

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

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28 **Abstract**

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

49

50 **Introduction**

51 The hippocampus plays a pivotal role in learning and memory (1). Gonadal steroids, including 17 β -
52 estradiol (E2), are powerful modulators of the hippocampal functions (2-4). Accordingly, E2 shapes
53 dendritic spine density of principal neurons during the estrous cycle (5), modulates neurogenesis (6,7),
54 alters neurotransmission (8), provides neuroprotection (9,10) and regulates the innate immune system
55 (11,12). In women, menopause is associated with increased risk of hippocampal decline and memory
56 disturbances (13,14). Estrogen replacement therapy (ERT) is effective in some, but not all women to
57 attenuate menopause-related memory disturbances. The effects of ERT on hippocampus and memory
58 are complex, and the effects depend on many parameters including estrogen responsiveness of the
59 individual, the chemical structure of the active estrogen compound, dosage and initiation of treatment,
60 among others (15). It has been proposed that a critical period exists for beneficial estrogen effects (16-
61 18). In accord with the hypothesis, perimenopausal and early postmenopausal hormone therapies are
62 often associated with improved cognitive functions later in life (19-22). However, the clinical practice
63 lacks an effective ERT to treat menopause-related decline of cognitive performance.

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

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

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

89

90 **Materials and Methods**

91 **Experimental animals**

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

104 2004 osmotic minipumps (DURECT, Cupertino, CA) filled either with E2 (0,33mg/ml in propylene-
105 glycol, n=8, E2 group) or vehicle only (n=8, control group) were implanted subcutaneously for 29
106 days in the scruff of the neck of the animals. We reported previously that these subcutaneous
107 treatments result 29.5 and 2.4 pg/mL serum E2 levels in E2- and vehicle-treated animals, respectively
108 (42). Body weight of control animals increased, while of E2-replaced animals decreased in accordance
109 with the anorexigenic effect of E2.

110 **Microarray using Affymetrix Rat Genome 230 PM Strip Arrays**

111 Hippocampi from 16 animals were prepared and total RNA was isolated and analyzed as
112 previously (12). RNA quality was measured by capillary electrophoresis using Nano RNA chips and
113 RNA integrity was determined by the software of 2100 Agilent Bioanalyzer (Santa Clara, CA, USA).
114 RNA samples displayed high RNA integrity numbers (RIN > 8.2). Eight samples were examined by
115 oligonucleotide microarray. Microarray experiments, including amplification, target labeling,
116 hybridization, staining and scanning, were carried out as described earlier (43). In brief, 25 ng of total
117 RNA Whole Transcriptome Amplification (WTA) library preparation and amplification for 17 cycles
118 were performed following the distributor's (Sigma-Aldrich) recommendations. 8 µg cDNA was
119 fragmented by DNaseI and biotinylated by terminal transferase obtained from the GeneChip Mapping
120 250K Nsp Assay Kit (Affymetrix Inc, Santa Clara, CA, USA). Hybridization, washing, staining and
121 scanning of Affymetrix Rat Genome 230 PM Strip arrays were performed following the
122 manufacturer's recommendations. Scanned images (DAT files) were transformed into intensities (CEL
123 files) using the AGCC software (Affymetrix). Data analysis, including GCRMA, statistical and data
124 mining work, were carried out as described earlier (42). Gene Ontology (GO) term enrichment was
125 analyzed using a public functional annotation tool (DAVID Bioinformatics Resources;
126 <http://david.abcc.ncifcrf.gov>) at default setting (44), but classification stringency was set to high.
127 Annotation clusters were ranked by their score number, termed enrichment score, calculated from the
128 modified Fisher's exact p-value of each GO-term.

129 **Real-time PCR Studies**

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

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

143

144 **Results**

145 **Oligonucleotide microarray revealed robust transcriptional activation in the hippocampus after** 146 **E2 replacement**

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

156 Nine genes, including transthyretin (*Ttr*), klotho (*Kl*), claudin (*Cldn2*), prolactin receptor
157 (*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 (*Colla2*), HT_{2C} receptor (*Htr2c*), secreted frizzled-related protein
162 (*Sfrp1*), α type VIII collagen (*Col8a2*), membrane frizzled-related protein (*Mfip*), 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 (*Colla1*).
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 metalloproteinase inhibitor (*Timp3*), aldehyde dehydrogenase (*Aldh1a2*), alpha-
182 2-macroglobulin (*A2m*), aquaporin 1 (*Aqp1*), *Colla1*, *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

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

187 **Classification of E2-induced genes**

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

191 *Cell adhesion*

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

196 *Cytoskeleton*

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

200 *Extracellular matrix (ECM)*

201 The ECM group composed of seventeen genes including six collagens (*Colla2*, *Col8a2*, *Col3a1*,
202 *Colla1*, *Col6a2*, *Col9a3*), four small leucine-rich proteoglycans (osteoglycin, decorin, lumican,
203 biglycan), two laminins (*Lama2*, *Lama3*), *Mgp*, prolargin (Prelp), cartilage associated protein (Crtap),
204 nidogen (Nid1) and fibulin-2 (Fbln2). Collagens and proteoglycans showed considerable changes in
205 response to E2.

