Hippocampal Gene Expression Is Highly Responsive to Estradiol Replacement in Middle-Aged Female Rats

Miklós Sárvári[1], Imre Kalló[1,2], Erik Hrabovszky[1], Norbert Solymosi[3], Annie Rodolosse[4], Csaba Vastagh[1], Herbert Auer[5], Zsolt Liposits[1,2]

[1]Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary;

[2]Faculty of Information Technology and Bionics, Pázmány Péter Catholic University, Budapest, Hungary;

[3]Faculty of Veterinary Science, Szent István University, Budapest, Hungary;

[4]Functional Genomics Core, Institute for Research in Biomedicine, Barcelona, Spain;

[5]Functional Genomics Consulting, Palleja, Spain.

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Corresponding author and person to whom reprint requests should be addressed:

Zsolt Liposits, MD, PhD, DSc

Institute of Experimental Medicine

Hungarian Academy of Sciences

Szigony utca 43., H-1083 Budapest, Hungary

E-mail: liposits@koki.hu

Phone: +3612109400 #171

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Abstract

In the hippocampus, estrogens are powerful modulators of neurotransmission, synaptic plasticity and neurogenesis. In women, menopause is associated with increased risk of memory disturbances, which can be attenuated by timely estrogen therapy. In animal models of menopause, 17β-estradiol (E2) replacement improves hippocampus-dependent spatial memory. Here, we explored the effect of E2 replacement on hippocampal gene expression in a rat menopause model. Middle-aged ovariectomized female rats were treated continuously for 29 days with E2 and then, the hippocampal transcriptome was investigated with Affymetrix expression arrays. Microarray data were analyzed by Bioconductor packages and web-based softwares, and verified with quantitative PCR. At standard fold change (FC) selection criterion, 156 genes responded to E2. All alterations but four were transcriptional activation. Robust activation (FC>10) occurred in the case of transthyretin, klotho, claudin 2, prolactin receptor, ectodin, coagulation factor V, insulin-like growth factor 2, Igfbp2 and sodium/sulfate symporter. Classification of the 156 genes revealed major groups including signaling (35 genes), metabolism (31 genes), extracellular matrix (17 genes) and transcription (16 genes). We selected 33 genes for further studies and all changes were confirmed by real-time PCR. The results suggest that E2 promotes retinoid, growth factor, homeoprotein, neurohormone and neurotransmitter signaling, changes metabolism, extracellular matrix composition, transcription, and induce protective mechanisms via genomic effects. We propose that these mechanisms contribute to effects of E2 on neurogenesis, neural plasticity and memory functions. Our findings provide further support for the rationale to develop safe estrogen receptor ligands for the maintenance of cognitive performance in postmenopausal women.

Introduction

The hippocampus plays a pivotal role in learning and memory (1). Gonadal steroids, including 17β-estradiol (E2), are powerful modulators of the hippocampal functions (2-4). Accordingly, E2 shapes dendritic spine density of principal neurons during the estrous cycle (5), modulates neurogenesis (6,7), alters neurotransmission (8), provides neuroprotection (9,10) and regulates the innate immune system (11,12). In women, menopause is associated with increased risk of hippocampal decline and memory disturbances (13,14). Estrogen replacement therapy (ERT) is effective in some, but not all women to attenuate menopause-related memory disturbances. The effects of ERT on hippocampus and memory are complex, and the effects depend on many parameters including estrogen responsiveness of the individual, the chemical structure of the active estrogen compound, dosage and initiation of treatment, among others (15). It has been proposed that a critical period exists for beneficial estrogen effects (16-18). In accord with the hypothesis, perimenopausal and early postmenopausal hormone therapies are often associated with improved cognitive functions later in life (19-22). However, the clinical practice lacks an effective ERT to treat menopause-related decline of cognitive performance.

E2 improves some aspects of cognitive performance in many rat studies. In young ovariectomized (OVX) female rats, E2 replacement (29 pg/mL of serum E2) increases working memory (23). In another study, E2 replacements (25 and 50 pg/mL of serum E2) enhance spatial memory, and improve adaptation to increasing amounts of working memory information (24). In the case of high memory demand, high-dose E2-replaced animals even outperform intact cycling females (24). The middle-aged OVX rat (25) is a widely used rodent model of menopause. In this model, immediate E2 replacement (20-25 pg/mL of serum E2) following OVX enhances acquisition during a working memory task, but treatment several month after OVX was ineffective (26). Orally administered E2 improves spatial memory, but E2 plus medroxyprogesterone-acetate treatment impairs performance (27). Additional studies also find that E2 improves hippocampus-dependent spatial memory (28-31). Current studies from the Daniel laboratory show that transient exposure of E2 after OVX (37 pg/mL of serum E2 levels) provides enduring benefits on the hippocampus and memory (32,33). Although many animal studies have demonstrated the positive effect of E2 on

cognitive performance, it should be noted that the effects are moderate and not all studies have found beneficial effects.

E2 exerts genomic nuclear actions (34) and nongenomic membrane actions (35). We hypothesize that genomic effects contribute to the impact of E2 on the hippocampus. Estrogen receptor-alpha (ERα), ERβ and GPR30 are expressed in the rat hippocampus at mRNA and protein levels (36-38). Genomic effects change with age which is partly due to the altered ratio of the ERα and ERβ subtypes (39). Relatively little information is currently available on the impact of E2 on hippocampal gene expression, and a comprehensive list of E2 regulated genes is still missing from the public domain. Therefore, elucidation of E2 target genes and estrogen driven regulatory mechanisms await further clarification. In this study, we identified 156 E2-regulated genes in middle-aged OVX rats and proposed major downstream regulatory mechanisms of E2 action, which underlie its impact on hippocampal functions of postmenopausal females.

Materials and Methods

Experimental animals

All studies were carried out with permission from the Animal Welfare Committee of the Institute of Experimental Medicine (IEM, Permission Number: A5769-01) and in accordance with the legal requirements of the European Community (Decree 86/609/EEC). Animal experimentation was conducted in accord with accepted standards. Female Harlan-Wistar female rats (n=16) were originally purchased from Toxicoop (Budapest, Hungary) and housed on a 12h light/12h dark cycle in the animal care facility of IEM. At their age of 9 month, they were retired as breeders and were housed individually for the subsequent months. Although environmental complexity may have an effect on the hippocampal formation of adult rats (40), any housing effect is excluded by comparing the E2-replaced group to the vehicle-treated one. Studies, which were performed in our animal facility, provided evidence that separate housing does not change basal corticosterone levels in rats (41). At their age of 13 months, the rats were deeply anesthetized and OVX bilaterally. Afterward, they were kept on phytoestrogen-free diet (Harlan Teklad Global Diets, Madison, WI). Ten days later, Alzet

2004 osmotic minipumps (DURECT, Cupertino, CA) filled either with E2 (0,33mg/ml in propylene-glycol, n=8, E2 group) or vehicle only (n=8, control group) were implanted subcutaneously for 29 days in the scruff of the neck of the animals. We reported previously that these subcutaneous treatments result 29.5 and 2.4 pg/mL serum E2 levels in E2- and vehicle-treated animals, respectively (42). Body weight of control animals increased, while of E2-replaced animals decreased in accordance with the anorexigenic effect of E2.

Microarray using Affymetrix Rat Genome 230 PM Strip Arrays

Hippocampi from 16 animals were prepared and total RNA was isolated and analyzed as previously (12). RNA quality was measured by capillary electrophoresis using Nano RNA chips and RNA integrity was determined by the software of 2100 Agilent Bioanalyzer (Santa Clara, CA, USA). RNA samples displayed high RNA integrity numbers (RIN > 8.2). Eight samples were examined by oligonucleotide microarray. Microarray experiments, including amplification, target labeling, hybridization, staining and scanning, were carried out as described earlier (43). In brief, 25 ng of total RNA Whole Transcriptome Amplification (WTA) library preparation and amplification for 17 cycles were performed following the distributor's (Sigma-Aldrich) recommendations. 8 µg cDNA was fragmented by DNAseI and biotinylated by terminal transferase obtained from the GeneChip Mapping 250K Nsp Assay Kit (Affymetrix Inc, Santa Clara, CA, USA). Hybridization, washing, staining and scanning of Affymetrix Rat Genome 230 PM Strip arrays were performed following the manufacturer's recommendations. Scanned images (DAT files) were transformed into intensities (CEL files) using the AGCC software (Affymetrix). Data analysis, including GCRMA, statistical and data mining work, were carried out as described earlier (42). Gene Ontology (GO) term enrichment was analyzed using a public functional annotation tool (DAVID Bioinformatics Resources; http://david.abcc.ncifcrf.gov) at default setting (44), but classification stringency was set to high. Annotation clusters were ranked by their score number, termed enrichment score, calculated from the modified Fisher's exact p-value of each GO-term.

Real-time PCR Studies

Custom TaqMan microfluidic cards (Applied Biosystems, Foster City, CA, USA) were designed to study mRNA expression by real-time PCR. Sixteen samples were investigated by PCR.

One μg of total RNA was used for reverse transcription. Reverse transcription and PCR were carried out as described earlier (12). The ViiA7 RUO 1.2.1 (Applied Biosystems) software and relative quantification against calibrator samples (ΔΔCt) were used for data evaluation. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl-transferase (*Hprt*) were used as housekeeping genes. Expression of these genes did not vary among treatment groups. A computed internal control corresponding to the geometric mean of cycle threshold (Ct) values of Gapdh and Hprt was used for Ct calculation. The use of TaqMan Gene Expression Assays and the equal amount of templates allowed us to categorize the genes according to mRNA expression levels by comparing their Ct values. E2-evoked genes were ranked into three arbitrary categories based on their abundant (Ct<25), moderate (25<Ct<28) or low (Ct>28) level of mRNA expression. PCR data evaluation and correlation analysis were performed as described previously (12).

Results

Oligonucleotide microarray revealed robust transcriptional activation in the hippocampus after E2 replacement

Two hundred and sixty-two transcripts showed the absolute FC higher than 1.5, which we considered as a criterion of E2 responsiveness. The 256 activated and 6 suppressed transcripts included 152 and 4 protein coding genes, respectively (**Supplemental Table 1**). The rest, 106 transcripts without currently known protein products, was not analyzed further. The heat map shows mRNA level of the top differentially expressed genes in the hippocampus of 4 vehicle- and 4 E2-treated OVX animals (**Fig. 1**). It shows small variability within treatment groups. As the color code indicates dark green, light green and orange represent very low, slight and moderate mRNA levels, respectively. The heat map shows a green to orange/red transition from left to right in color pattern illustrating the robust transcriptional activation in response to E2, as the leading trend.

