17β-Estradiol Modulates Gene Expression in the Female Mouse Cerebral Cortex

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Abstract

17β-estradiol (E2) plays critical roles in a number of target tissues including the mammary gland, reproductive tract, bone, and brain. Although it is clear that E2 reduces inflammation and ischemia-induced damage in the cerebral cortex, the molecular mechanisms mediating the effects of E2 in this brain region are lacking. Thus, we examined the cortical transcriptome using a mouse model system. Female adult mice were ovariectomized and implanted with silastic tubing containing oil or E2. After 7 days, the cerebral cortices were dissected and RNA was isolated and analyzed using RNA-sequencing. Analysis of the transcriptomes of control and E2-treated animals revealed that E2 treatment significantly altered the transcript levels of 88 genes. These genes were associated with long term synaptic potentiation, myelination, phosphoprotein phosphatase activity, mitogen activated protein kinase, and phosphatidylinositol 3-kinase signaling. E2 also altered the expression of genes linked to lipid synthesis and metabolism, vasoconstriction and vasodilation, cell-cell communication, and histone modification. These results demonstrate the far-reaching and diverse effects of E2 in the cerebral cortex and provide valuable insight to begin to understand cortical processes that may fluctuate in a dynamic hormonal environment.

Introduction

The effects of 17β-estradiol (E2) have been extensively studied in the female reproductive tract where it is required for reproductive competency. E2 also targets a variety of other tissues, including the mammary gland [1], bone [2,3], cardiovascular [4], and brain [5]. E2 plays several critical roles in brain development, such as influencing sexual dimorphism [6] and forming synapses [7]. In the cycling female, E2 is an important regulator of ovulation through its communication with the hypothalamus and pituitary [8,9]. E2 can also act on brain regions not associated with reproduction and can influence pain perception, locomotion, and mood [10].

Numerous experiments have demonstrated that E2 protects the brain from a variety of insults [11–13]. For example, E2 protects neuroblastoma cells from H2O2 [14] and beta amyloid [15,16] toxicity. Additionally, E2 decreases cellular damage in neurons that have been treated with excitotoxic levels of glutamate [17] and hippocampal slice cultures that have been exposed to oxygen and glucose deprivation [18]. In vivo, E2 reduces inflammation [19,20] and ischemia-induced damage [21,22] and this protection is most evident in the cerebral cortex.

In addition to its neuroprotective effects, E2 modulates synaptic plasticity [23], influences neurotransmission [24,25], and acts as a neurotrophin [26] to support brain homeostasis. These cumulative reports suggest that critical changes in gene expression in the brain are induced by E2. Although the cerebral cortex receives input from several brain regions and is essential for cognitive and executive functions [27], the mechanism by which E2 mediates its effects in the cerebral cortex are unclear. To better understand the molecular consequences of E2 in the cerebral cortex, we analyzed RNA sequencing (RNA-Seq) data from the cortices of oil- and E2-treated, ovariectomized female mice. This unbiased approach identified E2-regulated genes that provide insight into the multiple biological processes influenced by E2 treatment.

Materials and Methods

Animals and surgery

14 week old female C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained on a 12 hr light/dark schedule with access to water and food ad libitum. After 7 days, mice were anesthetized by inhalation of 4% isoflurane, bilaterally ovariectomized and then implanted subcutaneously with silastic tubing (0.062 in/0.125 in, inner/outer diameter, 1 in length; Dow Corning, Midland, MI) plugged at both ends with medical adhesive (Dow Corning). The silastic tubing, which remained in the mice for 7 days, contained either 35 μl of cottonseed oil or 35 μl of cottonseed oil with 180 μg/ml E2 and produced a low, physiological level of circulating E2 (~25 pg/ml) [21,28] that is equivalent to estrus levels in mice [29]. Ovariectomized mice were fed phytoestrogen-free chow and after 7 days, the mice were sacrificed, the brains were dissected, and cerebral cortices were harvested. This method of E2 treatment has been
RNA isolation

Total RNA was isolated from each cerebral cortex using Ambion RNAqueous according to the manufacturer’s protocol (Life Technologies, Grand Island, NY) and treated with Turbo DNA-free reagent (Ambion, Life Technologies, Austin, TX) to remove genomic DNA. RNA purity was assessed with native agarose gel electrophoresis and analysis of the 28S and 18S rRNA bands. RNA was of high purity, showed no degradation, and was free of DNA (Fig. S1).

RNA-Sequencing

RNA-seq was completed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois Urbana-Champaign. The TruSeq RNA sample prep kit (Illumina, San Diego, CA) and 1 μg of total RNA were used to make poly-A selected and barcoded RNA-Seq libraries for each cortical sample. cDNA libraries were pooled and quantified using real-time PCR with the Library Quantification kit (Kapa Biosystems, Woburn, MA). The libraries were sequenced using 3 lanes for 101 cycles with 7 additional cycles for the index read on the Illumina HiSeq2000 according to the manufacturer’s instructions. The RNA-Seq libraries produced over 255 million reads with each individual sample having more than 29 million reads. The data was then used to generate Fastq files using Casava 1.8.2.