206 *Immune*

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

210 *Metabolism*

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

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

217 *Signaling*

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

230 *Transcription*

231 This group comprised seven transcription factors (*Otx2*, TSC22 domain family *Tsc22d2*, ZIC family
232 member *Zic1* and *Zic4*, class E basic helix-loop-helix *Bhlhe40*, *Sp1*, butyrate response factor *Zfp3611*),
233 seven transcriptional modulators (*Msx1*, C2H2 Zn finger *Plagl1*, MyoD family inhibitor domain-
234 containing *Mdfic*, ETS domain-containing *Elk3*, ets variant *Etv3*, homeobox interacting protein
235 *Pbxip1*, homeobox protein *Zeb2*), as well as histone cluster 1 protein (*Hist1h4b*), and mRNA
236 stabilization factor ELAV-like protein (*Elavl1*).

237 **Results of real-time PCR study confirmed microarray data**

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

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

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

258

259 **Discussion**

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

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

272 **Transcriptional fingerprint of chronic E2 treatment in the hippocampus**

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

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

287 **E2 influences signaling mechanisms through genomic effects**

288 **1. Transthyretin and retinoid signaling**

289 Transthyretin transports retinol binding protein-retinol complex from the liver to target tissues.
290 It is expressed highly in the liver and the choroid plexus, but it is also present in the hippocampus
291 (49,50). Transthyretin is implicated in the maintenance of memory capacities as decreased
292 hippocampal expression of the gene is associated with the development of age-related memory
293 impairment (51). Transthyretin also sequesters beta-amyloid peptide (52). Therefore, robust increase
294 in *Ttr* expression following E2 replacement may lead to enhanced availability of retinoids and
295 accelerated clearance of neurotoxic peptides in the hippocampus. The rat and human *Ttr* genes

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

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

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

317 2. Klotho signaling

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

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

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

326 Insulin-like growth factor 2 plays a pivotal role in adult hippocampal functions such as
327 neurogenesis (68), memory consolidation and enhancement (69). Igf2 exerts its effects via the receptor
328 tyrosine kinase IGF-1R (70), which is strongly expressed in the hippocampus (71). The receptor is
329 often colocalized with the two ER subtypes (72). Through IGF-1R, Igf2 activates the ERK/MAPK and
330 PI3K-Akt signaling pathways resulting in phosphorylation and ligand-independent activation of ER α
331 (73,74). Association between ER- and IGF-1R- mediated signal transduction pathways is supported
332 further by a recent study demonstrating that subsequent IGF-1R antagonism terminates the memory
333 enhancing effect of short-term E2 treatment in OVX female rats (75). Based on the close linkage of
334 estrogen and insulin-like growth factor signaling, we propose that in the hippocampus, robust
335 activation of *Igf2* is one of the most important downstream events in E2 signaling.

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

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

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

349

350 **4. Neuropeptide signaling**

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

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

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

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

373 **5. Ectodin and Wnt signaling**

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

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

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

383 **6. Notch signaling**

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

389 **7. OTX2 homeoprotein signaling**

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

397 **E2 alters composition of the extracellular matrix**

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

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

412 **E2 modulates neurotransmission via gene expression**

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

414 HT_{2C} receptor is abundantly expressed in the CA1, CA2, CA3 pyramidal cell layer, and is also
415 expressed in the dentate gyrus (117). Serotonin regulates neuronal network excitability and modulates
416 adult neurogenesis in the hippocampus (118). Granule cells and interneurons in the dentate gyrus
417 receive serotonergic input from the brain stem (119). HT_{2C} is one of the receptor subtypes which
418 mediates the effect of serotonin on neurogenesis (119). Inhibition of the key enzyme of serotonin
419 synthesis, or lesion of serotonergic neurons decrease neurogenesis (120). Chronic use of fluoxetine
420 increases neurogenesis in adult rats (121,122). Strong evidence indicates the involvement of serotonin
421 in the neurogenic effects of E2 in the adult dentate gyrus (123). Serotonin dependence of physical
422 activity-induced adult hippocampal neurogenesis highlights further the importance of this mechanism
423 (124).

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

429 **2. GABAergic and glutamatergic neurotransmission**

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

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

439 3. **KCNE2**

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

448 **E2 tunes innate immune mechanisms**

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

453 **E2 may promote antioxidant mechanisms**

454 Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide are generated by
455 all cells during normal oxidative respiration. In the absence of control mechanisms, ROS cause
456 oxidative damage to proteins, lipids and DNA (137). It is known that E2 exerts antioxidant activity
457 (138,139). We demonstrated modest increase in the expression of extracellular superoxide dismutase,
458 glutathione S-transferases and glutathione peroxidase. Via transcriptional activation of *Sod3*, *Gstm2*,
459 *Gsta4* and *Gpx1*, E2 can modulate cellular respiration, scavenging of superoxide, catabolism of
460 hydrogen peroxide and metabolic detoxication of xenobiotics.

461 In summary, we provide evidence that the hippocampal transcriptome is highly responsive to
462 E2 replacement in middle-aged rats shortly after ovariectomy. E2 robustly activates transcription of
463 152 genes involved in key metabolic and signaling pathways, ECM structure, neurotransmission,
464 immune and antioxidant mechanisms. Main E2 target genes include transthyretin, klotho, insulin-like
465 growth factor 2, orthodenticle homeobox 2. The resulting expression profile suggests that E2
466 replacement evokes downstream events such as activation of retinoid, klotho, insulin-like growth
467 factor, Otx2 homeoprotein, serotonin signaling mechanisms, which promote plasticity, neurogenesis
468 and neuroprotection (**Fig. 3**). We propose that activation of the above mentioned mechanisms
469 contributes to the improvement of hippocampus-dependent memory performance after chronic
470 administration of E2 in middle-aged OVX rats (26,27,30-32). The findings provide further support for
471 the development of safe ER ligands to prevent memory disturbances and cognitive aging in
472 postmenopausal women.