Nine genes, including transthyretin (*Ttr*), klotho (*Kl*), claudin (*Cldn2*), prolactin receptor (*Prlr*), ectodin/wise (*Sostdc1*), coagulation factor V (*F5*), insulin-like growth factor 2 (*Igf2*), IGF

158 binding protein (*Igfbp2*) and sodium/sulfate symporter (*Slc13a4*) showed the most robust increase
159 (FC>10) in mRNA expression. We termed these prime E2 target genes (**Table 1**).

160 Twelve genes showed considerable activation of transcription (10>FC>4) and encoded folate
161 receptor (*Folr1*), α type I collagen (*Col1a2*), HT_{2C} receptor (*Htr2c*), secreted frizzled-related protein
162 (*Sfrp1*), α type VIII collagen (*Col8a2*), membrane frizzled-related protein (*Mfrp*), α type III collagen
163 (*Col3a1*), orthodenticle homeobox (*Otx2*), angiotensin-converting enzyme (*Ace*), midkine (*Mdk*),
164 ectonucleotide phosphodiesterase family member 2/autotaxin (*Enpp2*) and α type I collagen (*Col1a1*).
165 We named these substantial E2 target genes (**Table 1**).

166 Forty genes showed moderate (4>FC>2) activation (**Supplemental Table 1**) including
167 p57/Kip2 (*Cdkn1c*), prostaglandin D2 synthase (*Ptgds*), subfamily E potassium voltage-gated channel
168 (*Kcne2*), matrix metalloproteinase 2 (*Mmp2*), Msh homeobox 1 (*Msx1*), glycosylation-dependent cell
169 adhesion molecule (*Glycam1*), mannose receptor 1 (*Mrc1*), p21/CIP1 (*Cdkn1a*) and extracellular
170 superoxide dismutase (*Sod3*). We called these genuine E2 target genes.

171 **Functional annotation identified clusters of E2-regulated genes**

172 The 156 differentially expressed genes were analyzed using a functional gene annotation tool
173 DAVID (44), which generated a ranked list of clusters by grouping gene annotation terms based on
174 their similarity. Annotation clusters were ranked according to their enrichment score (ES), a factor
175 calculated from the adjusted *p*-values of terms. Top annotation clusters were arbitrarily named and
176 listed according to their enrichment scores (ES). Top clusters (**Supplemental Table 2**) with high ES
177 included response to hormones (4.23), pattern binding (3.95), cell motility (3.72), development (3.37),
178 retinoid (3.19) and collagen (2.08). Indeed, activation of several growth factors, homeobox proteins,
179 cell-cycle regulators and metabolic enzymes resembles earlier developmental stages. The 156 E2-
180 regulated genes were enriched at the highest confidence in the response to hormones cluster. This
181 cluster included TIMP metallopeptidase inhibitor (*Timp3*), aldehyde dehydrogenase (*Aldh1a2*), alpha-
182 2-macroglobulin (*A2m*), aquaporin 1 (*Aqp1*), *Col1a1*, *Cdkn1a*, glutathione peroxidase (*Gpx1*), *Igf1*,
183 *Igf2*, *Igfbp2*, matrix Gla protein (*Mgp*), matrix metalloproteinase 14 (*Mmp14*), *Mdk*, platelet-derived
184 growth factor receptor alpha (*Pdgfra*), *Ptgds* and retinol binding protein 4 (*Rbp4*). Noteworthy, that

the "response to hormones" cluster contained only two of the nine prime E2 target genes indicating partial knowledge on E2-responsive genes.

Classification of E2-induced genes

Classification of the 156 E2-regulated genes based on biological function revealed nine groups including cell adhesion, cytoskeleton, extracellular matrix, immune, metabolism, miscellaneous, signaling, transport and transcription (**Fig. 2**).

Cell adhesion

This group contained twelve genes including *Cldn1, Cldn2*, cingulin-like 1 (*Cgnl1*), glycosylation-dependent cell adhesion molecule, gap junction protein beta 2, ADAMTS-like 4, six transmembrane epithelial antigen, desmoglein, occludin (*Ocln*), lectin (*Lgals3bp*), InaD-like 2 (*Inadl2*) and anthrax receptor. Five genes (*Cldn1, Cldn2, Cgnl1, Inadl2, Ocln*) in the group encoded tight junction proteins.

Cytoskeleton

Cytoskeletal genes comprised ezrin (*Ezr*), tubulin beta (*Tubb4b*), alpha actin (*Acta2*), dynein light chain (*Dynlrb2*), vimentin (*Vim*), filamin A (*Flna*), vinculin (*Vcl*), tensin-1 (*Tns1*) and troponin C (*Tnnc2*). Their changes in mRNA expression were modest.

Extracellular matrix (ECM)

The ECM group composed of seventeen genes including six collagens (*Col1a2, Col8a2, Col3a1, Col1a1, Col6a2, Col9a3*), four small leucine-rich proteoglycans (osteoglycin, decorin, lumican, biglycan), two laminins (*Lama2, Lama3*), *Mgp*, prolargin (Prelp), cartilage associated protein (Crtap), nidogen (Nid1) and fibulin-2 (Fbln2). Collagens and proteoglycans showed considerable changes in response to E2.

Immune

This small set of genes contained mannose receptor (*Mrc1*), chemokine ligand (*Cxcl16*), lysozyme (*Lyz2*), interleukin 1 receptor (*Il1r1*), IFN-induced transmembrane protein (*Ifitm3*). These immune genes were likely of glial sources.

Metabolism

This group consisted thirty-one genes which were involved in retinol binding (*Rbp1* and *Rbp4, Crabp2* and stimulated by retinoic acid gene *Stra6*), retinoic acid synthesis (*Rdh10*, *Aldh1a2*), lipid messenger

synthesis (autotoxin *Enpp2*, prostaglandin D2 synthase, phospholipase *Pla2g5*), protection against oxidative stress (extracellular superoxide dismutase, phosphatidylinositol-4-phosphate 5-kinase (*Pip5k1b*), *Gpx1,* glutathione S-transferase *Gsta4* and *Gstm2*) as well as cargo transport (*Ttr, A2m, Tcn2*).

Signaling

This large set of genes included thirty-five genes that encode klotho (*Kl*), prolactin receptor (*Prlr*), ectodin/wise (*Sostdc1*), Igf2, Igfbp2, folate receptor (*Folr1*), HT_{2C} receptor, secreted frizzled-related protein (*Sfrp*), membrane frizzled-related protein (*Mfrp*), midkine (*Mdk*), p57/Kip2, annexin 2, disabled homolog 2, p21/CIP1, Kunitz type 2 inhibitor, follistatin-like 1, Notch ligand and receptor (Jag1, Notch2), regulatory subunit of protein phosphatase 1, connective tissue growth factor, Igf1, calmodulin-like 4, Igf binding protein-like 1, a disintegrin and metalloproteinase with thrombospondin motif 1, transforming growth factor β receptor 3, ADP-ribosylation factor-like 4, wntless homolog, vav guanine nucleotide exchange factor 3, GTPase activating protein with IQ motif, sumo-specific peptidase, platelet endothelial aggregation receptor, serine threonine kinase 39, ajuba LIM protein, platelet-derived growth factor receptor alpha, cocaine-amphetamine regulated transcript. E2 robustly activated *Kl*, *Prlr*, *Sostdc1*, *Igf2* and *Igfbp2*, and considerably induced *Folr1*, *Htr2c*, *Sfrp1*, *Mfrp* and *Mdk*.

Transcription

This group comprised seven transcription factors (*Otx2*, TSC22 domain family *Tsc22d2*, ZIC family member *Zic1* and *Zic4*, class E basic helix-loop-helix *Bhlhe40*, *Sp1*, butyrate response factor *Zfp36l1*), seven transcriptional modulators (*Msx1*, C2H2 Zn finger *Plagl1*, MyoD family inhibitor domain-containing *Mdfic*, ETS domain-containing *Elk3*, ets variant *Etv3*, homeobox interacting protein *Pbxip1*, homeobox protein *Zeb2*), as well as histone cluster 1 protein (*Hist1h4b*), and mRNA stabilization factor ELAV-like protein (*Elavl1*).

Results of real-time PCR study confirmed microarray data

Thirty-three genes were selected from the top of the microarray gene list for further studies using real-time PCR. The PCR study showed strong transcriptional activation in the hippocampus after E2 replacement (**Table 2**). All of the changes were confirmed and found to be statistically significant

($p<0.05$). The PCR study allowed us to perform correlation analysis and to determine the correlation coefficient (R) among gene pairs. The R matrix of the prime E2 target genes revealed correlation in several cases (**Supplemental Table 3**). Strong correlation ($R>0.98$) was shown in case of *Ttr-Igf2, Ttr-Igfbp2, Prlr-Sostdc1, Sostdc1-Slc13a4* and *Igf2-Igfbp2*. Three of the five gene pairs contain Igf2 suggesting that the protein product of activated Igf2 may evoke secondary transcriptional effects. The other two pairs include Sostdc1 suggesting the potential involvement of this BMP antagonist in secondary estrogenic effects. PCR experiments also provided information on the level of gene expression. In the vehicle treated OVX animals, we found considerable level ($Ct<25$) of mRNA expression for *Ttr, Enpp2, Ptgds, Gpx1*. Moderate ($25<Ct<28$) expression was detected in case of *Igf2, Igfbp2, Htr2c, Sfrp1, Ace, Mdk, Cdkn1c, Msx1, A2m, Sod3, Rdh10*. The rest of the genes were expressed weakly (data not shown). A complete list of gene symbols in alphabetical order and the corresponding gene names can be found in **Supplemental Table 4**.

Due to our special interest and their significant role in hippocampal regulation, we studied the putative estrogenic regulation of additional genes. The PCR study revealed that corticotropin releasing hormone receptor 2 (*Crhr2*), serum and glucocorticoid-regulated kinase 1 (*Sgk1*), Na-K-Cl cotransporter (*Slc12a2*), vesicular glutamate transporter 1 (*Slc17a7*) and vesicular inhibitory amino acid transporter (*Slc32a1*) were regulated by E2 (**Table 2**).