RNA-Seq alignment and statistics

Sequences were aligned using TopHat v. 1.4.1 [32] and Bowtie 1.0 [33]. The genome sequence index was mm10 from UCSC (http://hgdownload.soe.ucsc.edu/downloads.html). Raw read counts were tabulated for each sample at the gene level using the GTF gene model file for mm10 from UCSC and htseq-count, from HTSeq v0.5.3p9 (http://www-huber.embl.de/users/anders/HTSeq/doc/index.html) using the default “exon” feature type and “gene_id” attribute. The raw read counts were used in R 3.0.0 [34] for data preprocessing and statistical analysis using packages from Bioconductor [35] as indicated below. Data are available in the Array Express database under accession number E-MTAB-2762. Genes without 1 count per million (CPM) mapped reads in at least 4 samples, irrespective of group, were filtered out and 14,908 of the 37,482 genes passed this filter and were analyzed using edgeR 3.2.3 [36]. The raw count values were used in a negative binomial statistical model that accounted for the total library size for each sample and an extra TMM normalization factor for any biases due to changes in total RNA composition of the samples [37,38]. Tests for the pairwise comparisons were pulled from the model and separately adjusted for multiple testing using the False Discovery Rate (FDR) method [39].

Comparable expression values were generated from read counts using voom normalized values [40]. The voom values were scaled to the standard deviation of the mean, hierarchically clustered, and displayed as heatmaps. Additional annotation information (gene names, descriptions) was obtained from Ensembl Genes 71, Mus musculus genes (GRCm38.p1) database using the Ensembl gene IDs provided in the GTF gene model file.

Cytoescape (Version 3.0.1) was used in conjunction with the plug-in ClueGO (Version 2.0.7) for network creation [41,42], KEGG [43], Reactome [44], and Gene Ontology (biological process) [45] databases were used within the program for network categorization. Over-representation (enrichment) was calculated in the program using a right-sided hypergeometric test and Bonferroni step-down method for multiple test correction.

Transcriptomine from the Nuclear Receptor Signaling Atlas website was used to determine previously identified E2-regulated genes. 17β-estradiol was selected as the ligand and >1.1 fold change in either direction with p<0.05 significance was selected for ‘CNS, all tissues and cell lines’ and ‘all tissues, all cell lines’ RNA sources.

Real-Time PCR (RT-PCR)

RNA concentrations were measured and cDNA was synthesized using the iScript kit (Bio-Rad, Hercules, CA) as described by the manufacturer. cDNA was combined with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and forward and reverse primers (0.9 μM) for receptor transporter protein 1, Rpl1 (5'-CTGCCCTGCTTACATGG -3’ and 5’-TCACCTCTC-CATCTTTCCTG -3’), macrophage galactose N-acetyl-galactosamine specific lectin 2, Mgl2 (5’-GGACCAGAAAAGG-AAGATG -3’ and 5’-GAGATGACCAGCTAGGC -3’), NLR-pyrin domain containing 3, Nlrp3, (5’-CTGCCATCAGCTGCTGTG-3’), lysosome, Lyz2, (5’-TGAAAGCCTCTCCCTGACTC-3’ and 5’-ACGCGTTGATGTGATTGC -3’), succinate dehydrogenase complex, subunit A, flavoprotein, Sdha, (5’-GCTCACGGGTGTGCCGTTG-3’ and 5’-TTGCTCCTTTATCCGGTGTATGACG -3’), aldolase A fructose-bisphosphate, Aldoa, (5’-GAGAACAACCGAGAAGAC-3’ and 5’-CTTTGACCTGATCACTTGG -3’), or ribosomal protein L7, Rpl7, (5’-GGGCTGAGAATTCGCTGCTGAG -3’ and 5’-TTAATTGAGCGCTTGTGTGAGG-3’), RT-PCR was carried out using a Bio-Rad iQ5 multicolor Real-Time PCR Detection System. Samples were run in triplicate with each primer set along with a standard curve. Ct values were normalized to Rpl7 using the delta-delta Ct method. Combined data are expressed as the mean ± SEM and Student’s t-test was used to detect significant (p<0.05) differences.

Western Blotting

Extracts from cortical tissue were prepared using RIPA buffer (Thermo Scientific, Rockford, IL), protease inhibitors (Complete Mini, Roche, Mannheim, Germany) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set III, Calbiochem, San Diego, CA). Samples were homogenized using a Pro Homogenizer (ProScientific Inc., Oxford, CT) and protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo-Scientific) with bovine serum albumin as a standard. 30 μg of protein was loaded per lane on a 4–12% gradient acrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with an anti-phosphorylated ERK (p44/p42 MAPK, #9101, Cell Signaling Technology, Danvers, MA) or an anti-ERK (p44/p42 MAPK, #4695, Cell Signaling Technologies) specific antibody. Western blots were imaged and quantitated using the Licor Odyssey Infrared Imaging System and pERK was normalized to total ERK. Combined data are expressed as the mean ± SEM and Student’s t-test was used to detect significant (p<0.05) differences in the levels of pERK.
Results and Discussion

To identify E2-mediated alterations in the female cerebral cortical transcriptome, a comprehensive study was carried out using a mouse model system. Female mice were ovariectomized and implanted with silastic tubing containing oil alone or oil with E2, which produced a low, physiological level of E2 [21]. This method of E2 treatment has been used in several studies to demonstrate the anti-inflammatory and neuroprotective actions of E2 in the mouse cerebral cortex [19,21,30,31]. After 7 days, brains were dissected and total RNA was isolated from the cerebral cortices. The isolated RNA was poly-A selected, converted to cDNA, and analyzed using RNA-Seq.