473

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898 **Tables and Figures with Legends**

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

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901

902 Microarray analysis identified 156 E2-regulated genes. Expression of nine genes increased more than
 903 10-fold, and these were named prime target genes. Expression of twelve genes increased more than 4-
 904 fold but less than 10-fold, these were called substantial target genes. Probeset ID, code of Affymetrix
 905 Rat 230 Expression Array probeset; FC, fold change; p adj, adjusted p value.

906

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	<i>Ttr</i>	15,99	0,008	55,16	0,013
Rn00580123_m1	<i>Kl</i>	14,90	0,006	32,01	0,000
Rn02063575_s1	<i>Cldn2</i>	11,86	0,021	31,15	0,001
Rn01525459_m1	<i>Prlr</i>	35,22	0,000	29,47	0,001
Rn00596672_m1	<i>Sostdc1</i>	13,49	0,006	28,79	0,000
Rn01454518_m1	<i>Igf2</i>	7,474	0,017	12,05	0,002
Rn00565473_m1	<i>Igfbp2</i>	9,807	0,004	10,85	0,001
Rn01747911_m1	<i>Slc13a4</i>	7,210	0,001	10,71	0,001
Rn00591759_m1	<i>Folr1</i>	7,144	0,005	8,562	0,016
Rn00562748_m1	<i>Htr2c</i>	6,750	0,003	6,885	0,011
Rn01478472_m1	<i>Sfrp1</i>	5,926	0,002	6,092	0,081
Rn01414596_m1	<i>Otx2</i>	13,31	0,003	4,812	0,154
Rn00561094_m1	<i>Ace</i>	6,469	0,006	4,404	0,029
Rn00675549_g1	<i>Mdk</i>	4,573	0,003	4,180	0,108
Rn01505088_m1	<i>Enpp2</i>	4,749	0,003	4,128	0,000
Rn01463516_m1	<i>Slco1a5</i>	18,68	0,001	3,791	0,231
Rn01502044_g1	<i>Cdkn1c</i>	3,777	0,006	3,787	0,005
Rn00564605_m1	<i>Ptgds</i>	3,016	0,000	3,256	0,166
Rn02094913_s1	<i>Kcne2</i>	3,722	0,023	3,239	0,588
Rn00667535_m1	<i>Msx1</i>	3,152	0,003	2,801	0,200
Rn00592456_m1	<i>Slc6a13</i>	2,955	0,000	2,784	0,040
Rn00560589_m1	<i>A2m</i>	3,480	0,000	2,611	0,204
Rn00571516_m1	<i>Anxa2</i>	3,254	0,001	2,542	0,081
Rn00562834_m1	<i>Aqp1</i>	11,31	0,008	2,478	0,110
Rn01487342_m1	<i>Mrc1</i>	1,959	0,004	2,357	0,152
Rn00562884_m1	<i>Cox8b</i>	3,252	0,024	2,224	0,341
Rn01427989_s1	<i>Cdkn1a</i>	2,103	0,001	2,197	0,464
Rn00563570_m1	<i>Sod3</i>	1,874	0,002	2,081	0,089
Rn00568361_m1	<i>Crabp2</i>	1,999	0,004	1,942	0,407
Rn00588079_m1	<i>Aldh1a2</i>	3,651	0,000	1,902	0,155
Rn00710306_m1	<i>Igf1</i>	4,018	0,000	1,672	0,501
Rn00710727_m1	<i>Rdh10</i>	1,364	0,006	1,654	0,161
Rn00577994_g1	<i>Gpx1</i>	1,653	0,000	1,619	0,205
Rn00575617_m1	<i>Ctfr2</i>	11,418	0,001		
Rn01537468_g1	<i>Sgk1</i>	1,508	0,021		
Rn00582505_m1	<i>Slc12a2</i>	1,75	0,001		
Rn00587830_m1	<i>Slc17a7</i>	0,59	0,001		
Rn00824654_m1	<i>Slc32a1</i>	0,66	0,001		

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909

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.

