liu, C. C., Kanekiyo, T., Xu, H. & Bu, G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nat. Rev. Neurol.* 9, 106–118 (2013).
5. Farrer, L. A. et al. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium, *JAMA* 278, 1349–1356 (1997).
6. Rubinsztein, D. C. & Easton, D. F. Apolipoprotein E genetic variation and Alzheimer’s disease. a meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *Neuron* 10, 199–209 (1999).
7. Bales, K. R. et al. Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nat. Genet.* 17, 263–264 (1997).
8. Castano, E. M. et al. Fibribrogenesis in Alzheimer’s disease of amyloid beta peptides and apolipoprotein E. *Biochem. J.* 306(Pt 2), 599–604 (1995).
9. Rebeck, G. W., Reiter, J. S., Strickland, D. K. & Hyman, B. T. Apolipoprotein E in sporadic Alzheimer’s disease: allelic variation and receptor interactions. *Neuron* 11, 575–580 (1993).
10. Schmechel, D. E. et al. Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl Acad. Sci. U S Am.* 90, 9649–9653 (1993).
11. Ma, J., Yee, A., Brewer, H. B. Jr., Das, S. & Potter, H. Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments. *Nat. Chem. Biol.* 1, 92–94 (1995).
12. Castellano, J. M. et al. Human apoE isoforms differentially regulate brain amyloid-beta peptide clearance. *Sci. Transl. Med.* 3, 89ra57 (2011).
13. Holtzman, D. M. et al. Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer’s disease. *Proc. Natl Acad. Sci. U S Am.* 97, 2882–2887 (2000).
44. Bessman, S. P. The creatine phosphate energy shuttle–the molecular asymmetry of a “pool”.  
45. Chen, W., Zhu, X. H., Adriany, G. & Ugurbil, K. Increase of creatine kinase activity in the visual cortex of human brain during visual  
47. Kreft, M., Bak, L. K., Waagepetersen, H. S. & Schousboe, A. Aspects of astrocyte energy metabolism, amino acid neurotransmitter  
54. Pfleger, J., He, M. & Abdellatif, M. Mitochondrial complex II is a source of the reserve respiratory capacity that is regulated by  
49. Patel, A. B.  
39. Auestad, N., Korsak, R. A., Morrow, J. W. & Edmond, J. Fatty acid oxidation and ketogenesis by astrocytes in primary culture.  
40. Guzman, M. & Blazquez, C. Ketone body synthesis in the brain: possible neuroprotective effects.  
42. Murin, R. & Hamprecht, B. Metabolic and regulatory roles of leucine in neural cells.  
41. Bixel, M. G. & Hamprecht, B. Generation of ketone bodies from leucine by cultured astroglial cells.  
51. Celotto, A. M., Chiu, W. K., Van Voorhies, W. & Palladino, M. J. Modes of metabolic compensation during mitochondrial disease using the Drosophila model of ATP6 dysfunction.  
53. Celotto, A. M., Chiu, W. K., Van Voorhies, W. & Palladino, M. J. The contribution of GABA to glutamate/glutamine cycling and energy metabolism in the rat cortex in vivo.  
48. Patel, A. B. et al. Energy Metabolism of the Brain, Including the Cooperation between Astrocytes and Neurons, Especially in the Context of Glycogen Metabolism.  
47. Patel, A. B. et al. The contribution of GABA to glutamate/glutamine cycling and energy metabolism in the rat cortex in vivo.  
411–424 (2015).  
48. Patel, A. B. et al. The contribution of GABA to glutamate/glutamine cycling and energy metabolism in the rat cortex in vivo.  
102, 289–8302 (2005).  
115–129 e115 (2017).  
124, 725–733 (2004).  
4, 1376–1386 (1991).  
7, 33426–33432 (1999).  
10, 1464 (2017).  
8, 752–758 (1996).  
5, 38 (2013).  
98, 8838–8843 (2001).  
11, 702 (2017).  
12, 319–332 (1995).  
6, 35 (2009).  
6, 375 (2013).  
124, 1027–1036 (2017).  
287–294 (2015).  
102, 16964–16995 (2005).  
102, 161–193 (2015).  
4, 411–424 (2015).  
3, 519–523 (1987).  
128 (1991).  
39, 785–799 (2002).  
76, 829–8302 (2005).  
161–193 (2015).  
1376–1386 (1991).  
5, 375 (2013).  
25, 1027–1036 (2017).  
370–376 (2016).  
2677–2684 (2011).  
370–376 (2016).  
5, 375 (2013).  
6, 35 (2009).  
128 (1991).  
10, 1241–1252 (2006).  
16, 23995–23981 (2015).  
102, 5588–5593 (2005).  
125, 1241–1252 (2006).  
191, 288–295 (2011).  
121, 287–294 (2016).  
829–8302 (2005).  
124, 7372–7381 (2017).  
5, 375 (2013).  
25, 1027–1036 (2017).  
34, 465–484 (2015).  
7, 287–294 (2008).  
4, 279–284 (2008).
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Author contributions

This study was designed and managed by T.N., E.A.G., and K.E.D. Animal care and breeding was performed by H.F. Transcriptomics analysis was performed by A.A.D., in the laboratory of M.R.C. Metabolomics analysis was performed by Q.C. and T.N., in the laboratory of S.S.G. Seahorse analysis was performed by D.L., R.R.A., and M.P., in the laboratory of L.A.G. PIUmet analysis was performed by L.P., in the laboratory of E.E. 1H NMR spectroscopy was performed by K.S. and D.G. Additional molecular biology experiments and bioinformatics were performed by T.N., A.A. and H.A. Manuscript preparation was performed by T.N. and E.A.G., with input from K.E.D.
Competing interests
K.E.D is on the board of directors of Ceracuity LLC. The authors declare no competing interests.

Additional information
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