Featured Article

Estrogen activates Alzheimer’s disease genes

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Abstract

Introduction: Women are at increased risk for Alzheimer’s disease (AD), but the reason why remains unknown. One hypothesis is that low estrogen levels at menopause increases vulnerability to AD, but this remains unproven.

Methods: We compared neuronal genes upregulated by estrogen in ovariectomized female rhesus macaques with a database of 17,000 diverse gene sets and applied a rare variant burden test to exome sequencing data from 1208 female AD patients with the age of onset < 75 years and 2162 female AD controls.

Results: We found a striking overlap between genes upregulated by estrogen in macaques and genes downregulated in the human postmortem AD brain, and we found that estrogen upregulates the APOE gene and that progesterone acts antagonistically to estrogen genome-wide. We also found that female patients with AD have excess rare mutations in the early menopause gene MCM8.

Discussion: We show with genomic data that the menopausal loss of estrogen could underlie the increased risk for AD in women.

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1. Introduction

Two-thirds of approximately 5.4 million Americans with Alzheimer’s disease (AD) are women [1]. Women are at increased risk for AD, even after adjusting for age and education level [1–3]. Moreover, while APOE is the largest known genetic susceptibility factor for AD [4], women with the risk allele have much higher risk for disease than men [5–7]. Various lines of evidence suggest that the menopause transition underlies the increased vulnerability [8–12]. In vitro studies suggest that estrogen is neuroprotective against various cellular insults [13–18] and can also confer protection from amyloid β (Aβ) toxicity [19,20], whereas postmortem analyses reveal that women with AD have reduced brain estrogen levels [21]. Moreover, early surgical removal of ovaries has been linked to cognitive decline [22] and increased risk for AD-related dementia [23].

Hormone replacement therapy (HRT) can alleviate the adverse symptoms of menopause and has been shown to reduce the risk for dementia [10,11,24–28]. However, the overall outcomes are controversial due to the findings of the Women’s Health Initiative Memory Study (WHIMS) which showed that HRT increased the risk for dementia [29,30]. These seemingly contradictory research findings point to significant gaps in our understanding of the effects of estrogen on brain function [8,9]. Many of these knowledge gaps were recently highlighted by the think tank...
convened by the Women’s Alzheimer’s Research Initiative [9] and the Society for Women’s Health Research Interdisciplinary Network on AD expert panel [8]. These include the need to better understand how estrogen influences risk at the molecular level [8], the need to resolve discrepancies between animal models and human clinical trials [8,9], the need to understand the role of progesterone [9], and the need to better understand how APOE confers female-biased risk [8,9].

A recent hypothesis proposed to explain how menopause increases vulnerability in women suggests that the perimenopause transition is a “bioenergetic transition state” [31–33]. This hypothesis proposes that estrogen promotes glucose metabolism via mitochondria [34] and that the decline in estrogen levels during perimenopause leads to a switch away from glucose toward ketone bodies as a fuel source [35]. Although this hypothesis is supported by a number of animal models [32,34] and brain-imaging studies [31,36], the mechanism by which estrogen acts as the master regulator of glucose uptake and mitochondrial function remains unknown [37].

In this study, we use a unique genomic approach to address some of the recently highlighted research gaps [8,9]. We integrate multiple data sets to gather genomic evidence for AD sex-biased risk factors. Our data identify target genes of estrogen and progesterone action within neurons, shed light on the cause of female-biased risk of APOE, and provide support for the “bioenergetic transition state hypothesis” [31–33] by revealing a central role for mitochondrial function in AD.

2. Methods

2.1. Microarray gene expression data

We downloaded the data set GSE16169 from the Gene Expression Omnibus [38]. We took the mean of the expression values across the two individuals in each treatment and then calculated the estrogen versus placebo fold change by dividing the mean expression values from the estrogen-treated macaques to the mean expression values for the placebo-treated macaques. We calculated the progesterone versus estrogen fold change by dividing the mean expression value of macaques treated with both estrogen and progesterone by the mean expression value of the macaques treated only with estrogen (Please see attached Supplementary Material file for more details.)