916

917 **Legends to figures**

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

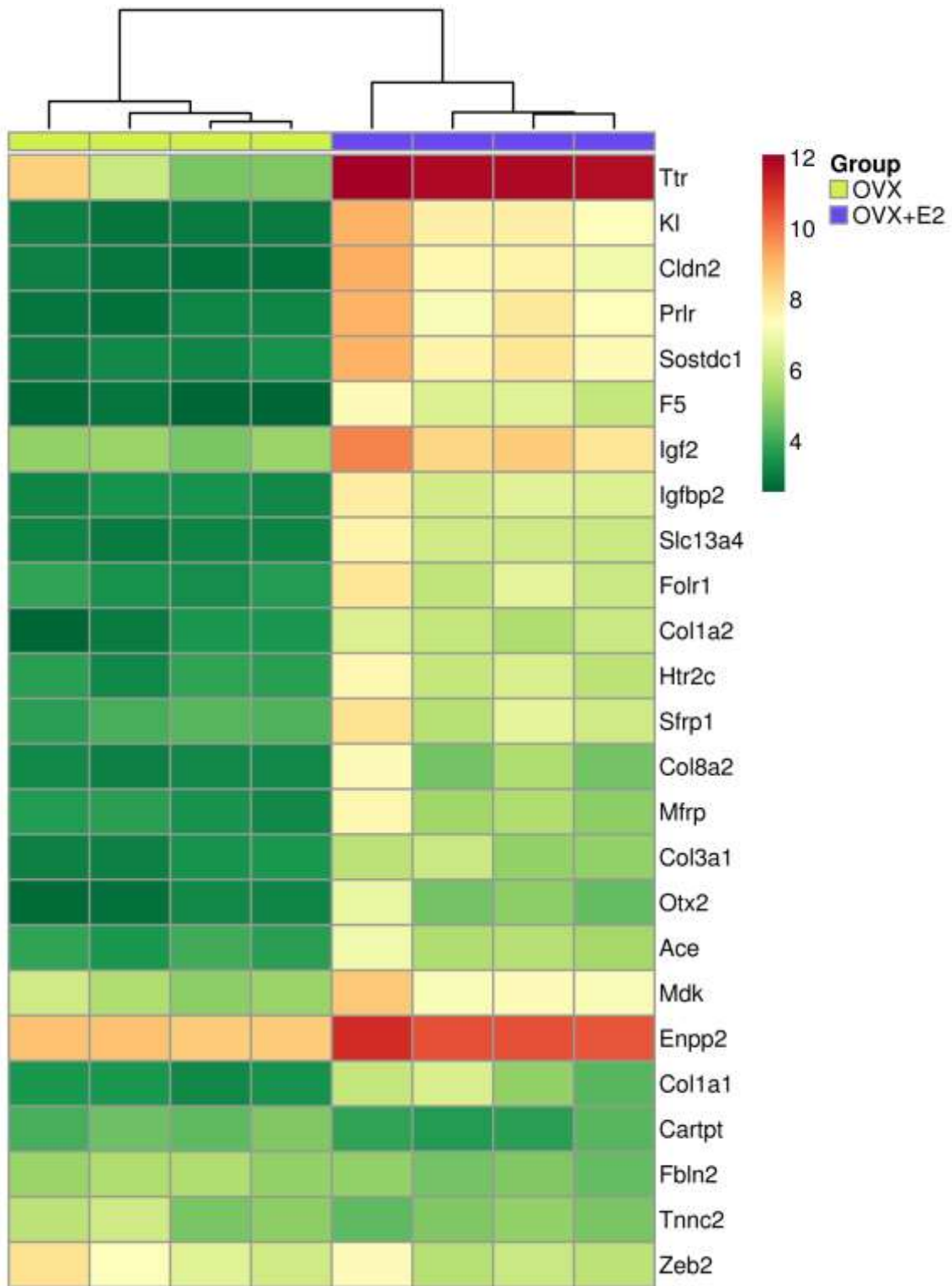
924 **Figure 2.** Classification of one hundred fifty-six genes revealed nine functional groups. These
925 groups include cell adhesion, cytoskeleton, ECM, immune, metabolism, miscellaneous, signaling,
926 transporter and transcription. Major functional groups are signaling and metabolism containing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

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

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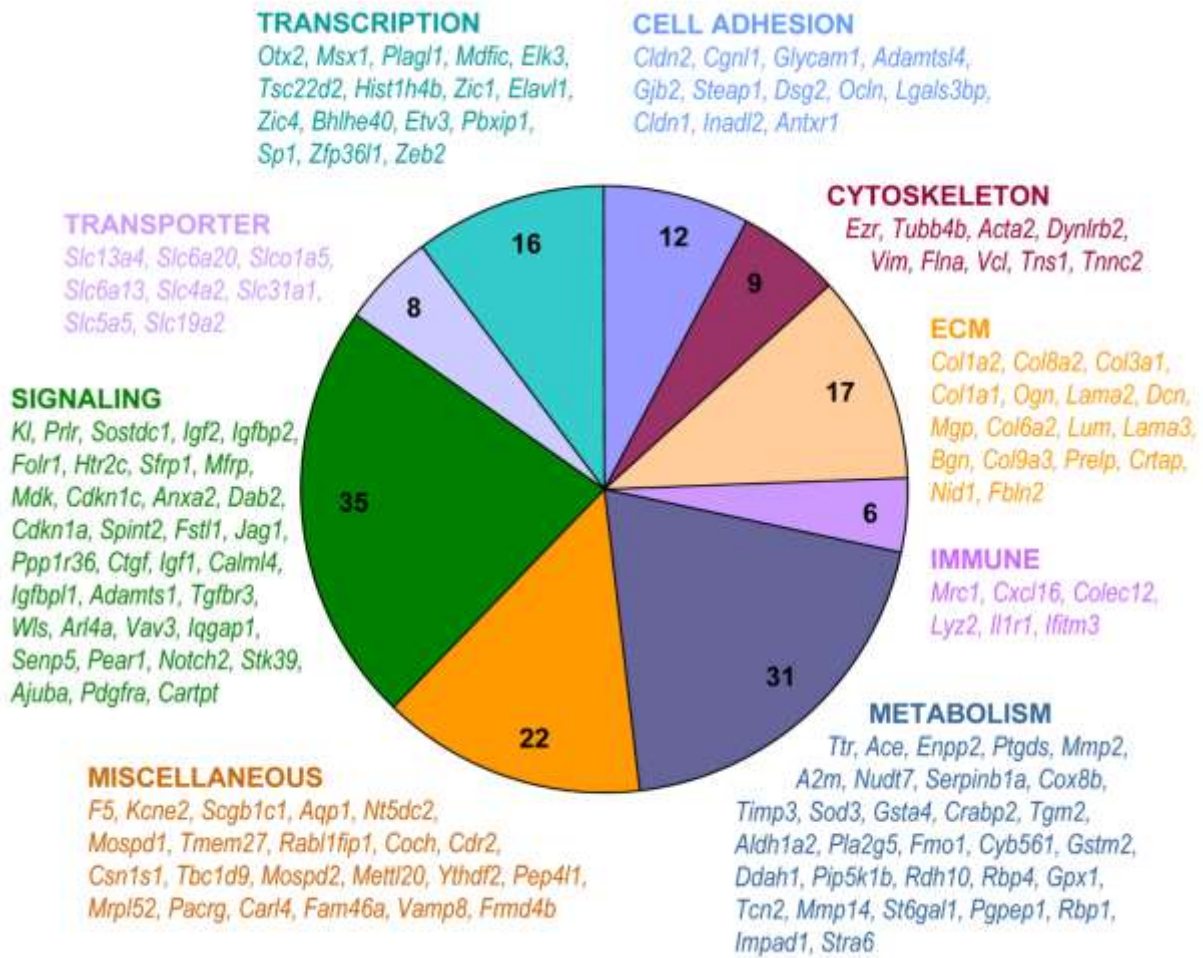
936 Figure 1