E2 significantly altered gene expression in cerebral cortex

E2 significantly (FDR $p<0.05$) altered the expression of 88 genes in the cerebral cortex (Table 1, Table S1). The expression of these genes is displayed on a heat map (Fig. 1A), where red indicates a significant increase and blue indicates a significant decrease in transcript levels. Interestingly, the number of genes decreased (48) by E2 treatment was slightly greater than the number of genes increased (40) by E2 treatment.

Of the 88 genes that responded to E2 treatment, 49 were altered 1.2 fold or more (FDR $p<0.05$, Fig. 1B). Again, the number of genes decreased (blue) by E2 treatment was greater than the number of genes increased (red) by E2 treatment suggesting that E2 is a more potent repressor than activator of gene expression in the cerebral cortex. However, both heatmaps demonstrated that E2 differentially regulates gene expression in the mouse cerebral cortex.

Validation of E2 regulated genes

To validate the RNA-Seq data, we examined a subset of E2-regulated genes using RT-PCR analysis. In agreement with the RNA-Seq data, E2 increased Rtp1, Mgl2, and Nlrp3 expression, decreased Fabp7 and Lys2 expression, and did not alter the expression of Sdha and Aldoa (Fig. 2, *$p<0.05$). Thus, RT-PCR analysis provided evidence of the accuracy of the RNA-Seq dataset.

E2-regulated genes

We were interested in determining if any of the 88 genes that were significantly altered by E2 in the cerebral cortex have previously been reported as E2-responsive genes. Using the genome-wide expression profiling database Transcriptome in the Nuclear Receptor Signaling Atlas, we found that each of the 88 genes except Rtp1, Gm20634, and 2410137F9r1 was listed as an E2-responsive gene in a variety of tissues or cultured cells [46], but only 5 genes, Aqp4, Bhlhe40, Ednrb, Erbb4 and Igfbp2, were designated as E2-responsive in the central nervous system. Two additional studies have reported that Gfap [47] and Slc13a3 [20] are E2-responsive in the brain. Thus, based on literature and database searches, the majority of the 88 genes identified in our dataset are novel, E2-regulated genes in the mouse cerebral cortex. Interestingly, earlier reports suggest that the gene expression profile of the hippocampus differs substantially from the gene expression profile of the cerebral cortex and that acute and chronic E2 treatments may differentially alter gene expression [48,49].

The 10 genes that declined most significantly in response to E2 treatment are shown in Table 2. The largest E2-mediated decrease was observed in cadherin-related family member 1 (Cdh1r1), which is a protocadherin in the cadherin superfamily, and functions as a calcium-dependent cell adhesion and signaling molecule [50]. The greatest E2-mediated increases in transcript levels are listed in Table 3. The expression of macrophage galactose N-acetyl-galactosamine specific lectin 2 (Mgl2), also referred to as CD301, was most significantly increased. The function of this gene in the cerebral cortex has not been described. However, microglia, the resident immune cells in the brain, often express multiple cluster of differentiation (CD) cell surface proteins [51]. Siglec1, another CD gene (CD169), is expressed on macrophages associated with the perivasculature in the rat brain [52]. These results suggest that E2 may be altering gene expression in immune cells.

Networks of E2-responsive biological processes and pathways

To begin to understand how E2 regulates the cerebral cortical transcriptome, the 88 E2-responsive genes were uploaded to ClueGO [42] to identify networks of biological processes and pathways that are altered by E2 treatment (Fig. 3 and Table 4). The nodes (filled circles) represent biological processes or pathways associated with the E2-regulated genes based on gene ontology terms [43], Reactome [44] and KEGG [43] databases. Related nodes are clustered together in color-coded networks and all of the nodes in a network are the same color. However, a node can participate in two networks and those nodes are white. The size of the node reflects the level of statistical significance of each of the E2-regulated biological processes or pathways. Thus larger nodes have increased statistical significance.

The largest network is comprised of 22 nodes (Fig. 3, blue nodes) and the 14 genes associated with these nodes are listed in Table 4. Genes associated with this network include glial fibrillary acidic protein (Gfap) and polo-like kinase 1 (Plk2), which play a role in long term synaptic potentiation. Synaptic plasticity, learning, and memory are linked to long term synaptic potentiation. Dual specificity phosphatase 4 (Dusp4) and sprouty-related, EVH1 domain containing 2 (Sprut2) are associated with the MAPK pathway, which is important in synaptic plasticity and also plays a role in cell signaling [53]. NUA1 family, SNF 1-like kinase (Nuak1) and tuberous sclerosis 1 (Tsc1) are associated with phosphoprotein phosphatase regulation and may be contributing to modulation of protein phosphorylation. Taken together, these results suggest that E2 is affecting important signal integration pathways in the cerebral cortex.

Unique to network 2 (green) were biological processes involved in vasoconstriction and vasodilation which included platelet derived growth factor B (Pdgfb) and endothelin receptor type B (Ednrb). Networks 1 and 2 shared genes involved in PI3K activity such as v-erb-a erythroblastic leukemia viral oncogene homolog 4 (Erbb4) and Pdgfb. The PI3K pathway is important for E2 signaling and inhibition of this pathway blocks downstream ERK activation by E2 in cortical neuron cultures [54]. Fatty acid synthesis is a critical function in the brain, which contains the second highest level of lipids in the body after adipose tissue [55]. Fatty acid metabolic processes were associated with insulin receptor substrate 2 (Irs2) and Mid1 interacting protein 1 (Midlip1) in network 3. Midlip1 enhances fatty acid synthesis and its overexpression in the liver causes triglyceride accumulation [56]. Irs2 is critical in regulating brain size, since the brains of Irs2 null mice are reduced by ~50% due to decreased neuronal proliferation [57].