Discussion

In animal models of menopause, E2 exposure exerts a strong and enduring impact on hippocampal functions, but the underlying mechanisms remain elusive. In this study, we compared the hippocampal transcriptomes of middle-aged, OVX E2-replaced and vehicle-treated rats, and identified 156 estrogen responsive genes. We conclude that in middle-aged, gonadal steroid deficient female rats *i*) the hippocampal transcriptome is highly responsive to E2; *ii*) major E2 target genes include *Ttr*, *Kl*, *Cldn2*, *Prlr*, *Sostdc1*, *Igf2*, *Igfbp2*, *Folr1*, *Htr2c*, *Sfrp1*, *Otx2*, *Ace*, *Mdk* and *Enpp2*; *iii*) top functional categories of E2-regulated gene clusters comprise signaling, metabolism, ECM and transcription; *iv*) E2 can induce downstream events consisting elevated retinoid, klotho, insulin-like growth factor, Otx2

homeoprotein and serotonin signaling; *v*) the above mentioned E2-activated genes and downstream signaling mechanisms are known to control neurogenesis, the processes of synaptic plasticity and protective mechanisms, all responsible for the integrity, networking and proper function of the hippocampal formation.

Transcriptional fingerprint of chronic E2 treatment in the hippocampus

To date, transcriptional effects of chronic E2 treatments have been investigated in the hippocampus of middle-aged OVX mice. In this model, cyclic estradiol-benzoate treatment modestly regulates 2089 probes (45). The discrepancy between the subtle mouse and robust rat estrogenic regulation of gene expression is not fully understood, but may reflect the distinct treatment paradigms and the different distribution and expression of ER in the two species at midlife (36,46).

We previously found in the frontal cortex that chronic E2 treatment caused moderate transcriptional changes including activation of dopamine receptors, signaling regulators, neuropeptides (42) and suppression of immune genes (42,47,48). On the other hand, E2 robustly activates transcription in the hippocampus. E2 target genes encoded signaling molecules, metabolic enzymes, ECM components and transcriptional regulators. Comparison of the E2-evoked changes revealed great difference in estrogen sensitivity of the two transcriptomes. In the two areas, several genes including signaling molecules (Igf2, Igfbp2, Cartpt), ECM genes (Col1a1, Col1a2, Col3a1, Lum, Ogn) and transcriptional regulators (Bhlhe40) showed opposite regulation. There was no overlap in the metabolism group. Immune related changes were similar in the frontal cortex (48) and the hippocampus (12).

E2 influences signaling mechanisms through genomic effects

1. **Transthyretin and retinoid signaling**

Transthyretin transports retinol binding protein-retinol complex from the liver to target tissues. It is expressed highly in the liver and the choroid plexus, but it is also present in the hippocampus (49,50). Transthyretin is implicated in the maintenance of memory capacities as decreased hippocampal expression of the gene is associated with the development of age-related memory impairment (51). Transthyretin also sequesters beta-amyloid peptide (52). Therefore, robust increase in *Ttr* expression following E2 replacement may lead to enhanced availability of retinoids and accelerated clearance of neurotoxic peptides in the hippocampus. The rat and human *Ttr* genes

contain a putative ERE sequence in the 5' flanking region between -3406 and -3392. In agreement with the robust transcriptional activation of *Ttr*, this distal ERE is functional and shows the characteristics of an E2-dependent enhancer-like element (53). This example highlights the importance of distal regulatory elements in E2-dependent transcriptional regulation (54).

Retinoic acid (RA), generated from retinol via a retinaldehyde intermediate, regulates transcription and intracellular signaling pathways. We demonstrated that E2 activates transcription of several genes associated with serum retinol transport (*Ttr*), retinol binding (*Rbp1*, *Rbp4*), membrane retinol receptor (*Stra6*), intracellular retinol binding (*Crabp2*), conversion of retinol to retinaldehyde (*Rdh10*) and its metabolism to RA (*Aldh1a2*). Transcriptional activation of these genes may result elevated RA levels in the hippocampus. RA and its receptors are essential for long-term potentiation and depression (55), play indispensable role in neurogenesis (56) and take part in the regulation of homeostatic synaptic plasticity (57). RA also increases *de novo* synthesis of E2 and testosterone in hippocampal slice cultures via transcriptional regulation of steroidogenic enzymes (58). These data suggest that increased expression of transthyretin and key enzymes of retinol metabolism contributes to the effects of E2 on neuronal plasticity, neurogenesis, and maintains local E2 synthesis in the hippocampus via retinoid signaling.

Midkine is a RA inducible growth factor, which promotes neurite extension, survival and migration of neurons (59). It is also expressed in neural stem and progenitor cells (NSPC) and enhances their growth and survival (60). E2 evoked transcriptional activation of *Mdk* in the hippocampus may promote neurite outgrowth (61), receptor clustering and synapse formation (62), neuronal survival and neurogenesis (63).

2. **Klotho signaling**

Klotho is a known aging suppressor. It is a transmembrane protein which is liberated from the plasma membrane by enzymatic cleavage. Its absence leads to premature aging and shortened life span in mice (64). Conversely, its overexpression extends life span and provides protection against oxidative stress (65). Klotho is expressed widespread in the rat brain including the hippocampus (66). In the brain, the expression of klotho decreases with age (67). We found that E2 robustly increases

klotho expression which may promote anti-aging klotho signaling. Our finding is the first evidence that klotho is a prime E2 target gene.

3. **Insulin-like growth factor signaling**

Insulin-like growth factor 2 plays a pivotal role in adult hippocampal functions such as neurogenesis (68), memory consolidation and enhancement (69). Igf2 exerts its effects via the receptor tyrosine kinase IGF-1R (70), which is strongly expressed in the hippocampus (71). The receptor is often colocalized with the two ER subtypes (72). Through IGF-1R, Igf2 activates the ERK/MAPK and PI3K-Akt signaling pathways resulting in phosphorylation and ligand-independent activation of ERα (73,74). Association between ER- and IGF-1R- mediated signal transduction pathways is supported further by a recent study demonstrating that subsequent IGF-1R antagonism terminates the memory enhancing effect of short-term E2 treatment in OVX female rats (75). Based on the close linkage of estrogen and insulin-like growth factor signaling, we propose that in the hippocampus, robust activation of *Igf2* is one of the most important downstream events in E2 signaling.

Igfbp2 controls Igf half life and receptor availability. Binding of Igfbp2 modulates affinity for glycosaminoglycans and activation of Igf receptors. It is noteworthy that E2 activates the transcription of seventeen ECM genes including four proteoglycans. Both *Igf2* and *Igfbp2* were found to respond to E2 in the hippocampus of young OVX rats (76).

Insulin-like growth factor 1 is produced mainly in the liver but also synthesized by neurons and astrocytes in the hippocampus, where it regulates neurotransmission (77,78) and neurogenesis (79). Accumulating evidence indicates a putative role of Igf1 in the improvement of anxiety and memory deficits (80,81).

Besides midkine, klotho, Igf2 and Igf1, E2 enhances mRNA expression of additional growth factors. Connective tissue growth factor (Ctgf) is a secreted, ECM-associated protein that plays a role in adhesion, migration, mitogenesis, differentiation and survival. Ctgf has been detected in layer VII neurons of the cerebral cortex and weakly in the hippocampus (82). Platelet-derived growth factor receptor α is an oligodendrocyte progenitor marker (83).

4. **Neuropeptide signaling**

Prolactin receptors mediate trophic actions of prolactin such as neurogenesis (84,85), myelinization (86) and neuroprotection (87). Estrogens are required for optimal prolactin response as OVX mice show reduced levels of pSTAT5 in the CNS (88). Prolactin receptor is robustly upregulated after E2 replacement in the hippocampus resulting in elevated sensitivity for the modulatory and trophic actions of prolactin.

E2 activates the transcription of Ace. Angiotensin-converting enzyme converts angiotensin I to angiotensin II (AII), and degrades vasoactive peptides. The hippocampus contains one of the highest levels of AII (89) indicating considerable level of basal Ace expression. In concert with this, our PCR study revealed moderate mRNA expression of Ace in the hippocampus. AII immuno-reactivity was detected in the CA1, CA3 and dentate gyrus (90), while its receptors were detected in the dentate gyrus (91). The marked increase of ACE expression may lead to elevation of AII level in the hippocampus which may affect learning and memory processes (92,93).

Corticotropin-releasing hormone receptor 2 is one of the two CRH receptors. In response to stress, rapid activation of the hypothalamic-pituitary-adrenal (HPA) axis is governed by hypothalamic CRH. The two CRH receptors inversely regulate stress sensitivity: CRHR1 facilitates while CRHR2 attenuates activation of the HPA axis (94). Sex hormones regulate the expression of CRH and CRH receptors (95). We found that E2 robustly increased the expression of *Crhr2* in the hippocampus. In concert with our finding, the *Crhr2* promoter contains ERE half sites and an androgen response element (96).

Cocaine- and amphetamine-regulated transcript (CART) is modestly expressed in the hippocampus, where it modulates voltage-gated Ca++ signaling in neurons (97). We showed that E2 slightly downregulates *Cart* in the hippocampus.

5. **Ectodin and Wnt signaling**

Ectodin/wise/USAG-1 is a Wnt/bone morphogenetic protein antagonist. By binding to BMPs, it regulates their signaling during cellular proliferation and differentiation (98,99). Secreted frizzled-related protein 1 is also a negative soluble regulator of Wnt signaling. It can bind Wnt proteins and

frizzled (Fz) receptors preventing Wnt binding to Fz (100). Mfrp, membrane-type frizzled-related protein is also a Wnt regulator.

Wntless/Gpr177 is a 7TM Wnt cargo receptor and essential component of the Wnt secretion machinery (101). Wntless shuttles Wnt to postsynaptic terminals (102). Wnt ligands and other Wnt signaling molecules modulate synaptic transmission (103). It is proposed that Wnt signaling plays a major role in the modulation of synaptic plasticity at mature synapses.

6. **Notch signaling**

Notch2 and Jagged1 interaction plays a role in cell-cell communication (104). We found that E2 enhanced mRNA expression of both Notch2 and Jagged1 which may result elevated Notch-mediated communication in the middle-aged female hippocampus after E2 replacement. Notch signaling plays a pivotal role in synaptic plasticity, learning and memory, and the maintenance of NCPCs (105,106).