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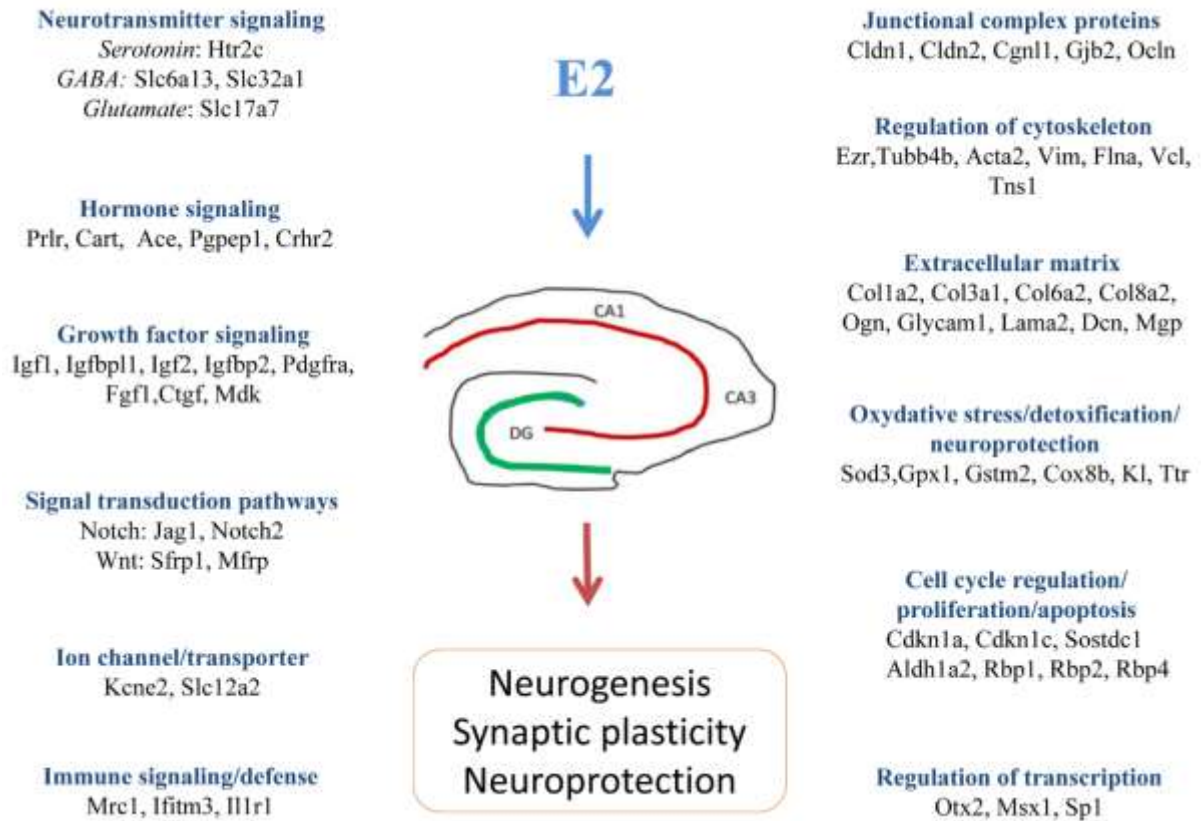
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949 **Supplemental Table 1** The list of E2-regulated genes in decreasing order

