

1 **Ovariectomy Alters Gene Expression of the Hippocampal Formation**
2 **in Middle-Aged Rats**
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28 **Abstract**

29 Ovarian hormones regulate the transcriptome of the hippocampus and modulate its functions. During
30 menopause, this complex signaling declines, leading to impaired learning and memory. This study was
31 undertaken to clarify the effects of long-term, surgical ovariectomy (OVX) on the rat hippocampal
32 transcriptome. At age of 13 months, intact control and ovariectomized groups were formed. All animals
33 were sacrificed 5 weeks after gonadectomy, hippocampal formations were dissected and processed for
34 transcriptome analysis. Microarray and PCR studies identified 252 and 61 genes, respectively, whose
35 expression was altered in the lack of ovarian hormones. Pathway analysis revealed impact on
36 neuroactive ligand-receptor interaction, endocannabinoid and estrogen signaling, among others.
37 Network and interaction analyses of proteins encoded by OVX-regulated genes revealed upregulation
38 of growth/ troph/transcription factor signaling assembly (*Mdk, Fgf1, Igf2, Ngf, Ngfr, Ntf3, Ntrk1, Otx2,*
39 *Hif1a, Esr1, Nr4a3*), peptides/peptide receptors (*Cartp, Kl, Ttr, Gnhr*), neurotransmission (*Grm1,*
40 *Gria4, Glis, Slc18a2, Kcj6*) and genes serving immune functions (*C3, Ccl2, Itgam, Il1b*). Downregulated
41 clusters included neuropeptides and their receptors (*Adcyap1, Cbln2, Cck, Cckbr, Crhr1 and 2, Oprd1,*
42 *Nts, Penk, Sstr1, Vip*), neurotransmitter signaling (*Htr2c, Chrna3, Chrm4, Grm8, Hrh3, Slc17a6*) and
43 potassium channels (*Kcnk9, Kcnj9, Kcnma1, Kcnc2*). Several transcription factors (*Rxra, Thrb*), solute
44 carriers and defense molecules (*Apitd1, Bcl2, Clql3, Ilr3a, Sod1, Sncb*) also underwent downregulation.
45 The findings indicate that surgical gonadectomy carried out at middle-age robustly changes the
46 hippocampal transcriptome that alters neurogenesis, synaptic plasticity, immune modulation causing
47 cognitive dysfunctions.

48

49 **Introduction**

50 The ovarian hormone supply is crucial for development of the brain and maintenance of its diverse
51 functions (1-3). The proper physiological performance of the ovary is guaranteed and regulated by the
52 hypothalamo-pituitary neuroendocrine unit (4). Aging results in a decrease in the production of ovarian
53 hormones, including the gonadal sex steroids estradiol and progesterone (5,6). During menopause, the
54 ovarian hormone supply to the brain, including the hippocampal formation, gradually weakens causing
55 impairments in learning ability, memory processing and spatial navigation (7,8). These events
56 negatively influence quality of life in menopause, therefore, various strategies of hormone replacement
57 therapy (HRT) have been introduced (9,10). Current efforts are aimed at HRTs with low health risk
58 consequence and high effectiveness (11-13). The timing of HRT has been found crucial (14,15) in order
59 to keep the brain's responsiveness to ovarian hormones and prevent structural disintegration of the
60 neural tissue. In experimental neuroscience, middle-aged, ovariectomized animals have been widely
61 used as models of menopause including primates (16,17) and rodents (18-23). The beneficial role of
62 HRT has been shown in counter-balancing hippocampus-related dysfunctions. These studies indicate
63 that declining ovarian hormone signaling to the hippocampal formation promotes neuroinflammation
64 (24), disturbs synaptic signaling and plasticity (25-29), alters neurogenesis (26,30,31) and decreases the
65 efficacy of cellular neuroprotective mechanisms (32-34).

66 Although many HRT studies used the ovariectomized animal model, the impact of surgical, long-term
67 OVX *per se* upon the hippocampal transcriptome and functions has not been fully elucidated yet. We
68 hypothesized that ablation of ovarian hormones by gonadectomy in middle-aged, female rats results in
69 malfunction of the related hormone receptors leading to modification of gene expression and altered
70 functions. Therefore, in this study, the hippocampal transcriptomes of intact control and long-term
71 ovariectomized, middle-aged rats were compared using microarray and quantitative real-time PCR. The
72 predicted networking of proteins encoded by OVX-regulated genes was also analyzed. We identified
73 clusters of differentially-regulated genes, indicating that gonadal hormone ablation might alter essential
74 hippocampal functions.