7. **OTX2 homeoprotein signaling**

OTX2, member of the homeoprotein family of transcription factors, can pass between cells by nonconventional mechanisms (107). The role of OTX2 in late postnatal periods and in adulthood is poorly understood. Maturation of fast-spiking interneurons is associated with periods when the visual cortex responds to environmental stimuli by physiological and morphological alterations (108). This critical period depends on the accumulation of OTX2 in parvalbumin-containing interneurons (109). We found considerable transcriptional activation of *Otx2* and propose that E2 may influence hippocampal plasticity through OTX2 signaling.

E2 alters composition of the extracellular matrix

E2 increased mRNA expression of components of the ECM (type I, III collagens) and matrix metalloproteinase 2 indicating a reorganization of the ECM in the hippocampus. The ECM provides a structural framework, acts as a storage deposit for growth factors and cytokines, regulates proliferation and differentiation of NSPCs, and allows migration of differentiated neurons to various areas of the hippocampal formation. Therefore, the structure and composition of the ECM influence neuronal plasticity and neurogenesis (110). NSPCs in the subgranular zone (SGZ) of the dentate gyrus proliferate throughout life (111,112). Signaling molecules such as hormones and growth factors

promote proliferation and differentiation of these cells (113), and their incorporation into the hippocampal network. Dividing cells and young, migrating neurons in the dentate gyrus express both ER subtypes (114). E2 modulates proliferation and survival of newly born cells in the dentate gyrus (115). Besides estrogens, these cells respond to other factors via ER, as ER antagonist ICI 182,780 blocks the effect of IGF1 on cell proliferation (116). As transthyretin and Igf2 are critical regulators of NSPCs (68), our results suggest that E2 controls neurogenesis, at least in part, via genomic effects including robust upregulation of *Ttr* and *Igf2*.

E2 modulates neurotransmission via gene expression

1. **HT_{2C} receptor and serotonergic neurotransmission**

HT_{2C} receptor is abundantly expressed in the CA1, CA2, CA3 pyramidal cell layer, and is also expressed in the dentate gyrus (117). Serotonin regulates neuronal network excitability and modulates adult neurogenesis in the hippocampus (118). Granule cells and interneurons in the dentate gyrus receive serotonergic input from the brain stem (119). HT_{2C} is one of the receptor subtypes which mediates the effect of serotonin on neurogenesis (119). Inhibition of the key enzyme of serotonin synthesis, or lesion of serotonergic neurons decrease neurogenesis (120). Chronic use of fluoxetine increases neurogenesis in adult rats (121,122). Strong evidence indicates the involvement of serotonin in the neurogenic effects of E2 in the adult dentate gyrus (123). Serotonin dependence of physical activity-induced adult hippocampal neurogenesis highlights further the importance of this mechanism (124).

The hippocampal network generates characteristic, state-dependent activity patterns including theta oscillations. It largely depends on cholinergic and GABAergic inputs from the medial septum (125). Theta oscillations are suppressed by serotonin via activation of HT_{2C} receptors (126). In conclusion, considerable increase of HT_{2C} receptor expression may influence network excitability, neurogenesis and theta oscillations.

2. **GABAergic and glutamatergic neurotransmission**

E2 regulated mRNA expression of two GABA (*Slc6a13*, *Slc32a1*) and a glutamate transporter (*Slc17a7*) gene. *Slc17a7* encodes vesicular glutamate transporter 1 (Vglut1), which is expressed in all principal cells of the hippocampus (127). *Slc32a1* encodes vesicular GABA transporter, which is

produced in GABAergic interneurons (128). The expression of these two transporters was slightly decreased resulting in altered presynaptic activity and synaptic function of both excitatory and inhibitory neurons (129,130). *Slc6a13* encodes GAT2, which is expressed in the cerebral cortex by neuronal, glial, ependymal and epithelial cells (131). As deletion of this gene neither affects growth, fertility and life span (132), the functional consequence of the modest upregulation of *Slc6a13* requires clarification.

3. **KCNE2**

The auxiliary β subunit for pore-forming potassium channel α subunits, KCNE2, regulates potassium channel voltage-dependence, gating, conductance, α subunit composition and pharmacology (133). KCNE2 also controls the function of neuronal hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels. Loss of *Kcne2* leads to decreased HCN channel function and increased neuronal excitability (134). E2 moderately increases mRNA expression of *Kcne2* which is in accordance with the presence of ERE in the *Kcne2* promoter (135). As HCN generates the pacemaker current I_h, upregulation of Kcne2 may result alterations in excitability, synaptic integration and rhythmic oscillatory activity (136).

E2 tunes innate immune mechanisms

E2 tunes the innate immune system and supports anti-inflammatory mechanisms in the hippocampus of middle-aged OVX rats (12). In this study, we identified additional immune related changes including upregulation of *Mrc1*, *Cxcl16*, *Colec12*, *Lyz2*, *Il1r1* and *Ifitm3*, which support further the immunomodulatory role of E2 in the limbic system.

E2 may promote antioxidant mechanisms

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide are generated by all cells during normal oxidative respiration. In the absence of control mechanisms, ROS cause oxidative damage to proteins, lipids and DNA (137). It is known that E2 exerts antioxidant activity (138,139). We demonstrated modest increase in the expression of extracellular superoxide dismutase, glutathione S-transferases and glutathione peroxidase. Via transcriptional activation of Sod3, Gstm2, Gsta4 and Gpx1, E2 can modulate cellular respiration, scavenging of superoxide, catabolism of hydrogen peroxide and metabolic detoxication of xenobiotics.

In summary, we provide evidence that the hippocampal transcriptome is highly responsive to E2 replacement in middle-aged rats shortly after ovariectomy. E2 robustly activates transcription of 152 genes involved in key metabolic and signaling pathways, ECM structure, neurotransmission, immune and antioxidant mechanisms. Main E2 target genes include transthyretin, klotho, insulin-like growth factor 2, orthodenticle homeobox 2. The resulting expression profile suggests that E2 replacement evokes downstream events such as activation of retinoid, klotho, insulin-like growth factor, Otx2 homeoprotein, serotonin signaling mechanisms, which promote plasticity, neurogenesis and neuroprotection (**Fig. 3**). We propose that activation of the above mentioned mechanisms contributes to the improvement of hippocampus-dependent memory performance after chronic administration of E2 in middle-aged OVX rats (26,27,30-32). The findings provide further support for the development of safe ER ligands to prevent memory disturbances and cognitive aging in postmenopausal women.

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Tables and Figures with Legends

Table 1 Lists of top E2 target genes in the hippocampus of middle-aged OVX rats

probeset ID	logFC	FC	p adj	symbol	gene name
		PRIME TARGET GENES			
1367598_PM_at	5,786	55,166	0,013	*Ttr*	transthyretin
1369361_PM_at	5,000	32,011	0,000	*Kl*	Klotho
1375933_PM_at	4,961	31,148	0,001	*Cldn2*	claudin 2
1370789_PM_a_at	4,881	29,468	0,001	*Prlr*	prolactin receptor
1379281_PM_at	4,848	28,794	0,000	*Sostdc1*	ectodin/wise
1374320_PM_at	3,915	15,082	0,000	*F5*	coagulation factor V
1367571_PM_a_at	3,591	12,054	0,002	*Igf2*	insulin-like growth factor 2
1367648_PM_at	3,439	10,845	0,001	*Igfbp2*	igf binding protein 2
1390532_PM_at	3,420	10,705	0,001	*Slc13a4*	sodium/sulfate symporters
		SUBSTANTIAL TARGET GENES			
1387889_PM_at	3,098	8,562	0,016	*Folr1*	folate receptor 1 (adult)
1387854_PM_at	2,927	7,605	0,001	*Col1a2*	collagen, type I, alpha 2
1376980_PM_at	2,783	6,885	0,011	*Htr2c*	5-HT receptor 2C
1383266_PM_at	2,607	6,092	0,081	*Sfrp1*	secreted frizzled-related
1374172_PM_at	2,379	5,202	0,248	*Col8a2*	collagen, type VIII, alpha 2
1377434_PM_at	2,358	5,128	0,164	*Mfrp*	membrane frizzled-related
1370959_PM_at	2,287	4,882	0,005	*Col3a1*	collagen, type III, alpha 1
1375465_PM_at	2,267	4,812	0,154	*Otx2*	orthodenticle homeobox 2
1387791_PM_at	2,139	4,404	0,029	*Ace*	angiotensin converting enz
1367682_PM_at	2,063	4,180	0,108	*Mdk*	midkine
1368536_PM_at	2,046	4,128	0,000	*Enpp2*	ectonuc pyrophosphatase
1388116_PM-at	2,019	4,052	0,155	*Col1a1*	collagen, type I, alpha 1

Microarray analysis identified 156 E2-regulated genes. Expression of nine genes increased more than 10-fold, and these were named prime target genes. Expression of twelve genes increased more that 4-fold but less than 10-fold, these were called substantial target genes. Probeset ID, code of Affymetrix Rat 230 Expression Array probeset; FC, fold change; p adj, adjusted p value.