Pathways involved in lipid synthesis were also present in network 3 (red) and included genes ELOVL family member 5, elongation of long chain fatty acids (Elovl5) and hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/ enoyl-Coenzyme A hydratase alpha subunit (Hadha) and the
### Table 1. 88 E2-responsive genes in the cerebral cortex.

| Gene symbol | Description                                           | FDR p-value | Fold change |
|-------------|--------------------------------------------------------|-------------|-------------|
| 2410137F16rlk | RIKEN cDNA 2410137F16 gene                           | 0.020       | 1.7         |
| Adcy9       | adenylate cyclase 9                                   | 0.019       | 1.2         |
| Agxt2l1     | alanine-glyoxylate aminotransferase 2-like 1          | 3.1E-05     | 1.8         |
| Ankrd33b    | ankyrin repeat domain 33B                             | 0.0071      | 1.2         |
| Apln        | apelin                                                | 0.0043      | –1.3        |
| Aqp4        | aquaporin 4                                           | 0.00059     | –1.3        |
| Bcas1       | breast carcinoma amplified sequence 1                 | 3.1E-05     | –1.3        |
| Bihne40     | basic helix-loop-helix family, member e40             | 0.0040      | 1.2         |
| Btbd17      | BTB (POZ) domain containing 17                       | 0.020       | –1.2        |
| C8orf2      | C8orf2 antigen                                        | 0.0058      | –1.3        |
| Cdhr1       | cadherin-related family member 1                      | 0.038       | –2.8        |
| Cmmt5       | CKLF-like MARVEL transmembrane domain containing 5    | 0.0038      | –1.2        |
| Cnp         | collagen, type XIX, alpha 1                           | 0.010       | –1.2        |
| Col19a1     | collagen, type XIX, alpha 1                           | 0.048       | 1.3         |
| Cpeb1       | cytoplasmic polyadenylation element binding protein 1 | 0.030       | 1.1         |
| Cryab       | crystallin, alpha B                                   | 0.0017      | –1.2        |
| Dusp4       | dual specificity phosphatase 4                        | 0.038       | 1.3         |
| Ednrb       | endothelin receptor type B                            | 0.011       | –1.3        |
| Elf52       | leucine rich repeat and fibronectin type III, extracellular 2 | 0.019 | 1.1 |
| Elovl5      | ELOVL family member 5, elongation of long chain fatty acids (yeast) | 0.011 | –1.2 |
| Erbb4       | v-erb-a erythroleukemia viral oncogene homolog 4 (avian) | 0.044 | 1.1 |
| Fa2h        | fatty acid 2-hydroxylase                              | 0.041       | –1.2        |
| Fabp7       | fatty acid binding protein 7, brain                   | 0.0071      | –1.6        |
| Fam107a     | family with sequence similarity 107, member A         | 0.016       | 1.2         |
| Fbxo33      | F-box protein 33                                       | 0.026       | 1.1         |
| Fcrl5       |Fc receptor-like 5, scavenger receptor                 | 0.030       | 1.2         |
| Flnb        | filamin, beta                                         | 0.046       | 1.1         |
| Gpdp5       | glycerophosphodiester phosphodiesterase domain containing 5 | 0.029 | –1.2 |
| Gfap        | glial fibrillary acidic protein                       | 0.0013      | –1.3        |
| Gja1        | gap junction protein, alpha 1                         | 0.044       | –1.1        |
| Gjc2        | gap junction protein, gamma 2                         | 0.00059     | –1.3        |
| Gtp         | glycolipid transfer protein                           | 0.023       | –1.2        |
| Gm20634     | predicted gene 20634                                   | 0.019       | –1.3        |
| Gsn         | gelsolin                                              | 0.0071      | –1.2        |
| Hadha       | hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit   | 0.019       | –1.1        |
| Herc1       | hec domain and RCC1-like domain 1                     | 0.039       | 1.1         |
| Hist1h2bc   | histone cluster 1, H2bc                               | 0.029       | –1.2        |
| Hivep3      | human immunodeficiency virus type 1 enhancer binding protein 3 | 0.010 | 1.2 |
| Igfbp2      | insulin-like growth factor binding protein 2          | 0.00090     | –1.5        |
| Igfbp1      | insulin-like growth factor binding protein-like 1     | 0.023       | –2.3        |
| Irs2        | insulin receptor substrate 2                         | 0.0022      | 1.3         |
| Jam2        | junction adhesion molecule 2                          | 0.011       | –1.2        |
| Lyz2        | lysozyme 2                                            | 0.0043      | –1.7        |
| Mag         | myelin-associated glycoprotein                        | 0.0017      | –1.3        |
| Mgl2        | macrophage galactose N-acetyl-galactosamine specific lectin 2 | 2.6E-06 | 3.5 |
| Mid1p1      | Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish)) | 0.038 | –1.1 |
| Mill1       | myeloid/lymphoid or mixed-lineage leukemia 1          | 0.0020      | 1.2         |
| Mvd         | mevalonate (diphospho) decarboxylase                  | 0.048       | –1.2        |
| Myoc        | myocilin                                              | 0.0017      | –1.4        |
| Ndufa3      | NADH dehydrogenase (ubiquinone) alpha subcomplex, 3   | 0.022       | –1.2        |
solute carrier 25 (Slc25a1). Regulation of ligase activity was associated with Mid1ip1 and tribbles homolog 2 (Trib2). In addition to its role in fatty acid synthesis, Mid1ip1 interacts with Mid1, a ubiquitin ligase and microtubule associated protein [58,59]. Trib2 functions as an adaptor for protein degradation through interactions with the E3-ubiquitin ligase Cop1 [60].