2.2. Exome sequencing data

To maximize the power to detect, we only considered white Europeans because they had the largest sample size. We extracted out white Europeans using the self-described race and ethnicity annotation. We then removed samples that were outliers on heterozygosity and outliers on race as determined by principle component analysis, cryptic relatedness, and other quality-control metrics. We filtered out all APOE ε4 homozygote individuals and only retained cases with age of onset < 75 years. We then extracted out singletons and filtered for deleterious variants, which we defined as loss of function, nonsense-mediated decay, annotated as ‘HIGH’ impact by SnpEff (version 4.3) [39] or annotated as both missense and probably damaging by Polyphen [40]. We only retained singletons not present in thousand genomes [41] or ExAC [42]. We then developed a rare variant association test similar to what was applied to copy number variations in autism [43], but we modified it for deleterious singleton SNPs. The female comparison involved female cases (n = 1208) versus female controls (n = 2162), the male comparison involved male cases (n = 953) versus male controls (n = 1495), and total comparison involved total cases (n = 2161) versus total controls (n = 3657). We calculated false discovery rate by randomizing the case-control status as described in the study by Pinto et al. [43] 1000 times. The rare variant association test was applied to all protein-coding genes after filtering out genes with less than three samples in either cases or controls with deleterious singletons.

3. Results

3.1. Macaque neuronal gene expression data

Rhesus macaques have been used to model menopause because they mimic the hormonal changes experienced by women throughout their life course [44]. We obtained microarray gene expression data from laser-captured serotonergic neurons from six female rhesus macaques (with ovaries removed) that were deposited in the Gene Expression Omnibus [38]. In this study, two macaques received estrogen, two received placebo, and two received estrogen with progesterone. We identified estrogen responsive genes by comparing gene expression patterns in macaques receiving estrogen with the gene expression patterns of macaques receiving placebo. We performed an unbiased genome-wide analysis to explore the role of estrogen in neurons, we did this by obtaining expression values from 15,517 genes which we ranked by calculating the fold change (FC) between estrogen and placebo treatments (Fig. 1, Supplementary Table 1).

3.2. Estrogen upregulates genes that are downregulated in AD

To identify any cellular and molecular pathways that may be enriched among estrogen-regulated genes, we compared the genes with greater than two-fold (FC > 2) upregulation (504 genes) with all 17,779 gene sets in the MSigDB [45–47] (a database of annotated gene sets from a variety of biological studies). We performed a hypergeometric ratio test to assess enrichment and found that ranked eighth, with an adjusted P value < 4.8 × 10−12, was a data set corresponding to genes downregulated in the hippocampus.
of patients with AD [48] (Supplementary Table 2). Among the 1027 genes identified in the AD study by Blalock et al. [48], 84 genes overlapped with the 504 estrogen upregulated genes (FC > 2). To assess the robustness of these results, we performed the same analysis using genes with FC greater than 1.5 (1222 genes) (Supplementary Table 3). This time, the same AD data set from the study by Blalock et al. [48], was ranked first (P value < 2.7 × 10^{-45}), with 226 genes intersecting estrogen upregulated and AD downregulated gene sets (Supplementary Table 4). We compared the overlap of all 4 pairwise comparisons of estrogen upregulated and downregulated genes with Alzheimer’s upregulated and downregulated genes and confirmed that the largest overlap was between estrogen upregulated and Alzheimer’s downregulated genes (P value < 8.14 × 10^{-15}, Supplementary Fig. 1, Supplementary Table 5). To estimate the false discovery rate, we compared the overlap of 100 randomized gene sets [45] and found that none of the 100 randomized gene sets had statistically significant (P value < .05) overlap with the AD downregulated gene signature from the study by Blalock et al. [48]. These results reveal that genes upregulated by estrogen are highly enriched for genes that are downregulated in human postmortem AD brains.

### 3.3. Mitochondrial genes link estrogen to AD risk

To gain insight into underlying mechanisms linking estrogen to AD risk, we compared the 84 genes that intersect estrogen upregulated and AD downregulated genes to pathways in MSigDB [45] and found enrichment for mitochondrial function. Ten pathways were annotated to mitochondrial-related descriptions including oxidative phosphorylation (P value < 2.8 × 10^{-66}), mitochondrial databases (P value < 7.6 × 10^{-5}), and the tricarboxylic acid cycle (P value < .001) (Supplementary Table 6). To
confirm that this enrichment is driven by genes downregulated in AD, we compared the overlap of estrogen upregulated genes (FC > 2) with mitochondrial genes, which we obtained from MitoCarta [49] (Supplementary Table 7), and found that the proportion of mitochondrial genes in the 84 intersecting genes was much greater than the proportion of mitochondrial genes in the 504 estrogen upregulated genes (26% vs. 11%, P value < .0007, Supplementary Material, Supplementary Table 7). These results suggest that mitochondrial pathways are an important mechanism linking estrogen loss to AD risk.