907 **Table 2** Comparison of PCR and microarray data on hippocampal gene expression

REAL-TIME PCR				MICROARRAY	
Assay ID	**symbol**	**RQ**	**p**	**FC**	**p adj**
Rn00562124_m1	*Ttr*	15,99	0,008	55,16	0,013
Rn00580123_m1	*Kl*	14,90	0,006	32,01	0,000
Rn02063575_s1	*Cldn2*	11,86	0,021	31,15	0,001
Rn01525459_m1	*Prlr*	35,22	0,000	29,47	0,001
Rn00596672_m1	*Sostdc1*	13,49	0,006	28,79	0,000
Rn01454518_m1	*Igf2*	7,474	0,017	12,05	0,002
Rn00565473_m1	*Igfbp2*	9,807	0,004	10,85	0,001
Rn01747911_m1	*Slc13a4*	7,210	0,001	10,71	0,001
Rn00591759_m1	*Folr1*	7,144	0,005	8,562	0,016
Rn00562748_m1	*Htr2c*	6,750	0,003	6,885	0,011
Rn01478472_m1	*Sfrp1*	5,926	0,002	6,092	0,081
Rn01414596_m1	*Otx2*	13,31	0,003	4,812	0,154
Rn00561094_m1	*Ace*	6,469	0,006	4,404	0,029
Rn00675549_g1	*Mdk*	4,573	0,003	4,180	0,108
Rn01505088_m1	*Enpp2*	4,749	0,003	4,128	0,000
Rn01463516_m1	*Slco1a5*	18,68	0,001	3,791	0,231
Rn01502044_g1	*Cdkn1c*	3,777	0,006	3,787	0,005
Rn00564605_m1	*Ptgds*	3,016	0,000	3,256	0,166
Rn02094913_s1	*Kcne2*	3,722	0,023	3,239	0,588
Rn00667535_m1	*Msx1*	3,152	0,003	2,801	0,200
Rn00592456_m1	*Slc6a13*	2,955	0,000	2,784	0,040
Rn00560589_m1	*A2m*	3,480	0,000	2,611	0,204
Rn00571516_m1	*Anxa2*	3,254	0,001	2,542	0,081
Rn00562834_m1	*Aqp1*	11,31	0,008	2,478	0,110
Rn01487342_m1	*Mrc1*	1,959	0,004	2,357	0,152
Rn00562884_m1	*Cox8b*	3,252	0,024	2,224	0,341
Rn01427989_s1	*Cdkn1a*	2,103	0,001	2,197	0,464
Rn00563570_m1	*Sod3*	1,874	0,002	2,081	0,089
Rn00568361_m1	*Crabp2*	1,999	0,004	1,942	0,407
Rn00588079_m1	*Aldh1a2*	3,651	0,000	1,902	0,155
Rn00710306_m1	*Igf1*	4,018	0,000	1,672	0,501
Rn00710727_m1	*Rdh10*	1,364	0,006	1,654	0,161
Rn00577994_g1	*Gpx1*	1,653	0,000	1,619	0,205
Rn00575617_m1	*Crhr2*	11,418	0,001		
Rn01537468_g1	*Sgk1*	1,508	0,021		
Rn00582505_m1	*Slc12a2*	1,75	0,001		
Rn00587830_m1	*Slc17a7*	0,59	0,001		
Rn00824654_m1	*Slc32a1*	0,66	0,001		

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910 Expression of selected genes was determined in the hippocampus of middle-aged OVX rats treated
911 either with vehicle (OVX) or E2 (OVX+E2) by quantitative real-time PCR. Comparison of PCR
912 results with microarray data showed high similarity which provides a strong confirmation of the
913 microarray data. Relative quantity ($RQ=2^{-\Delta\Delta Ct}$) was used to characterize expression of a given gene in

914 E2 treated animals compared to controls and was determined from seven independent experiments.
915 One-way ANOVA identified statistically significant ($p<0.05$) changes for all genes. P, p value.
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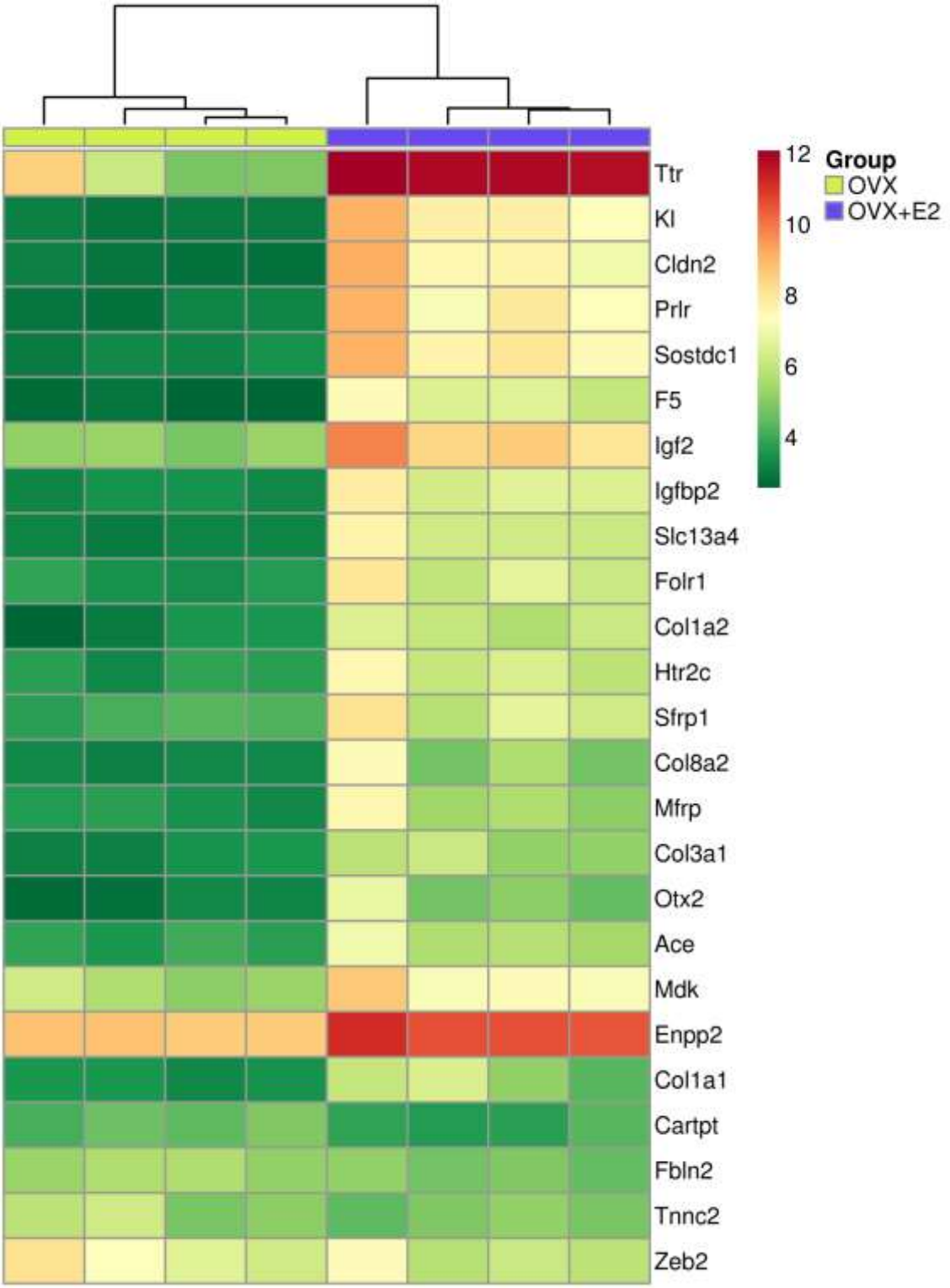
Legends to figures

Figure 1. Heat map depicting top 25 differentially expressed genes in the hippocampus of middle-aged ovariectomized (OVX) and middle-aged ovariectomized rats treated with E2 (OVX+E2). On the left, four columns represent expression data from vehicle-treated rats, four columns on the right show data from E2-replaced animals. In general, weakly expressed genes became moderately expressed. The two exceptions included transthyretin (Ttr) and autotaxin (Enpp2), they became abundantly expressed. Downregulated genes were weakly transcribed.

Figure 2. Classification of one hundred fifty-six genes revealed nine functional groups. These groups include cell adhesion, cytoskeleton, ECM, immune, metabolism, miscellaneous, signaling, transporter and transcription. Major functional groups are signaling and metabolism containing thirty-five and thirty-one genes, respectively.

Figure 3. Schematic illustration of the E2 fingerprint on the hippocampal transcriptome of the middle-aged ovariectomized female rat. E2 modifies gene expression of most cellular constituents of the hippocampal formation resulting in changes in neurogenesis, synaptic plasticity and neuroprotection as evidenced in the literature. The most widely characterized regulatory mechanisms of the hippocampus involving the corresponding target genes are depicted in the left and right columns of the scheme. CA1-CA3, sector 1-3 of *Cornu Ammonis*; DG, dentate gyrus.

936 Figure 1



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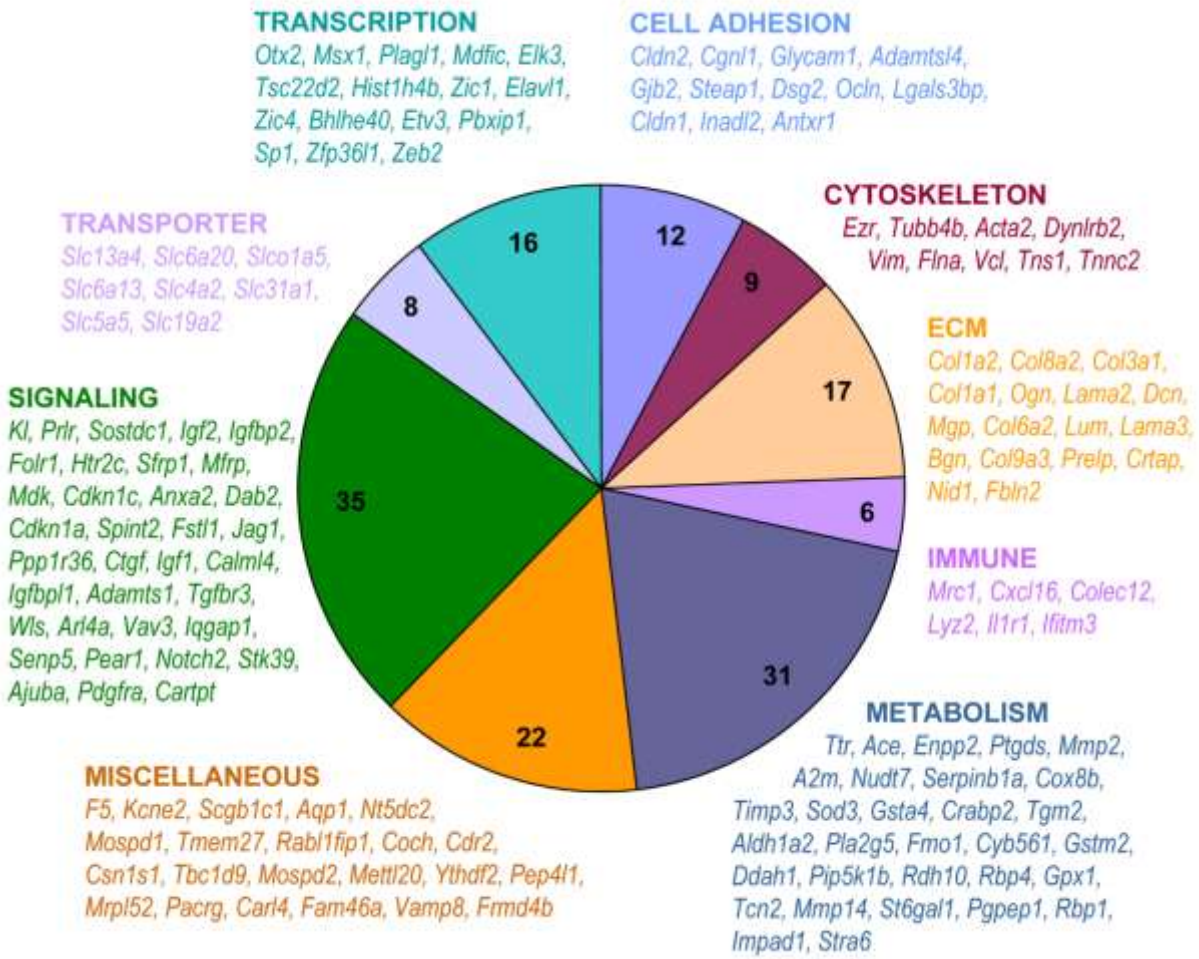
940 Figure 2