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

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

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1371847_PM_at	0,761	1,695	0,684	<i>Crtap</i>	cartilage associated protein
1370952_PM_at	0,760	1,694	0,515	<i>Gstm2</i>	glutathione S-transferase mu 2
1390937_PM_at	0,759	1,692	0,212	<i>Ppp1r36</i>	protein phosphatase 1, regulatory subunit 36
1367631_PM_at	0,758	1,691	0,049	<i>Ctgf</i>	connective tissue growth factor
1370333_PM_a_at	0,741	1,672	0,501	<i>Igf1</i>	insulin-like growth factor 1
1375026_PM_at	0,739	1,669	0,737	<i>Calml4</i>	calmodulin-like 4
1387111_PM_at	0,734	1,663	0,649	<i>Ddah1</i>	dimethylarginine dimethylaminohydrolase 1
1379352_PM_at	0,731	1,660	0,156	<i>Pip5k1b</i>	phosphatidylinositol-4-phosphate 5-kinase, type I, beta
1390715_PM_at	0,729	1,657	0,490	<i>Igfbpl1</i>	insulin-like growth factor binding protein-like 1
1393351_PM_at	0,726	1,654	0,161	<i>Rdh10</i>	retinol dehydrogenase 10 (all-trans)
1368223_PM_at	0,726	1,654	0,082	<i>Adamts1</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 1
1371518_PM_at	0,722	1,649	0,372	<i>Nid1</i>	nidogen 1
1372726_PM_at	0,713	1,639	0,495	<i>Hist1h4b</i>	histone cluster 1, H4b
1390912_PM_at	0,705	1,631	0,506	<i>Pcp4l1</i>	Purkinje cell protein 4-like 1
1388654_PM_at	0,702	1,627	0,230	<i>Mrp152</i>	mitochondrial ribosomal protein L52
1370750_PM_a_at	0,700	1,625	0,219	<i>Il1r1</i>	interleukin 1 receptor, type I
1369524_PM_at	0,698	1,622	0,154	<i>Zic1</i>	Zic family member 1
1367574_PM_at	0,697	1,621	0,311	<i>Vim</i>	vimentin
1371762_PM_at	0,695	1,619	0,480	<i>Rbp4</i>	retinol binding protein 4, plasma
1367576_PM_at	0,695	1,619	0,205	<i>Gpx1</i>	glutathione peroxidase 1
1387484_PM_at	0,692	1,616	0,418	<i>Tgfr3</i>	transforming growth factor, beta receptor III
1367960_PM_at	0,684	1,607	0,211	<i>Arl4a</i>	ADP-ribosylation factor-like 4A
1383129_PM_at	0,680	1,602	0,612	<i>Wls</i>	wntless homolog (Drosophila)
1377721_PM_at	0,678	1,600	0,501	<i>Pacrg</i>	Park2 co-regulated
1387995_PM_a_at	0,677	1,599	0,513	<i>Ifitm3</i>	interferon induced transmembrane p
1375892_PM_at	0,676	1,598	0,780	<i>Elavl1</i>	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
1369020_PM_at	0,675	1,597	0,441	<i>Slc5a5</i>	solute carrier family 5 (sodium iodide symporter), member 5
1367765_PM_at	0,673	1,594	0,273	<i>Tcn2</i>	transcobalamin 2
1392677_PM_at	0,669	1,589	0,204	<i>Zic4</i>	Zic family member 4
1393605_PM_at	0,660	1,580	0,328	<i>Vav3</i>	vav 3 guanine nucleotide exchange f
1379483_PM_at	0,658	1,578	0,850	<i>Bhlhe40</i>	basic helix-loop-helix family, member e40
1388762_PM_at	0,654	1,573	0,156	<i>Iqgap1</i>	IQ motif containing GTPase activating protein 1
1391262_PM_at	0,647	1,566	0,775	<i>Senp5</i>	Sumo1/sentrin/SMT3 specific peptidase 5
1388342_PM_at	0,645	1,564	0,868	<i>Etv3</i>	ets variant 3
1379868_PM_at	0,640	1,558	0,657	<i>Pbxip1</i>	pre-B-cell leukemia homeobox interacting protein 1
1373790_PM_at	0,638	1,556	0,345	<i>Car14</i>	carbonic anhydrase 14
1367860_PM_a_at	0,632	1,550	0,313	<i>Mmp14</i>	matrix metalloproteinase 14
1371880_PM_at	0,628	1,545	0,775	<i>Sp1</i>	Sp1 transcription factor
1370907_PM_at	0,628	1,545	0,205	<i>St6gal1</i>	ST6 beta-galactosamide alpha-2,6-sialyltransferase 1
1384591_PM_at	0,627	1,544	0,306	<i>Pear1</i>	platelet endothelial aggregation receptor 1
1394940_PM_at	0,626	1,544	0,566	<i>Fam46a</i>	family with sequence similarity 46, member A
1371382_PM_at	0,624	1,541	0,239	<i>Flna</i>	filamin A, alpha
1398362_PM_at	0,614	1,531	0,441	<i>Notch2</i>	notch 2
1372905_PM_at	0,611	1,527	0,814	<i>Vcl</i>	vinculin
1371954_PM_at	0,606	1,522	0,642	<i>Tns1</i>	tensin 1
1369970_PM_at	0,603	1,519	0,269	<i>Vamp8</i>	vesicle-associated membrane protein 8
1375215_PM_x_at	0,601	1,517	0,779	<i>Pgpep1</i>	pyroglutamyl-peptidase I
1387059_PM_at	0,599	1,514	0,449	<i>Stk39</i>	serine threonine kinase 39