3.4. Estrogen upregulates APOE and other synapse genes

We found estrogen upregulates several amyloid and synapse-related genes. Ranked third in estrogen-induced fold change out of the 15,517 genes, with a log₂ fold change of 4.44, was the AD susceptibility gene APOE (Table 1). Because a key feature of AD is synapse loss [50,51], we hypothesized that estrogen responsive genes may be enriched for synaptic function. To test this, we compared synaptic genes (obtained from SynaptomeDB [52,53], number of genes = 1644) with the 504 estrogen upregulated genes (FC > 2) (Supplementary Table 4) and found a significant overlap of 140 genes (28%, P value < 2.2e⁻¹⁶), which remained significant even after randomization. We also quantified the overlap between the mitochondrial and synapse enrichment and found that synapse genes (n = 1644) overlapped with mitochondrial genes (n = 988) by 253 genes, which is statistically significant with a hypergeometric ratio test (P value < 6.17e⁻⁴⁴). This suggests that estrogen’s role in synapse function could at least in part be mediated by the mitochondria.

3.5. Progesterone acts antagonistically to estrogen genome-wide

HRT often includes a progesterone component, although not much is known about how progesterone influences gene expression in the brain. Interestingly, we found that estrogen downregulates the progesterone receptor (PGR) by nearly 6-fold (Fig. 2, Supplementary Table 1), and when we compared the expression values of macaques treated with both estrogen and progesterone with macaques treated with estrogen only, we found that adding progesterone antagonized estrogen-induced gene expression on a genome-wide scale (Figure 1, Fig. 3). The mean expression of the 504 estrogen upregulated genes nearly halved after adding progesterone (t-test P value = 6.968e⁻¹⁴), and the mean expression of the estrogen downregulated genes increased by more than two-fold after adding progesterone (t-test P value = .001277). We found that progesterone antagonized the expression of APOE, other known AD risk genes [54] (Supplementary Material), the 84 genes intersecting estrogen upregulated and Alzheimer’s downregulated genes sets, and mitochondrial [49] and synapse genes [52,53] (Fig. 3).

3.6. Rare variant burden test on exome sequencing data

By linking APOE, mitochondrial, and synapse genes to estrogen action in neurons, the macaque experiment provided insight into the mechanism by which estrogen loss at menopause could increase vulnerability to AD in women. To validate our genomic evidence linking menopause to AD risk, we sought to analyze an independent genomic data set. We obtained exome sequencing data from the Alzheimer’s disease sequencing project [55]. Our aim was to assess whether any differences in mutation patterns in female AD cases compared with female AD controls could link menopause to increased vulnerability to AD in women. To enrich for AD cases with a strong genetic component, we only considered cases with the age of onset <75 years, which left us with a total of 2161 cases and 3657 controls, in which 1208 were female cases and 2162 were female controls.

To maximize our chances of uncovering genes that disrupt core biology, we focused on ultra-rare mutations known as singletons (mutations only seen once within the data set). These mutations are likely to exert strong biological effects because they have been kept at low frequency by purifying selection. We applied rigorous filtering on these singletons to enrich for causal mutations, which we defined as loss of function, nonsense-mediated decay, annotated as ‘HIGH’ impact by SNPEff (version 4.3) [39] or annotated as both missense and probably damaging by PolyPhen [40] (Please see Supplementary Tables for mutation coordinates).
We developed a rare variant collapsing burden test similar to that applied to copy number variations in autism [43] but modified it for deleterious singleton single-nucleotide variants (Supplementary Table 8). Currently, many sequencing studies use the optimal test within the family of sequence kernel association tests [56,57] to identify disease-associated genes; however, we chose to use our rare variant collapsing burden test because simulations have shown that when most variants are likely to be causal, the collapsing burden test outperforms the optimal test within the family of sequence kernel association tests [56,57].

3.7. Validation of our ultra-rare variant association test

We first aimed to validate our rare variant burden test by performing it on all AD cases (n = 2161) and all AD controls (n = 3657). When we did this, we found our top hit to be SORL1 and our third top hit to be ABCA7 (Fig. 4, Supplementary Table 9). This finding confirmed the validity of our method because both SORL1 and ABCA7 are well-known AD risk loci [54,58,59]. Interestingly, our macaque neuronal gene expression data also identified SORL1 to be highly regulated by estrogen (Fig. 2, Supplementary Table 1).