Gelsolin (Gsn), which encodes an actin binding protein involved in signaling and cytoskeletal remodeling [61] is associated with negative regulation of protein complex disassembly.

Three networks (4–6) were not connected to any of the other networks. Genes previously reported to be estrogen responsive in various cell types were included in network 4 (orange). The genes in this group included crystallin alpha b (Cryab), aquaporin 4 (Aqp4), and insulin-like growth factor binding protein 2 (Igfbp2), which have been reported as E2-responsive genes in the mouse uterus [62], cultured rat cortical neurons [63], and rat hippocampal tissue, respectively [64,65]. Myelin is essential for proper nerve conduction [66] and network 5 (yellow) contained several genes associated with myelination. Fatty acid 2-hydroxylase (Fa2h), plasma membrane proteolipid (Pllp), UDP galactosyltransferase 8A (Ugt8a), and tuberous sclerosis 1 (Tst1) have been associated with oligodendrocytes, which produce myelin. Network 6 (purple)

### Table 1. Cont.

| Gene symbol | Description                                      | FDR p-value | Fold change |
|-------------|--------------------------------------------------|-------------|-------------|
| Nlrp3       | NLR family, pyrin domain containing 3            | 0.0020      | 2.0         |
| Nov         | nephroblastoma overexpressed gene                | 0.010       | 1.3         |
| Nauk1       | NUAK family, SNF1-like kinase, 1                 | 0.0013      | 1.2         |
| Olfm1       | olfactomedin-like 3                               | 7.0E-06     | −1.4        |
| Pcnt        | pericentrin (kendrin)                            | 0.038       | 1.1         |
| Pdgfb       | platelet derived growth factor, B polypeptide    | 8.8E-08     | 1.3         |
| Phf15       | PHD finger protein 15                             | 0.0013      | 1.2         |
| Phf21b      | PHD finger protein 21B                            | 0.021       | 1.2         |
| Plk2        | polo-like kinase 2                                | 0.022       | 1.1         |
| Pllp        | plasma membrane proteolipid                      | 0.033       | −1.2        |
| Prdx6       | peroxiredoxin 6                                  | 0.043       | −1.1        |
| Ptn         | pleiotrophin                                     | 0.016       | −1.2        |
| Pttn7       | protein tyrosine phosphatase, non-receptor type 7| 0.046       | −1.7        |
| Rtp1        | receptor transporter protein 1                    | 0.0043      | 2.8         |
| Serpinb1a   | serine (or cysteine) peptidase inhibitor, clade B, member 1a | 0.011 | −1.5 |
| Siglec1     | sialic acid binding Ig-like lectin 1, sialoadhesin | 0.048 | 1.9 |
| Slc13a3     | solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 | 0.014 | −1.4 |
| Slc25a1     | solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 | 0.019 | −1.2 |
| Slc38a3     | solute carrier family 38, member 3               | 0.048       | −1.2        |
| Sntb2       | syntrophin, basic 2                              | 0.012       | 1.3         |
| Sowahb      | sosondowah ankyrin repeat domain family member B | 0.039       | 1.2         |
| Spred2      | sprouty-related, EVH1 domain containing 2        | 0.023       | 1.1         |
| Srm4        | serine/arginine repetitive matrix 4              | 0.0017      | 1.2         |
| Tcn2        | transcobalamin 2                                 | 0.022       | −1.2        |
| Tet3        | tet methylcytosine dioxygenase 3                 | 0.016       | 1.1         |
| Tfc         | transferrin receptor                             | 0.0017      | 1.2         |
| Tgfb1       | transforming growth factor, beta receptor 1      | 7.1E-07     | 1.3         |
| Tmem116     | transmembrane protein 116                        | 0.038       | 1.4         |
| Top2a       | topoisomerase (DNA) II alpha                     | 0.022       | −1.7        |
| Trib2       | tribbles homolog 2 (Drosophila)                  | 0.017       | 1.2         |
| Trp53inp2   | transformation related protein 53 inducible nuclear protein 2 | 0.038 | −1.1 |
| Tsc1        | tuberous sclerosis 1                             | 0.017       | 1.1         |
| Tst         | thiosulfate sulfurtransferase, mitochondrial     | 0.0052      | −1.2        |
| Ugt8a       | UDP galactosyltransferase 8A                      | 0.038       | −1.3        |
| Unc5b       | unc-5 homolog B (C. elegans)                     | 0.021       | −1.2        |
| Vcam1       | vascular cell adhesion molecule 1                | 0.016       | −1.3        |
| Vstm4       | V-set and transmembrane domain containing 4      | 0.038       | −1.4        |
| Vwf         | Von Willebrand factor homolog                     | 3.1E-05     | 1.6         |

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Figure 1. Heatmaps comparing cerebral cortices from oil- and E2-treated mice. Hierarchical clustering was used to visualize the transcript levels of (A) 88 genes that were significantly altered (FDR $p < 0.05$) by E2 treatment or (B) 49 genes that were altered 1.2 fold or more by E2 treatment (FDR $p < 0.05$). Each column represents cortical tissue from one mouse (8 mice total) and rows indicate genes. Colors symbolize increased (red) or decreased (blue) transcript levels.