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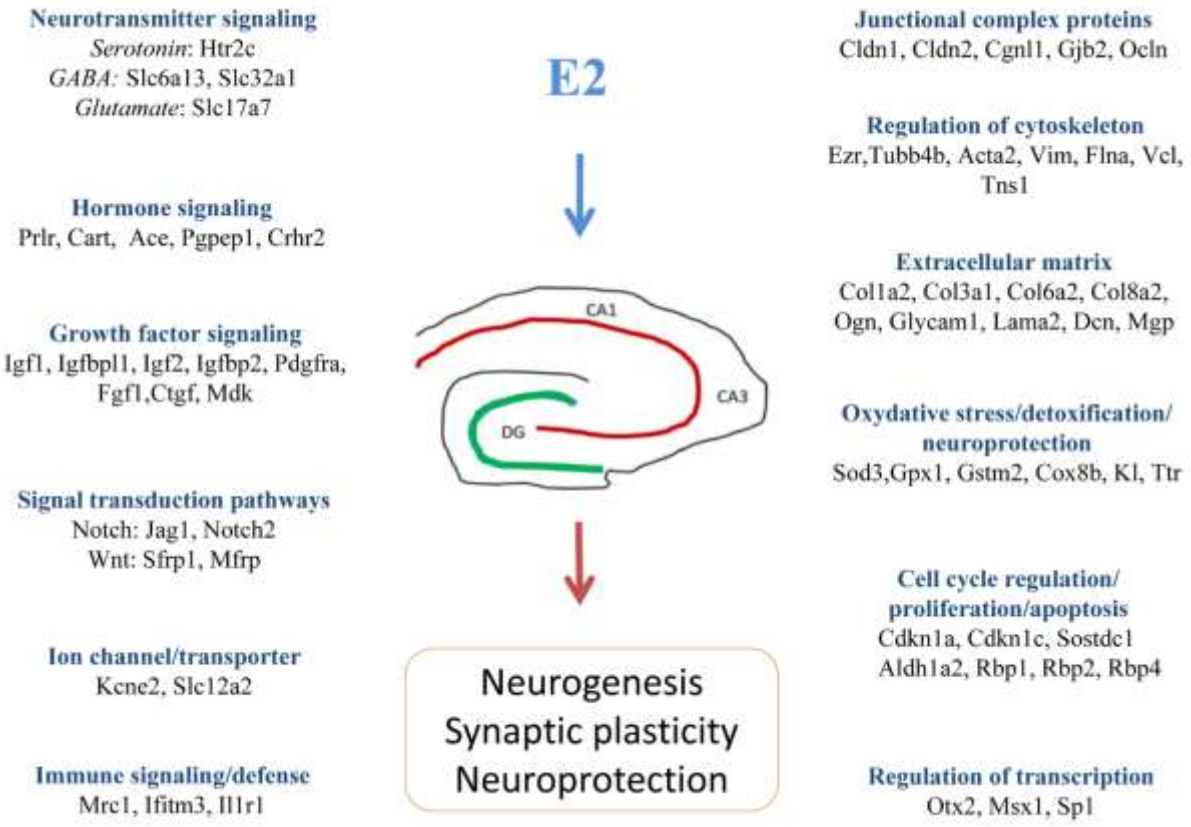
944 Figure 3



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948 **SUPPLEMENTUM**

949 **Supplemental Table 1** The list of E2-regulated genes in decreasing order

probeset ID	logFC	FC	adj. P	symbol	gene name
1367598_PM_at	5,786	55,166	0,013	*Ttr*	transthyretin
1369361_PM_at	5,000	32,011	0,000	*Kl*	Klotho
1375933_PM_at	4,961	31,148	0,001	*Cldn2*	claudin 2
1370789_PM_a_at	4,881	29,468	0,001	*Prlr*	prolactin receptor
1379281_PM_at	4,848	28,794	0,000	*Sostdc1*	sclerostin domain containing 1
1374320_PM_at	3,915	15,082	0,000	*F5*	coagulation factor V
1367571_PM_a_at	3,591	12,054	0,002	*Igf2*	insulin-like growth factor 2
1367648_PM_at	3,439	10,845	0,001	*Igfbp2*	igf binding protein 2
1390532_PM_at	3,420	10,705	0,001	*Slc13a4*	sodium/sulfate symporters
1387889_PM_at	3,098	8,562	0,016	*Folr1*	folate receptor 1 (adult)
1387854_PM_at	2,927	7,605	0,001	*Col1a2*	collagen, type I, alpha 2
1376980_PM_at	2,783	6,885	0,011	*Htr2c*	5-HT receptor 2C
1383266_PM_at	2,607	6,092	0,081	*Sfrp1*	secreted frizzled-related protein
1374172_PM_at	2,379	5,202	0,248	*Col8a2*	collagen, type VIII, alpha 2
1377434_PM_at	2,358	5,128	0,164	*Mfrp*	membrane frizzled-related protein
1370959_PM_at	2,287	4,882	0,005	*Col3a1*	collagen, type III, alpha 1
1375465_PM_at	2,267	4,812	0,154	*Otx2*	orthodenticle homeobox 2
1387791_PM_at	2,139	4,404	0,029	*Ace*	angiotensin converting enzyme
1367682_PM_at	2,063	4,180	0,108	*Mdk*	midkine
1368536_PM_at	2,046	4,128	0,000	*Enpp2*	ectonucleotide pyrophosphatase
1388116_PM_at	2,019	4,052	0,155	*Col1a1*	collagen, type I, alpha 1
1369705_PM_at	1,928	3,805	0,001	*Slc6a20*	solute carrier family 6, member 20
1368606_PM_at	1,923	3,791	0,231	*Slco1a5*	sol carrier organic anion transporter family, member 1a5
1372299_PM_at	1,921	3,787	0,005	*Cdkn1c*	cyclin-dependent kinase inhibitor 1C
1389107_PM_at	1,909	3,755	0,040	*Cgnl1*	cingulin-like 1
1385248_PM_a_at	1,817	3,523	0,005	*Ogn*	osteoglycin
1367851_PM_at	1,703	3,256	0,166	*Ptgds*	prostaglandin D2 synthase (brain)
1394343_PM_s_at	1,696	3,239	0,588	*Kcne2*	K+ channel, Isk-related family
1370301_PM_at	1,521	2,870	0,164	*Mmp2*	matrix metallopeptidase 2
1393436_PM_at	1,520	2,869	0,662	*Scgb1c1*	secretoglobin, family 1C, member 1
1368302_PM_at	1,486	2,801	0,200	*Msx1*	msh homeobox 1
1387372_PM_at	1,477	2,784	0,040	*Slc6a13*	neurotransmitter transporter, GABA
1368337_PM_at	1,462	2,754	0,036	*Glycam1*	glycosylation dependent CAM 1
1367794_PM_at	1,385	2,611	0,204	*A2m*	alpha-2-macroglobulin
1367584_PM_at	1,346	2,542	0,081	*Anxa2*	annexin A2
1390404_PM_at	1,332	2,517	0,253	*Lama2*	laminin, alpha 2
1370956_PM_at	1,319	2,494	0,002	*Dcn*	decorin
1369625_PM_at	1,309	2,478	0,110	*Aqp1*	aquaporin 1
1371849_PM_at	1,278	2,425	0,081	*Nt5dc2*	5'-nucleotidase domain containing 2
1389251_PM_at	1,269	2,410	0,361	*Nudt7*	nudix (nucleoside diphosphate linked moietyX)-type motif 7
1377034_PM_at	1,243	2,366	0,262	*Serpinb1a*	serine/cysteine proteinase inhibitor, clade B, member 1a
1376861_PM_at	1,241	2,364	0,164	*Mospd1*	motile sperm domain containing 1
1392648_PM_at	1,237	2,357	0,152	*Mrc1*	mannose receptor, C type 1
1367568_PM_a_at	1,208	2,309	0,078	*Mgp*	matrix Gla protein
1367739_PM_at	1,153	2,224	0,341	*Cox8b*	cytochrome c oxidase, subunit VIIIb
1368202_PM_a_at	1,151	2,221	0,141	*Dab2*	disabled homolog 2 (Drosophila)
1371369_PM_at	1,136	2,198	0,441	*Col6a2*	collagen, type VI, alpha 2