75

76 **Material and Methods**

77 **Experimental animals**

78 All experiments were performed with permission from the Animal Welfare Committee of the Institute
79 of Experimental Medicine (IEM, Permission Number: A5769-01) and in accordance with regulations of
80 the European Community (Decree 86/609/EEC). Female Harlan-Wistar rats were purchased from
81 Toxicoop (Budapest, Hungary) and housed on a 12h light/12h dark cycle in the animal care facility of
82 IEM. The rats were used as breeders and retired at their age of 8 months, then housed individually for
83 the subsequent months. At their age of 13 months, 14 animals were deeply anesthetized and sham-
84 operated or ovariectomized bilaterally (18,35). Two experimental animal groups were formed: intact
85 control group (n=6) and ovariectomized group (n=8). Both groups were kept on phytoestrogen-free diet
86 (Harlan Teklad Global Diets, Madison, WI). Intact middle-aged female rats show the initial signs of
87 reproductive aging manifested in persistent vaginal cornification, tonic estrogen secretion and low
88 plasma levels of progesterone (36). Accordingly, our middle-aged intact females had low serum E2 level
89 (12 pg/mL). In contrast, ovariectomized middle-aged animals had only residual serum E2 (2 pg/mL),
90 significantly decreased uterus weight and significantly increased body weight indicating progressing
91 reproductive senescence and clear signs of menopause. Five weeks after the surgical intervention all
92 animals were terminated, the brains were quickly removed from the skull and placed into an ice-cold rat
93 brain matrix. A three millimeter thick coronal slice was dissected with two blades positioned at bregma
94 levels -3.8 and -6.8. Between these levels the targeted part of the hippocampal formation was easily
95 separable from the neighboring brain areas (the thalamus and the corpus callosum), it was lifted from
96 the slice and processed for transcriptome analysis.

97 *Affymetrix Rat Genome 230PM Strip Arrays*

98 Hippocampal formations from 14 animals were prepared and total RNA was isolated and analyzed as
99 described previously (24). RNA quality was measured and samples displayed high RNA integrity
100 numbers (RIN > 8.2) on Agilent's Bioanalyzer Nano RNA chips (Santa Clara, CA, USA). Four samples
101 from each group were individually examined by oligonucleotide microarray including amplification,
102 target labeling, hybridization, staining and scanning steps, which were carried out as described earlier
103 (26). In brief, using 25 ng of total RNA Whole Transcriptome Amplification (WTA) library preparation

104 and amplification for 17 cycles were performed following distributor's (Sigma-Aldrich)
105 recommendations. 8 µg cDNA was fragmented by DNase I and biotinylated by terminal transferase
106 obtained from the GeneChip Mapping 250K Nsp Assay Kit (Affymetrix Inc, Santa Clara, CA, USA).
107 Hybridization, washing, staining and scanning of Affymetrix Rat Genome 230 PM Strip arrays were
108 performed following the manufacturer's recommendations. Scanned images (DAT files) were
109 transformed into intensities (CEL files) using the AGCC software (Affymetrix).

110 **Quantitative real-time PCR**

111 Custom TaqMan microfluidic cards (Applied Biosystems, Foster City, CA, USA) were designed to
112 study mRNA expression by real-time PCR. Fourteen samples were examined. Reverse transcription and
113 PCR were carried out by using Applied Biosystems' High Capacity cDNA Reverse Transcription Kit
114 and TaqMan Universal PCR Master Mix II, respectively. PCR data evaluation were performed as
115 described previously (24). The ViiA7 RUO 1.2.1 (Applied Biosystems) software and relative
116 quantification against calibrator samples ($\Delta\Delta Ct$) were used for data evaluation. Intact control was the
117 calibrator sample. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine
118 phosphoribosyl-transferase (*Hprt*) were used as housekeeping genes. Expression of these genes did not
119 vary among experimental groups. A computed internal control corresponding to the geometric mean of
120 cycle threshold (Ct) values of *Gapdh* and *Hprt* was used for ΔCt calculation. Relative quantity (RQ)
121 was calculated according to $RQ=2^{(-\Delta\Delta Ct)}$ equation.

122 **Data analysis**

123 Microarray data analysis, including GC robust multi-array average (GCRMA), statistical and data
124 mining work, were carried out as published earlier (25,26). In brief, raw data were preprocessed for
125 analysis using GCRMA. For selection of differentially expressed genes, fold change values were used
126 and linear models combined with empirical Bayesian methods were applied. Obtained p-values were
127 adjusted by the false discovery rate (FDR)-based method. Genes that met the selection criterion of fold
128 change (FC) >1.5 were considered OVX-regulated. For statistical analysis of real-time PCR results we
129 used one-way ANOVA. Pathway analysis was constructed by using the KEGG database;
130 <http://www.genome.jp/kegg>). Putative protein-protein interactions were evaluated by the web-based
131 STRING 10 platform (<http://string-db.org>), as reported previously (25).