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Figure 2. Validation of transcripts altered by E2 treatment. (A) Quantitative real-time PCR was conducted with gene-specific primers. The normalized fold change ± SEM was calculated using the delta-delta Ct method with Rpl7 as a control gene. The Student’s t-test was used to detect significant differences in oil- and E2- treated animals (4 animals/treatment, *$p<0.05$).

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included DNA topoisomerase 2A (Top2a) and basic helix loop helix family, member 40 (Bhlhe40) which are both involved in chromosome separation. However, Top2a has also been reported to be expressed in cortical neurons [67] and Bhlhe40, also known as Stra13, has been implicated in neuronal differentiation [68]. Thus the roles of these genes extend beyond chromosomal separation.

The network analysis provided insight into the diverse array of functions that were affected by E2 treatment. However, this analysis is constrained by existing information in the databases used, which consequently did not include all of the 88 genes we identified. Thus we utilized literature searches to provide a more comprehensive picture of the pathways and processes that were affected by E2 treatment.

Signaling Pathways

The MAP kinase pathway plays a critical role in neuronal plasticity and survival [53] and E2 has been implicated in inducing rapid signaling through this pathway in neuroblastoma cells, primary cortical neurons, cortical explants, and the cerebral cortex in vivo [54,69–71]. To determine whether MAP kinase signaling was activated after longer E2 treatment, we examined the level of phosphorylated extracellular regulated kinase (pERK) in the cortices of mice that had been treated with oil or E2 for 7 days. In fact, the level of pERK was significantly increased in the E2-treated animals (Fig. 4) demonstrating that E2 modulation of pERK and MAP kinase signaling is not limited to acute exposures (5–30 min), but is still enhanced after a longer treatment time.

We identified several E2-regulated genes in the cerebral cortex that modulate the MAP kinase signaling pathway. Dual specificity phosphatase 4 (Dusp4) dephosphorylates ERK [72] and is increased by E2 in breast cancer cells [73]. Thus the E2-mediated increase in Dusp4 expression could lead to decreased ERK phosphorylation. Interestingly, expression of protein tyrosine phosphatase, non-receptor type 7 (Ptpn7) was decreased with E2 treatment and may also be involved in ERK dephosphorylation [74]. In addition, expression of a repressor of MAP kinase activity, Spred2, [75] was modestly increased with E2 treatment. The fine-tuned expression of these genes by E2 highlights the balance that is needed between phosphorylation and dephosphorylation in the MAP kinase pathway [74]. Dusp4, Ptpn7 and Spred2 are novel, E2-responsive modulators of MAP kinase in the cerebral cortex.

Cerebral cortex microvasculature

The brain is one of the most highly perfused organs in the body [76]. Proper brain function relies on maintenance of an extensive network of capillaries that form the cerebral microvasculature, which supplies oxygen and nutrients to meet the demands of this highly metabolic tissue. The microvasculature is comprised of endothelial cells surrounded by an extracellular matrix and a

Table 2. E2-regulated genes with the most decreased expression.

| Gene symbol | Description                                | Fold decrease | FDR p value |
|-------------|--------------------------------------------|---------------|-------------|
| Cdhr1       | cadherin-related family member 1           | −2.8          | 0.038       |
| Igfbp1      | insulin-like growth factor binding protein-like 1 | −2.3          | 0.023       |
| Ptpn7       | protein tyrosine phosphatase, non-receptor type 7 | −1.7          | 0.046       |
| Top2a       | topoisomerase (DNA) II alpha               | −1.7          | 0.022       |
| Ly22        | lysozyme 2                                 | −1.7          | 0.0043      |
| Fabp7       | fatty acid binding protein 7, brain        | −1.6          | 0.0013      |
| Serpinb1a   | serine (or cysteine) peptidase inhibitor, clade B, member 1a | −1.5          | 0.011       |
| Igfbp2      | insulin-like growth factor binding protein 2 | −1.5          | 0.0009      |
| Olfml3      | olfactomedin-like 3                        | −1.4          | 7.00E-06    |
| Myoc        | myocilin                                   | −1.4          | 0.0017      |

Table 3. E2-regulated genes with the most increased expression.

| Gene symbol | Description                                | Fold increase | FDR p value |
|-------------|--------------------------------------------|---------------|-------------|
| Mgl2        | macrophage galactose N-acetyl-galactosamine specific lectin 2 | 3.5           | 2.60E-06    |
| Rtp1        | receptor transporter protein 1             | 2.8           | 0.0043      |
| Nlrp3       | NLR family, pyrin domain containing 3      | 2             | 0.002       |
| Siglec1     | sialic acid binding Ig-like lectin 1, sialoadhesin | 1.9           | 0.048       |
| Aght2l1     | alanine-glyoxylate aminotransferase-2-like 1 | 1.8           | 3.10E-05    |
| 2410137F16Rik | RIKEN cDNA 2410137F16 gene                  | 1.7           | 0.02        |
| Vwf         | Von Willebrand factor homolog              | 1.6           | 3.10E-05    |
| Tmem116     | transmembrane protein 116                  | 1.4           | 0.038       |
| Pdgfb       | platelet derived growth factor, B polypeptide | 1.3           | 8.80E-08    |
| Snthb2      | syntrophin, basic 2                        | 1.3           | 0.012       |
variety of cell types including neurons, astrocytes, microglia, and pericytes [51]. This complex network is referred to as the “neurovascular unit” since cooperation between these cells is necessary to maintain microvascular function [77]. Tight junctions between endothelial cells, together with the surrounding astrocyte end feet and pericytes form the blood-brain barrier (BBB), which carefully regulates the exchange of nutrients, water and other molecules. A dysfunctional BBB can lead to neurodegeneration and is the hallmark of several brain injuries [78].