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1367939_PM_at	0,597	1,513	0,164	<i>Rbp1</i>	retinol binding protein 1, cellular
1373122_PM_at	0,595	1,511	0,219	<i>Ajuba</i>	ajuba LIM protein
1396109_PM_at	0,595	1,511	0,649	<i>Antxr1</i>	anthrax toxin receptor 1
1395036_PM_at	0,594	1,510	0,608	<i>Impad1</i>	inositol monophosphatase domain containing 1
1390525_PM_a_at	0,593	1,509	0,600	<i>Stra6</i>	stimulated by retinoic acid gene 6
1369959_PM_at	0,592	1,508	0,468	<i>Zfp36l1</i>	zinc finger protein 36, C3H type-like 1
1390464_PM_at	0,591	1,506	0,863	<i>Frm4b</i>	FERM domain containing 4B
1390863_PM_at	0,590	1,505	0,570	<i>Slc19a2</i>	solute carrier family 19 (thiamine transporter), member 2
1370941_PM_at	0,589	1,504	0,110	<i>Pdgfra</i>	platelet derived growth factor receptor, alpha polypeptide
1368585_PM_at	-0,583	0,667	0,509	<i>Cartpt</i>	CART prepropeptide
1389533_PM_at	-0,615	0,653	0,398	<i>Fbln2</i>	fibulin 2
1372195_PM_at	-0,667	0,630	0,779	<i>Tnnc2</i>	troponin C type 2 (fast)
956 1393795_PM_at	-0,733	0,602	0,862	<i>Zeb2</i>	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	
	response to steroid hormone	0.002		Timp3, Aldh1a2, A2m, Aqp1, Col1a1,
	response to hormone stimulus	0.012		Cdkn1a, Gpx1, Igf1, Igf2, Igfbp2, Mgp,
	response to endogenous stimulus	0.024		Mmp14, Mdk, Pdgfra, Ptgds, Rbp4
2	Pattern binding		3.950	
	glycosaminoglycan binding	0.004		Adamtsl4, Bgn, Cln12, Ctgt, Dcn, Flstl1,
	pattern binding	0.006		Mrc1, Mdk, Pla2, Tgfbr3
	polysaccharide binding	0.006		
	heparin binding	0.010		
3	Cell motility		3.726	
	regulation of locomotion	0.009		Htr2c, Ace, Cxcl16, Enpp2, Igf1, Jub,
	regulation of cell migration	0.011		Lama2, Lama3, Pdgfra, Tgfbr3, Vcn
	regulation of cell motion	0.020		
4	Development		3.368	
	lung development	0.020		Sp1, Aldh1a2, Ctgf, Mgp, Mmp14, Pdgfra,
				Rbp4, Rdh10
5	Retinoid		3.195	
	vitamin A	0.001		Aldh1a2, Rbp2, Ptgds, Rbp1, Rbp4, Ttr
	retinoid binding	0.007		
	isoprenoid binding	0.010		
	retinol binding	0.050		
6	Collagen		2.085	
	fibrillar collagen	0.013		Anxa2, Col1a1, Col1a2, Col3a1, Cln12,
	collagen	0.050		Lum

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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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972 **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	

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

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symbol	gene name
<i>A2m</i>	alpha-2-macroglobulin
<i>Ace</i>	angiotensin converting enzyme
<i>Acta2</i>	smooth muscle alpha-actin
<i>Adamts1</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 1
<i>Adamtsl4</i>	ADAMTS-like 4
<i>Ajuba</i>	ajuba LIM protein
<i>Aldh1a2</i>	aldehyde dehydrogenase 1 family
<i>Antxr1</i>	anthrax toxin receptor 1
<i>Anxa2</i>	annexin A2
<i>Aqp1</i>	aquaporin 1
<i>Arl4a</i>	ADP-ribosylation factor-like 4A
<i>Bgn</i>	biglycan
<i>Bhlhe40</i>	basic helix-loop-helix family, member e40
<i>Calml4</i>	calmodulin-like 4
<i>Car14</i>	carbonic anhydrase 14
<i>Cartpt</i>	CART prepropeptide
<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A
<i>Cdkn1c</i>	cyclin-dependent kinase inhibitor 1C
<i>Cdr2</i>	cerebellar degeneration-related 2
<i>Cgnl1</i>	cingulin-like 1
<i>Cldn1</i>	claudin 1
<i>Cldn2</i>	claudin 2
<i>Coch</i>	coagulation factor C homolog, cochlin
<i>Col1a1</i>	collagen, type I, alpha 1
<i>Col1a2</i>	collagen, type I, alpha 2
<i>Col3a1</i>	collagen, type III, alpha 1
<i>Col6a2</i>	collagen, type VI, alpha 2
<i>Col8a2</i>	collagen, type VIII, alpha 2
<i>Col9a3</i>	procollagen, type IX, alpha 3
<i>Colec12</i>	collectin sub-family member 12
<i>Cox8b</i>	cytochrome c oxidase, subunit VIIIb
<i>Crabp2</i>	cellular retinoic acid binding protein 2
<i>Crtap</i>	cartilage associated protein
<i>Ctgf</i>	connective tissue growth factor
<i>Cxcl16</i>	chemokine (C-X-C motif) ligand 16
<i>Cyb561</i>	cytochrome b-561
<i>Csn1s1</i>	casein alpha s1
<i>Dab2</i>	disabled homolog 2 (Drosophila)
<i>Dcn</i>	decorin
<i>Ddah1</i>	dimethylarginine dimethylaminohydrolase 1
<i>Dsg2</i>	desmoglein 2
<i>Dynlrb2</i>	dynein light chain roadblock-type 2
<i>Elav1</i>	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
<i>Elk3</i>	ELK3, ETS-domain protein
<i>Enpp2</i>	ectonucleotide pyrophosphatase
<i>Etv3</i>	ets variant 3
<i>Ezr</i>	ezrin