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1388674_PM_at	1,136	2,197	0,464	*Cdkn1a*	cyclin-dependent kinase inhibitor 1A
1387013_PM_at	1,130	2,189	0,656	*Tmem27*	transmembrane protein 27
1367749_PM_at	1,129	2,186	0,078	*Lum*	lumican
1368082_PM_at	1,123	2,178	0,204	*Slc4a2*	solute carrier family 4, member 2
1372064_PM_at	1,118	2,170	0,108	*Cxcl16*	chemokine (C-X-C motif) ligand 16
1387122_PM_at	1,112	2,162	0,204	*Plagl1*	pleiomorphic adenoma gene-like 1
1370538_PM_at	1,096	2,137	0,606	*Lama3*	laminin, alpha 3
1373386_PM_at	1,095	2,136	0,498	*Gjb2*	gap junction protein, beta 2
1389836_PM_a_at	1,082	2,116	0,012	*Timp3*	TIMP metallopeptidase inhibitor 3
1368046_PM_at	1,070	2,100	0,309	*Slc31a1*	solute carrier family 31, member 1
1368322_PM_at	1,057	2,081	0,089	*Sod3*	superoxide dismutase 3, extracellular
1377790_PM_at	1,048	2,068	0,318	*Rab11fip1*	RAB11 family interacting protein 1
1372426_PM_at	1,033	2,046	0,408	*Adamtsl4*	ADAMTS-like 4
1393706_PM_at	1,000	2,000	0,643	*Steap1*	six transmembrane epithelial antigen
1382083_PM_at	0,992	1,989	0,204	*Coch*	coagulation factor C homolog, cochlin
1374139_PM_at	0,992	1,989	0,559	*Cdr2*	cerebellar degeneration-related 2
1374752_PM_at	0,983	1,977	0,511	*Mdfic*	MyoD family inhibitor domain contain
1372297_PM_at	0,967	1,954	0,443	*Gsta4*	glutathione S-transferase alpha 4
1370391_PM_at	0,958	1,942	0,407	*Crabp2*	cellular retinoic acid binding protein 2
1370875_PM_at	0,949	1,930	0,129	*Ezr*	ezrin
1388557_PM_at	0,946	1,926	0,164	*Tubb4b*	tubulin, beta 4B class IVb
1380596_PM_at	0,931	1,907	0,204	*Dsg2*	desmoglein 2
1378753_PM_at	0,931	1,906	0,575	*Ocln*	occludin
1368003_PM_at	0,928	1,902	0,155	*Aldh1a2*	aldehyde dehydrogenase 1 family
1388320_PM_at	0,923	1,895	0,253	*Spint2*	Kunitz type serine peptidase inhibitor
1387946_PM_at	0,915	1,885	0,566	*Lgals3bp*	lectin, galactoside-binding, soluble
1372818_PM_at	0,893	1,857	0,017	*Colec12*	collectin sub-family member 12
1388183_PM_at	0,890	1,853	0,802	*Csn1s1*	casein alpha s1
1369943_PM_at	0,877	1,836	0,040	*Tgm2*	transglutaminase 2, C polypeptide
1370857_PM_at	0,867	1,824	0,121	*Acta2*	smooth muscle alpha-actin
1370068_PM_at	0,863	1,819	0,505	*Pla2g5*	phospholipase A2, group V
1367594_PM_at	0,854	1,807	0,149	*Bgn*	biglycan
1387053_PM_at	0,850	1,802	0,161	*Fmo1*	flavin containing monooxygenase 1
1368822_PM_at	0,837	1,786	0,218	*Fstl1*	follistatin-like 1
1389617_PM_at	0,834	1,782	0,743	*Elk3*	ELK3, ETS-domain protein
1383895_PM_at	0,832	1,780	0,108	*Dynlrb2*	dynein light chain roadblock-type 2
1396150_PM_at	0,828	1,775	0,639	*Cldn1*	claudin 1
1377631_PM_at	0,813	1,757	0,443	*Col9a3*	procollagen, type IX, alpha 3
1387886_PM_at	0,802	1,743	0,049	*Prelp*	proline/arginine-rich end leucine-rich
1398484_PM_at	0,795	1,735	0,253	*Tbc1d9*	TBC1 domain family, member 9
1390989_PM_at	0,795	1,735	0,345	*Mospd2*	motile sperm domain containing 2
1398383_PM_at	0,791	1,730	0,262	*Cyb561*	cytochrome b-561
1368725_PM_at	0,783	1,720	0,638	*Jag1*	jagged 1
1382171_PM_at	0,779	1,716	0,805	*Tsc22d2*	TSC22 domain family, member 2
1370154_PM_at	0,779	1,715	0,082	*Lyz2*	lysozyme 2
1376200_PM_at	0,778	1,715	0,271	*Mettl20*	methyltransferase like 20
1371960_PM_at	0,771	1,706	0,402	*Ythdf2*	YTH domain family, member 2
1374743_PM_at	0,766	1,701	0,004	*Inadl2*	InaD-like 2 (Drosophila)

1371847_PM_at	0,761	1,695	0,684	*Crtap*	cartilage associated protein
1370952_PM_at	0,760	1,694	0,515	*Gstm2*	glutathione S-transferase mu 2
1390937_PM_at	0,759	1,692	0,212	*Ppp1r36*	protein phosphatase 1, regulatory subunit 36
1367631_PM_at	0,758	1,691	0,049	*Ctgf*	connective tissue growth factor
1370333_PM_a_at	0,741	1,672	0,501	*Igf1*	insulin-like growth factor 1
1375026_PM_at	0,739	1,669	0,737	*Calml4*	calmodulin-like 4
1387111_PM_at	0,734	1,663	0,649	*Ddah1*	dimethylarginine dimethylaminohydrolase 1
1379352_PM_at	0,731	1,660	0,156	*Pip5k1b*	phosphatidylinositol-4-phosphate 5-kinase, type I, beta
1390715_PM_at	0,729	1,657	0,490	*Igfbpl1*	insulin-like growth factor binding protein-like 1
1393351_PM_at	0,726	1,654	0,161	*Rdh10*	retinol dehydrogenase 10 (all-trans)
1368223_PM_at	0,726	1,654	0,082	*Adamts1*	ADAM metallopeptidase with thrombospondin type 1 motif, 1
1371518_PM_at	0,722	1,649	0,372	*Nid1*	nidogen 1
1372726_PM_at	0,713	1,639	0,495	*Hist1h4b*	histone cluster 1, H4b
1390912_PM_at	0,705	1,631	0,506	*Pcp4l1*	Purkinje cell protein 4-like 1
1388654_PM_at	0,702	1,627	0,230	*Mrpl52*	mitochondrial ribosomal protein L52
1370750_PM_a_at	0,700	1,625	0,219	*Il1r1*	interleukin 1 receptor, type I
1369524_PM_at	0,698	1,622	0,154	*Zic1*	Zic family member 1
1367574_PM_at	0,697	1,621	0,311	*Vim*	vimentin
1371762_PM_at	0,695	1,619	0,480	*Rbp4*	retinol binding protein 4, plasma
1367576_PM_at	0,695	1,619	0,205	*Gpx1*	glutathione peroxidase 1
1387484_PM_at	0,692	1,616	0,418	*Tgfbr3*	transforming growth factor, beta receptor III
1367960_PM_at	0,684	1,607	0,211	*Arl4a*	ADP-ribosylation factor-like 4A
1383129_PM_at	0,680	1,602	0,612	*Wls*	wntless homolog (Drosophila)
1377721_PM_at	0,678	1,600	0,501	*Pacrg*	Park2 co-regulated
1387995_PM_a_at	0,677	1,599	0,513	*Ifitm3*	interferon induced transmembrane p
1375892_PM_at	0,676	1,598	0,780	*Elavl1*	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
1369020_PM_at	0,675	1,597	0,441	*Slc5a5*	solute carrier family 5 (sodium iodide symporter), member 5
1367765_PM_at	0,673	1,594	0,273	*Tcn2*	transcobalamin 2
1392677_PM_at	0,669	1,589	0,204	*Zic4*	Zic family member 4
1393605_PM_at	0,660	1,580	0,328	*Vav3*	vav 3 guanine nucleotide exchange f
1379483_PM_at	0,658	1,578	0,850	*Bhlhe40*	basic helix-loop-helix family, member e40
1388762_PM_at	0,654	1,573	0,156	*Iqgap1*	IQ motif containing GTPase activating protein 1
1391262_PM_at	0,647	1,566	0,775	*Senp5*	Sumo1/sentrin/SMT3 specific peptidase 5
1388342_PM_at	0,645	1,564	0,868	*Etv3*	ets variant 3
1379868_PM_at	0,640	1,558	0,657	*Pbxip1*	pre-B-cell leukemia homeobox interacting protein 1
1373790_PM_at	0,638	1,556	0,345	*Car14*	carbonic anhydrase 14
1367860_PM_a_at	0,632	1,550	0,313	*Mmp14*	matrix metallopeptidase 14
1371880_PM_at	0,628	1,545	0,775	*Sp1*	Sp1 transcription factor
1370907_PM_at	0,628	1,545	0,205	*St6gal1*	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1
1384591_PM_at	0,627	1,544	0,306	*Pear1*	platelet endothelial aggregation receptor 1
1394940_PM_at	0,626	1,544	0,566	*Fam46a*	family with sequence similarity 46, member A
1371382_PM_at	0,624	1,541	0,239	*Flna*	filamin A, alpha
1398362_PM_at	0,614	1,531	0,441	*Notch2*	notch 2
1372905_PM_at	0,611	1,527	0,814	*Vcl*	vinculin
1371954_PM_at	0,606	1,522	0,642	*Tns1*	tensin 1
1369970_PM_at	0,603	1,519	0,269	*Vamp8*	vesicle-associated membrane protein 8
1375215_PM_x_at	0,601	1,517	0,779	*Pgpep1*	pyroglutamyl-peptidase I
1387059_PM_at	0,599	1,514	0,449	*Stk39*	serine threonine kinase 39

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	1367939_PM_at	0,597	1,513	0,164	*Rbp1*	retinol binding protein 1, cellular
	1373122_PM_at	0,595	1,511	0,219	*Ajuba*	ajuba LIM protein
	1396109_PM_at	0,595	1,511	0,649	*Antxr1*	anthrax toxin receptor 1
	1395036_PM_at	0,594	1,510	0,608	*Impad1*	inositol monophosphatase domain containing 1
	1390525_PM_a_at	0,593	1,509	0,600	*Stra6*	stimulated by retinoic acid gene 6
	1369959_PM_at	0,592	1,508	0,468	*Zfp36l1*	zinc finger protein 36, C3H type-like 1
	1390464_PM_at	0,591	1,506	0,863	*Frmd4b*	FERM domain containing 4B
	1390863_PM_at	0,590	1,505	0,570	*Slc19a2*	solute carrier family 19 (thiamine transporter), member 2
	1370941_PM_at	0,589	1,504	0,110	*Pdgfra*	platelet derived growth factor receptor, alpha polypeptide
	1368585_PM_at	-0,583	0,667	0,509	*Cartpt*	CART prepropeptide
	1389533_PM_at	-0,615	0,653	0,398	*Fbln2*	fibulin 2
	1372195_PM_at	-0,667	0,630	0,779	*Tnnc2*	troponin C type 2 (fast)
956	1393795_PM_at	-0,733	0,602	0,862	*Zeb2*	zinc finger E-box binding homeobox 2

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958 In data analysis we applied the standard selection criterion of absolute FC>1.5. Affymetrix probeset

959 ID, logFC, FC, adjusted p value, gene symbol and name are displayed.

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962 **Supplemental Table 2** Clusters of E2 regulated genes identified by functional annotation