Previous studies have shown that E2 decreases BBB permeability and thereby limits ischemic damage [79]. We identified several genes involved in BBB regulation that were altered by E2 treatment. Pdgfb transcript levels were increased by E2 treatment. Since Pdgfb binds to the Pdgfb receptor on pericytes [80] and mice with low Pdgfb levels have a dysfunctional BBB [81], Pdgfb is

| Biological process GO term or Pathway                                                                 | Genes                     |
|------------------------------------------------------------------------------------------------|---------------------------|
| Network 1  Regulate centrosome cycle                                                                | Gja1, Plk2                |
| Inactivation of MAPK activity                                                                       | Dusp4, Spred2             |
| Cell-cell junction assembly                                                                        | Gja1, Ugt1a               |
| Regulation of tissue remodeling                                                                     | Gja1, Tfrc                |
| Regulation of mRNA splicing, via spliceosome                                                        | Gja1, Srrm4               |
| Carbohydrate derivative transport                                                                  | Gja1, Gltp                |
| Regulation of signal transduction by p53 class mediator                                             | Gja1, Spred2              |
| Long-term potentiation                                                                             | Gfap, Plk2                |
| Regulation of cell junction assembly                                                                | Gja1, Tsc1                |
| Lens development in camera-type eye                                                                 | Cryab, Gja1, Tgfβ1        |
| Regulation of phosphoprotein phosphatase activity                                                  | Nua1, Tsc1                |
| rRNA transport                                                                                     | Gja1, Tsc1, Tst            |
| Network 2  Cerebellum morphogenesis                                                                 | Herc1, Pcnt               |
| Vasoconstriction                                                                                   | Apln, Ednrb, Pdgfb        |
| Vasodilation                                                                                        | Apln, Cnp, Pdgfb          |
| Network 3  Fatty acid elongation                                                                    | Elovl5, Hadha             |
| Triglyceride biosynthesis                                                                           | Elovl5, Slc25a1           |
| Histone H4 acetylation                                                                             | Mll1, Phf15               |
| Histone H3-K4 methylation                                                                          | Mll1, Tet3                |
| Regulation of ligase activity                                                                       | Mid1ip1, Trib2            |
| Lysine degradation                                                                                  | Hadha, Mll1               |
| Negative regulation of protein complex disassembly                                                 | Gsn, Mid1ip1              |
| Network 4  Response to estradiol stimulus                                                           | Aqp4, Cryab, Igfbp2       |
| Vasopressin-regulated water reabsorption                                                            | Adcy9, Aqp4               |
| Network 5  Myelination                                                                              | Fa2h, Pllp, Tsc1, Ugt1a   |
| Network 6  Meiotic chromosome separation                                                            | Bhlhe40, Top2a            |
| Network 1 and 2  Olfactory lobe development                                                          | Erbb4, Pcnt               |
| Regulation of phosphatidylinositol 3-kinase activity                                                 | Erbb4, Pdgfb              |
| Tissue regeneration                                                                                 | Erbb4, Gja1               |
| Regulation of lipid kinase activity                                                                 | Erbb4, Pdgfb              |
| Network 2 and 3  Positive regulation of fatty acid metabolic process                                | Irs2, Mid1ip1             |
| Fatty acid beta oxidation                                                                           | Hadha, Irs2               |
| Regulation of polysaccharide metabolic process                                                      | Irs2, Pdgfb               |

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necessary for pericyte proliferation and maintenance [82,83] and a functional neurovascular unit. Therefore, E2 may be acting to stimulate synthesis of Pdgfb in pericytes and endothelial cells, which could enhance autocrine and paracrine signaling to support BBB function.

E2 decreased the expression of Aqp4, a water transporter present on astrocyte end feet. These findings are in agreement with a previous report which indicated that E2 decreases Aqp4 expression and reduces hypoxia-induced swelling of rat cortical astrocytes in vitro [63]. E2 also decreased the expression of two solute carriers, Slc13a3, a sodium decarboxylate cotransporter and Slc38a3, an amino acid transporter, that have been associated with the BBB [84]. Together, the E2 mediated reduction in Aqp4, Slc13a3, and Slc38a3 could alter the exchange of water and solutes at the BBB and help to maintain fluid balance and homeostasis in the brain.

Von Willebrand Factor (Vwf) was increased by E2 treatment. Vwf is highly expressed in endothelial cells of brain microvasculature [85] and Vwf-null mice have increased damage compared to their wild-type counterparts after exposure to hypoxia and reoxygenation [86], suggesting that this factor is necessary for BBB adaptability and may help the brain to recover from an hypoxic event.