<i>F5</i>	coagulation factor V
<i>Fam46a</i>	family with sequence similarity 46, member A
<i>Fbln2</i>	fibulin 2
<i>Flna</i>	filamin A, alpha
<i>Fmo1</i>	flavin containing monooxygenase 1
<i>Folr1</i>	folate receptor 1 (adult)
<i>Frm4b</i>	FERM domain containing 4B
<i>Fstl1</i>	follicle-stimulating-like 1
<i>Gjb2</i>	gap junction protein, beta 2
<i>Glycam1</i>	glycosylation dependent CAM 1
<i>Gpx1</i>	glutathione peroxidase 1
<i>Gsta4</i>	glutathione S-transferase alpha 4
<i>Gstm2</i>	glutathione S-transferase mu 2
<i>Hist1h4b</i>	histone cluster 1, H4b
<i>Htr2c</i>	5-HT receptor 2C
<i>Ifitm3</i>	interferon induced transmembrane p
<i>Igf1</i>	insulin-like growth factor 1
<i>Igf2</i>	insulin-like growth factor 2
<i>Igfbp2</i>	igf binding protein 2
<i>Igfbpl1</i>	insulin-like growth factor binding protein-like 1
<i>Il1r1</i>	interleukin 1 receptor, type I
<i>Impad1</i>	inositol monophosphatase domain containing 1
<i>Inadl2</i>	InaD-like 2 (Drosophila)
<i>Iqgap1</i>	IQ motif containing GTPase activating protein 1
<i>Jag1</i>	jagged 1
<i>Kcne2</i>	K ⁺ channel, Isk-related family
<i>Kl</i>	Klotho
<i>Lama2</i>	laminin, alpha 2
<i>Lama3</i>	laminin, alpha 3
<i>Lgals3bp</i>	lectin, galactoside-binding, soluble
<i>Lum</i>	lumican
<i>Lyz2</i>	lysozyme 2
<i>Mdfic</i>	MyoD family inhibitor domain contain
<i>Mdk</i>	midkine
<i>Mettl20</i>	methyltransferase like 20
<i>Mfrp</i>	membrane frizzled-related protein
<i>Mgp</i>	matrix Gla protein
<i>Mmp14</i>	matrix metalloproteinase 14
<i>Mmp2</i>	matrix metalloproteinase 2
<i>Mospd1</i>	motile sperm domain containing 1
<i>Mospd2</i>	motile sperm domain containing 2
<i>Mrc1</i>	mannose receptor, C type 1
<i>Mrpl52</i>	mitochondrial ribosomal protein L52
<i>Msx1</i>	msh homeobox 1
<i>Nid1</i>	nidogen 1
<i>Notch2</i>	notch 2
<i>Nt5dc2</i>	5'-nucleotidase domain containing 2
<i>Nudt7</i>	nudix (nucleoside diphosphate linked moietyX)-type motif 7

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<i>Ocln</i>	occludin
<i>Ogn</i>	osteoglycin
<i>Otx2</i>	orthodenticle homeobox 2
<i>Pacrg</i>	Park2 co-regulated
<i>Pbxip1</i>	pre-B-cell leukemia homeobox interacting protein 1
<i>Pcp4l1</i>	Purkinje cell protein 4-like 1
<i>Pdgfra</i>	platelet derived growth factor receptor, alpha polypeptide
<i>Pear1</i>	platelet endothelial aggregation receptor 1
<i>Pgpep1</i>	pyroglutamyl-peptidase I
<i>Pip5k1b</i>	phosphatidylinositol-4-phosphate 5-kinase, type I, beta
<i>Pla2g5</i>	phospholipase A2, group V
<i>Plagl1</i>	pleiomorphic adenoma gene-like 1
<i>Ppp1r36</i>	protein phosphatase 1, regulatory subunit 36
<i>Prelp</i>	proline/arginine-rich end leucine-rich
<i>Prlr</i>	prolactin receptor
<i>Ptgds</i>	prostaglandin D2 synthase (brain)
<i>Rab11fip1</i>	RAB11 family interacting protein 1
<i>Rbp1</i>	retinol binding protein 1, cellular
<i>Rbp4</i>	retinol binding protein 4, plasma
<i>Rdh10</i>	retinol dehydrogenase 10 (all-trans)
<i>Scgb1c1</i>	secretoglobin, family 1C, member 1
<i>Senp5</i>	Sumo1/sentrin/SMT3 specific peptidase 5
<i>Serpinh1a</i>	serine/cysteine proteinase inhibitor, clade B, member 1a
<i>Sfrp1</i>	secreted frizzled-related protein
<i>Slc13a4</i>	sodium/sulfate symporters
<i>Slc19a2</i>	solute carrier family 19 (thiamine transporter), member 2
<i>Slc31a1</i>	solute carrier family 31, member 1
<i>Slc4a2</i>	solute carrier family 4, member 2
<i>Slc5a5</i>	solute carrier family 5 (sodium iodide symporter), member 5
<i>Slc6a13</i>	neurotransmitter transporter, GABA
<i>Slc6a20</i>	solute carrier family 6, member 20
<i>Slco1a5</i>	sol carrier organic anion transporter family, member 1a5
<i>Sod3</i>	superoxide dismutase 3, extracellular
<i>Sostdc1</i>	sclerostin domain containing 1
<i>Sp1</i>	Sp1 transcription factor
<i>Spint2</i>	Kunitz type serine peptidase inhibitor
<i>St6gal1</i>	ST6 beta-galactosamide alpha-2,6-sialyltransferase 1
<i>Steap1</i>	six transmembrane epithelial antigen
<i>Stk39</i>	serine threonine kinase 39
<i>Stra6</i>	stimulated by retinoic acid gene 6
<i>Tbc1d9</i>	TBC1 domain family, member 9
<i>Tcn2</i>	transcobalamin 2
<i>Tgfb3</i>	transforming growth factor, beta receptor III
<i>Tgm2</i>	transglutaminase 2, C polypeptide
<i>Timp3</i>	TIMP metalloproteinase inhibitor 3
<i>Tmem27</i>	transmembrane protein 27
<i>Tnnc2</i>	troponin C type 2 (fast)
<i>Tns1</i>	tensin 1

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