3.8. Identification of MCM8 as a female-specific risk factor

To identify female-specific risk factors, we performed our rare variant burden test to compare female AD cases (n = 1208) with female AD controls (n = 2162). We identified 50 genes with both false discovery rate and P value < .05 (Table 2, Supplementary Table 10). Of these 50 genes, 45 contained excess mutations in cases and 5 contained excess mutations in controls. The genes with excess mutations in cases included MCM8, which we found to have five missense variants predicted to be damaging by PolyPhen [40] (Supplementary Table 11, Fig. 4). This finding provides robust independent support that menopause could underlie AD vulnerability in women because MCM8 has been strongly associated with primary ovarian insufficiency [60] and early menopause in women [61]. We also found excess mutations in female AD cases in the CCT7 and MCAT genes; which is interesting because our macaque data reveal CCT7 to have the highest estrogen-induced fold change in neurons (Table 1, Supplementary Table 1) and MCAT is a mitochondrial enzyme. To identify potential protective factors, we looked for genes with excess mutations in controls and found the brain size gene ASPM, which had eight missense variants predicted by PolyPhen to be damaging [40] and one stop-gain mutation (Fig. 4, Supplementary Table 12). We also performed our rare variant burden test on male cases (n = 953) and male controls (n = 1495) (Supplementary Table 13) and did not detect statistically significant excess of deleterious singletons in MCM8, ASPM, CCT7, or MCAT.

4. Discussion

Here, we use a unique genomic approach to uncover evidence consistent with estrogen loss at menopause underlying increased vulnerability to AD in women. We link estrogen to mitochondrial and synapse function and...
Figure 3. Antagonistic impact of progesterone in various gene sets. (A) GWAs identified AD risk genes [54] (Supplementary Material). (B) 84 genes intersecting estrogen upregulated and Alzheimer downregulated. (C) Synapse genes [52, 53]. (D) Mitochondrial genes [49].
demonstrate that estrogen upregulates APOE and show that progesterone acts antagonistically to estrogen genome-wide. Finally, we use an independent exome sequencing data set to demonstrate that female AD cases have excess rare, deleterious mutations in the early menopause gene MCM8.

AD is a disorder of the synapse [50,51]. In fact, cognitive decline has been shown to correlate most closely with synapse loss [51]. Our finding that estrogen upregulates synapse genes fits with those of prior imaging studies, which reveal that hippocampal size correlates with estrogen levels throughout the menstrual cycle [62,63]. Our data also reveal a central role for mitochondria. We show that estrogen upregulates mitochondrial genes, and our exome data reveal excess mutations in the mitochondrial enzyme MCAT (Table 2), which is also associated with the reduction of amyloid β production [64]. These findings are consistent with the bioenergetic state transition hypothesis [31–33], which links metabolism deficits in the brains of postmenopausal women [31]. Importantly, our data also reveal a convergence of mitochondrial and synapse genes, which is consistent with the notion that mitochondria could influence synapse growth [65], possibly to help meet synaptic ATP requirements [66].

One of the strengths of our study is that we reveal the target genes of estrogen and progesterone action within neurons. These data reveal both APOE and SORL1 to be highly estrogen responsive (Fig. 2) and, curiously, both APOE and SORL1 are associated with female-specific risk for AD [5–7,67]—suggesting that estrogen may interact with genetic mutations to confer sex-biased risk.

We used genomic data spanning multiple data types to cross validate our findings on estrogen loss and AD risk. We found excess deleterious singleton mutations in the ovarian failure [60,68–71] and early menopause [61,72–76] gene MCM8 (Table 2, Supplementary Table 11), providing robust support for our hypothesis that estrogen loss at menopause confers increased vulnerability to AD in women. This finding fits with previous studies that have linked surgical menopause to doubled lifetime risk for dementia [23], increased risk for AD neuropathology [22] and cognitive decline [22].

Our method also provided the opportunity to identify potential protective genetic factors. Our finding of excess rare,
deleterious singletons in the brain size gene ASPM [77] (Supplementary Tables 10,12) is interesting because ASPM has also been linked to the human-specific evolution of brain size [78,79] and because ASPM may represent a neural substrate [80] for the “brain reserve” hypothesis [81–84], which reasons that large brain size can protect against cognitive decline [83].