E2 has antinflammatory effects on the vasculature [87]. We found that E2 treatment decreased Vcam1 expression in the cerebral cortex. Vcam1 attracts leukocytes and monocytes to inflamed endothelial cells [88]. It has been proposed that E2 may decrease inflammation of endothelial cell cultures that have been subjected to an inflammatory agent by decreasing Vcam1 expression [89]. In addition, the E2 induced increase of Tgfb receptor 1 (Tgfbr1) may increase the sensitivity of Tgfb signaling, thus reducing inflammation [90]. The combined effects of E2 on maintaining the BBB (Pdgfb, Aqp4, Slc13a3, Slc38a3, Vwf) and reducing inflammation (Vcam1, Tgfbr1) could help to protect the cerebral cortex from injury.

Figure 3. Networks of E2-regulated genes. ClueGO analysis classified the 88 E2-regulated genes into 6 networks. White nodes indicate that a biological process is associated with two networks. Node size indicates the statistical significance of the biological process represented. Thus, larger nodes indicate greater statistical significance.
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Oligodendrocytes and Myelin

Oligodendrocytes insulate neuronal axons by extending processes that produce lipid rich myelin. Myelin ensheathment of axons is important for nerve conduction and the loss of myelin leads to neurodegeneration. We were surprised at the number of oligodendrocyte-associated genes that were E2 responsive. Expression of myocillin (Myoc), myelin-associated glycoprotein (Mag), UDP galactosyltransferase 8a (Ugt8a), fatty acid 2-hydrolase (Fa2h), 2', 3'-cyclic nucleotide 3' phosphodiesterase (Cnp), CKLF-like MARVEL transmembrane domain containing 5 (Cmtm5) and plasma membrane proteolipid (Pllp) were all decreased by E2 treatment. A previous study found that turnover of oligodendrocytes in female rodents was increased and that Cnp protein expression was less than in males [91]. The decreased expression of these genes could indicate that E2 increases oligodendrocyte turnover rates in the cerebral cortex as well. However, much remains to be learned about the molecular consequences of decreased expression in this subset of oligodendrocyte-associated genes.

Neurite extension

Neurite outgrowth is important for neuronal development, communication and function [92]. Impairment of neurite extension is associated with aging and neurodegeneration [93]. E2 can increase neurite extension in a variety of brain regions through several pathways including growth factor signaling, PI3K, and MAP kinase pathways [94]. We identified several genes involved in neurite extension that were altered by E2 treatment. E2 treatment modestly increased expression of Erbb4, which encodes a transmembrane protein that binds to neuregulin 1 (Nrg1). Erbb4-Nrg1 signaling enhances neurite outgrowth through activation of the PI3K and MAP kinase pathways [95]. The E2-mediated increase in Erbb4 in the cerebral cortex could enhance neurogenesis.

The expression of Igfbp11 and Igfbp2 was decreased by E2 treatment. Igfbp11 and Igfbp2 bind and sequester growth factors such as Igf1 [96]. Although Igfbp11 is expressed in the developing mouse forebrain [97], the role of Igfbp11 in the cerebral cortex has not been examined. In breast cancer cells, a decrease in Igfbp11 has been associated with an increase in Igf1 levels [98]. E2

**Figure 4. E2 increases pERK protein levels.** (A) Western blot analysis was used to monitor pERK and total ERK levels in the cortices of mice that had been treated with oil or E2 for 7 days. (B) pERK values were normalized to total ERK and are displayed as the normalized fold change ± SEM. The Student’s t-test was used to detect significant differences in oil- and E2-treated animals (*p<0.05). The number of animals in each treatment group is indicated at the base of each bar.
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decreases Igfbp2 in the hippocampus [64] which can modulate Igf1 signaling pathways [59]. Moreover, Igf1 and E2 act synergistically to promote neurite outgrowth [100]. The E2-mediated decrease in expression of both Igfbp1 and Igfbp2 could potentially allow growth factors such as Igf1 to circulate and synergistically to promote neurite outgrowth [100]. The E2-Igf1 signaling pathways [99]. Moreover, Igf1 and E2 act decreases Igfbp2 in the hippocampus [64] which can modulate Gfap in our studies suggests that E2 supports neurite extension, outgrowth [47,101]. The E2-mediated decrease in expression of Gfap in our studies suggests that E2 supports neurite extension, and could prevent an age-related increase in Gfap expression.

**Overall Implications**

E2 alters gene expression through classical pathways that involve binding of the E2-occupied receptor to DNA, E2 can also act through non-classical pathways, by activation of membrane-associated proteins and rapid signaling pathways such as MAP kinase and PI3K, both of which have been shown to be important in the brain [102,103]. It has been suggested that cross-talk occurs amongst the various E2 signaling pathways [104,105] and that the cumulative E2-activation of several pathways may be required for effective E2-mediated neuroprotection [102].

Our study reflects the complex nature of E2 action and suggests that multiple signaling pathways in the cerebral cortex converge to orchestrate a diverse array of molecular events including those related to cerebrovascular function, neurite outgrowth, and brain homeostasis. The molecular impact of E2 treatment has particular relevance when considering the physiological consequences of menopause and estrogen replacement therapy. Further understanding of these events may provide insight into mechanisms responsible for estrogen-mediated gene expression and promote development of targeted treatments that support brain homeostasis.

**Supporting Information**

Figure S1 RNA gel demonstrating intact RNA samples. Native agarose gel electrophoresis was used to resolve the intact 28s and 18s rRNA bands. 2 μg of RNA were run per lane. (PDF)

Table S1 88 E2-responsive genes in the cerebral cortex. (XLSX)

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**Author Contributions**

Conceived and designed the experiments: AN YZ. Performed the experiments: GH YZ. Analyzed the data: GH AN. Contributed to the writing of the manuscript: GH AN.
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