	CLUSTER	p	ES	GENES
1	**Response to hormones**		4.230	Timp3, Aldh1a2, A2m, Aqp1, Col1a1, Cdkn1a, Gpx1, Igf1, Igf2, Igfbp2, Mgp, Mmp14, Mdk, Pdgfra, Ptgds, Rbp4
	response to steroid hormone	0.002		
	response to hormone stimulus	0.012		
	response to endogenous stimulus	0.024		
2	**Pattern binding**		3.950	Adamtsl4, Bgn, Cln12, Ctgt, Dcn, Flstl1, Mrc1, Mdk, Pla2, Tgfbr3
	glycosaminoglycan binding	0.004		
	pattern binding	0.006		
	polysaccharide binding	0.006		
	heparin binding	0.010		
3	**Cell motility**		3.726	Htr2c, Ace, Cxcl16, Enpp2, Igf1, Jub, Lama2, Lama3, Pdgfra, Tgfbr3, Vcn
	regulation of locomotion	0.009		
	regulation of cell migration	0.011		
	regulation of cell motion	0.020		
4	**Development**		3.368	Sp1, Aldh1a2, Ctgf, Mgp, Mmp14, Pdgfra, Rbp4, Rdh10
	lung development	0.020		
5	**Retinoid**		3.195	Aldh1a2, Rbp2, Ptgds, Rbp1, Rbp4, Ttr
	vitamin A	0.001		
	retinoid binding	0.007		
	isoprenoid binding	0.010		
	retinol binding	0.050		
6	**Collagen**		2.085	Anxa2, Col1a1, Col1a2, Col3a1, Clcn12, Lum
	fibrillar collagen	0.013		
	collagen	0.050		

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967 DAVID Functional Annotation Clustering Tool (http://david.abcc.ncifcrf.gov) was used for functional
968 annotation clustering of the 156 genes regulated by E2 at high stringency. P, adjusted p value; ES,
969 enrichment score.

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Supplemental Table 3 Correlation analysis of prime E2 target genes.

	Ttr	Kl	Cldn2	Prlr	**Sostdc1**	**Igf2**	Igfbp2	**Slc13a4**
Ttr		0,962	0,572	0,924	0,959	**0,991**	**0,987**	0,964
Kl	0,962		0,569	0,859	0,894	0,970	0,962	0,911
Cldn2	0,572	0,569		0,637	0,625	0,550	0,560	0,590
Prlr	0,924	0,859	0,637		**0,981**	0,918	0,910	**0,976**
Sostdc1	0,959	0,894	0,625	**0,981**		0,950	0,952	**0,992**
Igf2	**0,991**	0,970	0,550	0,918	0,950		**0,995**	0,961
Igfbp2	**0,987**	0,962	0,560	0,910	0,952	**0,995**		0,955
Slc13a4	0,964	0,911	0,590	**0,976**	**0,992**	0,961	0,955	

Real-time PCR data from eight independent experiments were analyzed by the Statistica software. The correlation coefficient (R) matrix showed great variations in R values. Strong association (R>0.98) was revealed in case of *Igf2-Igfbp2*, *Sostdc1-Slc13a4*, *Igf2-Ttr*, *Ttr-Igfbp2* and *Prlr-Sostdc1*.

Supplemental Table 4 List of abbreviations

symbol	gene name
A2m	alpha-2-macroglobulin
Ace	angiotensin converting enzyme
Acta2	smooth muscle alpha-actin
Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
Adamtsl4	ADAMTS-like 4
Ajuba	ajuba LIM protein
Aldh1a2	aldehyde dehydrogenase 1 family
Antxr1	anthrax toxin receptor 1
Anxa2	annexin A2
Aqp1	aquaporin 1
Arl4a	ADP-ribosylation factor-like 4A
Bgn	biglycan
Bhlhe40	basic helix-loop-helix family, member e40
Calml4	calmodulin-like 4
Car14	carbonic anhydrase 14
Cartpt	CART prepropeptide
Cdkn1a	cyclin-dependent kinase inhibitor 1A
Cdkn1c	cyclin-dependent kinase inhibitor 1C
Cdr2	cerebellar degeneration-related 2
Cgnl1	cingulin-like 1
Cldn1	claudin 1
Cldn2	claudin 2
Coch	coagulation factor C homolog, cochlin
Col1a1	collagen, type I, alpha 1
Col1a2	collagen, type I, alpha 2
Col3a1	collagen, type III, alpha 1
Col6a2	collagen, type VI, alpha 2
Col8a2	collagen, type VIII, alpha 2
Col9a3	procollagen, type IX, alpha 3
Colec12	collectin sub-family member 12
Cox8b	cytochrome c oxidase, subunit VIIIb
Crabp2	cellular retinoic acid binding protein 2
Crtap	cartilage associated protein
Ctgf	connective tissue growth factor
Cxcl16	chemokine (C-X-C motif) ligand 16
Cyb561	cytochrome b-561
Csn1s1	casein alpha s1
Dab2	disabled homolog 2 (Drosophila)
Dcn	decorin
Ddah1	dimethylarginine dimethylaminohydrolase 1
Dsg2	desmoglein 2
Dynlrb2	dynein light chain roadblock-type 2
Elavl1	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
Elk3	ELK3, ETS-domain protein
Enpp2	ectonucleotide pyrophosphatase
Etv3	ets variant 3
Ezr	ezrin

F5	coagulation factor V
Fam46a	family with sequence similarity 46, member A
Fbln2	fibulin 2
Flna	filamin A, alpha
Fmo1	flavin containing monooxygenase 1
Folr1	folate receptor 1 (adult)
Frmd4b	FERM domain containing 4B
Fstl1	follistatin-like 1
Gjb2	gap junction protein, beta 2
Glycam1	glycosylation dependent CAM 1
Gpx1	glutathione peroxidase 1
Gsta4	glutathione S-transferase alpha 4
Gstm2	glutathione S-transferase mu 2
Hist1h4b	histone cluster 1, H4b
Htr2c	5-HT receptor 2C
Ifitm3	interferon induced transmembrane p
Igf1	insulin-like growth factor 1
Igf2	insulin-like growth factor 2
Igfbp2	igf binding protein 2
Igfbpl1	insulin-like growth factor binding protein-like 1
Il1r1	interleukin 1 receptor, type I
Impad1	inositol monophosphatase domain containing 1
Inadl2	InaD-like 2 (Drosophila)
Iqgap1	IQ motif containing GTPase activating protein 1
Jag1	jagged 1
Kcne2	K+ channel, Isk-related family
Kl	Klotho
Lama2	laminin, alpha 2
Lama3	laminin, alpha 3
Lgals3bp	lectin, galactoside-binding, soluble
Lum	lumican
Lyz2	lysozyme 2
Mdfic	MyoD family inhibitor domain contain
Mdk	midkine
Mettl20	methyltransferase like 20
Mfrp	membrane frizzled-related protein
Mgp	matrix Gla protein
Mmp14	matrix metallopeptidase 14
Mmp2	matrix metallopeptidase 2
Mospd1	motile sperm domain containing 1
Mospd2	motile sperm domain containing 2
Mrc1	mannose receptor, C type 1
Mrpl52	mitochondrial ribosomal protein L52
Msx1	msh homeobox 1
Nid1	nidogen 1
Notch2	notch 2
Nt5dc2	5'-nucleotidase domain containing 2
Nudt7	nudix (nucleoside diphosphate linked moietyX)-type motif 7

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Ocln	occludin
Ogn	osteoglycin
Otx2	orthodenticle homeobox 2
Pacrg	Park2 co-regulated
Pbxip1	pre-B-cell leukemia homeobox interacting protein 1
Pcp4l1	Purkinje cell protein 4-like 1
Pdgfra	platelet derived growth factor receptor, alpha polypeptide
Pear1	platelet endothelial aggregation receptor 1
Pgpep1	pyroglutamyl-peptidase I
Pip5k1b	phosphatidylinositol-4-phosphate 5-kinase, type I, beta
Pla2g5	phospholipase A2, group V
Plagl1	pleiomorphic adenoma gene-like 1
Ppp1r36	protein phosphatase 1, regulatory subunit 36
Prelp	proline/arginine-rich end leucine-rich
Prlr	prolactin receptor
Ptgds	prostaglandin D2 synthase (brain)
Rab11fip1	RAB11 family interacting protein 1
Rbp1	retinol binding protein 1, cellular
Rbp4	retinol binding protein 4, plasma
Rdh10	retinol dehydrogenase 10 (all-trans)
Scgb1c1	secretoglobin, family 1C, member 1
Senp5	Sumo1/sentrin/SMT3 specific peptidase 5
Serpinb1a	serine/cysteine proteinase inhibitor, clade B, member 1a
Sfrp1	secreted frizzled-related protein
Slc13a4	sodium/sulfate symporters
Slc19a2	solute carrier family 19 (thiamine transporter), member 2
Slc31a1	solute carrier family 31, member 1
Slc4a2	solute carrier family 4, member 2
Slc5a5	solute carrier family 5 (sodium iodide symporter), member 5
Slc6a13	neurotransmitter transporter, GABA
Slc6a20	solute carrier family 6, member 20
Slco1a5	sol carrier organic anion transporter family, member 1a5
Sod3	superoxide dismutase 3, extracellular
Sostdc1	sclerostin domain containing 1
Sp1	Sp1 transcription factor
Spint2	Kunitz type serine peptidase inhibitor
St6gal1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1
Steap1	six transmembrane epithelial antigen
Stk39	serine threonine kinase 39
Stra6	stimulated by retinoic acid gene 6
Tbc1d9	TBC1 domain family, member 9
Tcn2	transcobalamin 2
Tgfbr3	transforming growth factor, beta receptor III
Tgm2	transglutaminase 2, C polypeptide
Timp3	TIMP metallopeptidase inhibitor 3
Tmem27	transmembrane protein 27
Tnnc2	troponin C type 2 (fast)
Tns1	tensin 1

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Tsc22d2	TSC22 domain family, member 2
Ttr	transthyretin
Tubb4b	tubulin, beta 4B class IVb
Vamp8	vesicle-associated membrane protein 8
Vav3	vav 3 guanine nucleotide exchange f
Vcl	vinculin
Vim	vimentin
Wls	wntless homolog (Drosophila)
Ythdf2	YTH domain family, member 2
Zeb2	zinc finger E-box binding homeobox 2
Zfp36l1	zinc finger protein 36, C3H type-like 1
Zic1	Zic family member 1
Zic4	Zic family member 4