Our finding that progesterone acts antagonistically to estrogen genome-wide is particularly provocative. Cell culture studies have suggested progesterone to be antagonistic [85–87], but our data highlight that the antagonism may be genome-wide. The seemingly contradictory findings from longitudinal studies of HRT use may be resolved by the consideration that progesterone acts antagonistically to estrogen. For instance, a large Finnish study of 230,580 women [88] found HRT containing both estrogen and progesterone seemed to increase risk for dementia, whereas estrogen-only HRT reduced the risk. This is consistent with the recent analyses [89] of two clinical trials, the Kronos Early Estrogen Prevention Study–Cognitive and Affective Study (KEEPS-Cogs) and the Early vs. Late Intervention Trial with Estradiol-Cognitive Endpoints (ELITE-Cog) [89], which found that taking HRT between the ages of 50 and 54 years is not cognitively detrimental, whereas taking HRT between the ages of 65 and 79 years was associated with reductions in global cognition, working memory, and executive function [89].

4.1. Limitations

Our data are valuable for gaining much needed clarity on the role of estrogen in AD risk; however, there are some limitations. First, we relied on macaque gene expression data to understand estrogen response in the human brain—human data would have been better, but no such datasets exist, and the experiments required to get such datasets mean they are unlikely to exist in the future. Macaques are preferable [90] to rodents because nonhuman primates more accurately recapitulate AD-relevant gene expression in the human brain [91]. Another potential limitation is the limited sample size of each treatment, which is why we focused on fold change, as other statistical tests were not feasible. However, although our sample size may be limiting, the APOE gene signal was captured by three probes, and the data were obtained from laser-captured neurons, so are unlikely to be confounded by expression from other cell types. Another potential limitation could be the different sample sizes in the exome data for men compared with women, which may lead to differential power to detect. We focused our analysis on females, which actually had more samples than males, however, not finding an association in males, may be due to decreased power to detect rather than an actual biological difference.
4.2. Future work

Our study is timely as it fits well with recent reports that have linked longer reproductive periods and more months being pregnant with reduced risk for AD [89]. Future work in this field may include stratifying female samples by HRT use to quantify the proportion of risk attributed to genetic mutations versus loss of estrogen and performing genome-wide association studies to uncover any association between common variants in MCM8 and AD risk in women.

4.3. Conclusions

Here, we endeavored to use genomic data to address the knowledge gaps surrounding why women have increased risk for AD, and in doing so, we address the controversy initiated by the Women’s Health Initiative Memory Study [29,30]. Our comprehensive, integrative, genomic analyses lead us to conclude that estrogen loss is likely to contribute to AD vulnerability—suggesting that increased risk for AD in women may be attributed to menopause.

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The three LSSCs are the Human Genome Sequencing Center at the Baylor College of Medicine (U54 HG003273), the Broad Institute Genome Center (U54HG003067), and the Washington University Genome Institute (U54HG003079). Biological samples and associated phenotypic data used in primary data analyses were stored at the study investigators’ institutions and at the National Cell Repository for Alzheimer’s Disease (NCRAD, U24AG021886) at Indiana University funded by NIA. Associated phenotypic data used in primary and secondary data analyses were provided by the study investigators, the NIA-funded Alzheimer’s Disease Centers (ADCS), and the National Alzheimer’s Coordinating Center (NACC, U01AG016976) and the National Institute on Aging Alzheimer’s Disease Data Storage Site (NIAGADS, U24AG041689) at the University of Pennsylvania, funded by NIA, and at the Database for Genotypes and Phenotypes (dbGaP) funded by NIH.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.trci.2019.09.004.
1. Systematic review: We searched the literature, recent conference abstracts, YouTube, and review papers by the think tank convened by the Women’s Alzheimer’s Research Initiative and the Society for Women’s Health Research Interdisciplinary Network on Alzheimer’s disease (AD) to evaluate the accumulated knowledge related to whether estrogen loss contributes to AD risk in women.

2. Interpretation: Previous findings have been based on observational and randomized control studies. We took a different approach of gene expression and genetic mutation data. Our findings provide quantitative evidence for the role of estrogen in AD risk, with particular emphasis on mitochondrial function.

3. Future directions: Future work can involve stratifying women by HRT use to determine whether the genetic signal is stronger in women without HRT and performing genome-wide association studies to see if common variants in MCM8 confer increased risk for AD.

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