132

133 **Results**

134 **Modification of hippocampal transcriptome in response to long-term OVX**

135 *Microarray study*

136 The comparison of hippocampal transcriptomes from middle-aged intact (M) and middle-aged
137 ovariectomized (M-OVX) rats revealed that chronic ablation of gonadal hormones by surgical OVX
138 results in differential expression of genes. At FC > 1.5 criterion, more than 200 genes were differentially
139 regulated. Downregulated genes (145) outnumbered upregulated (107) ones. The full list of genes is
140 shown in **Supplemental Table 1** providing the probe set ID, gene symbol and name, corresponding FC
141 and adjusted P values. Top up- and downregulated genes selected at FC >1.7 are listed in **Table 1**. Top
142 upregulated genes encode peptides like cocaine- and amphetamine-regulated transcript (*Cartpt*) and
143 transthyretin (*Ttr*), peptidases (*Sppl2a*, *Usp25*, *Prcp*), adhesion proteins (*Vcl*, *Pcdh20*), proteo- and
144 glucosaminoglycans (*Spock3*, *Fndc3a*), ion channel (*Trpm7*), signaling molecule (*Ptpn3*) and
145 transcription factors (*Hif1a*, *Klf3*) among others. In the top downregulated category, differentially
146 regulated genes encode potassium channels (*Kcnma1*, *Kcnj9*, *Kcnk9*), serotonin receptor (*Htr2c*),
147 neuropeptide (*Adcyap1*), members of G-protein coupled signaling mechanisms (*Gpr123*, *Rgs17* and
148 *Rgs4*), retinoid acid signaling molecule (*Crabp1*), components of growth factor signaling (*Igfbp4*, *Nov*),
149 members of solute carrier family (*Slc39a7*, *Slc17a6*) and transcription factor (*Etv1*).

150 *Quantitative real-time PCR study*

151 In this study, the putative differential expression of 62 genes was examined by TaqMan-based PCR.
152 Target selection was aimed at validation of the microarray result and also getting further insight to basic
153 molecular mechanisms of neuronal networks of the hippocampus affected by OVX with special
154 reference to synaptic plasticity, neurogenesis and immune modulation. The PCR results are listed in
155 **Table 2** and grouped in the aforementioned functional categories. In the clusters of synaptic plasticity
156 and neurogenesis, both up- and downregulated genes were noted, on the other hand, most defense genes
157 were upregulated. Neuropeptide and neurotransmitter signaling mechanisms shape neuronal plasticity
158 of the hippocampal formation, accordingly, a large number of genes encoding various components of
159 these signaling pathways were seen robustly changed by long-term OVX. In case of peptide signaling,

160 *Cartpt, Ttr, Cck, Vip* and *Penk* were involved. Altered expression of genes encoding neuropeptide
161 receptors was revealed for *Crh1r* and *Crh2r, Gnhr, Cckbr, Sstr1* and *Oprd1*. Regarding the involvement
162 of classic neurotransmitter signaling mechanisms, differential expression was found in case of
163 cholinergic (*Chrm4, Chrna3*), glutamatergic (*Grm8, Slc17a6*), serotonergic (*Htr2c*) and histaminergic
164 (*Hrh3*) systems. Neurogenesis in the subgranular layer of the hippocampal formation is highly regulated
165 by growth and troph factors, and this process is mirrored in changes of transcriptional activity. OVX
166 influences both mechanisms with a particular strong impact on growth/troph hormone signaling. To the
167 most important contributors of this cluster belong nerve growth factor (*Ngf, Ngfr, Ntf3, Ntrk*), insulin-
168 like growth factor (*Igf2, Igfbp4*) and fibroblast growth factor (*Fgf1*) signaling systems. Regarding the
169 regulation of transcription, *Otx2* as classical transcription factor, whereas *Esr1, Rxra* and *Thrb* as ligand-
170 activated nuclear transcription factor genes responded to OVX. Alteration in the expression of *Sgk1*
171 mRNA has extraordinary importance because of its wide-based regulatory potential. The gene cluster
172 affiliated with processes of immune response and defense, covered predominantly upregulated genes
173 like *C3, Il1b, Tlr3, Tlr4, RT1-A1, Ccl2* and *Aif1* among others.

174 *KEGG pathway analysis of OVX-regulated genes*

175 The top KEGG pathways changing in response to chronic OVX in middle-aged rats were summarized
176 in **Table 3**. Neuroactive ligand-receptor interaction (ID: 4080, counts: 15), retrograde endocannabinoid
177 signaling (ID: 4723, counts: 9), transcriptional misregulation in cancer (ID: 5202, counts 10), apoptosis
178 (ID: 4210, counts: 7) and proteoglycans in cancer (ID: 5205, counts 11) pathways appeared at false
179 discovery rate lesser than 0.005. Estrogen (ID: 4915) and thyroid (ID:4919) hormone signaling pathways
180 also emerged. The influence on cholinergic (ID: 4725), glutamatergic (ID: 4724), adrenergic (ID: 4262)
181 and serotonergic (ID: 4726) signaling pathways was also raised.

182 **Predicted networking of hippocampal proteins encoded by OVX-regulated genes**

183 In order to elucidate the interrelationship of genes, the predicted interaction and networking of proteins
184 encoded by OVX-regulated changes were performed using the STRING 10 platform. The analysis was
185 based on microarray and PCR results, carried out at confidence value 0.6. and the non-interacting genes
186 were omitted. The networking of proteins coded by OVX-regulated genes is depicted in **Supplementary**
187 **Figure 1**. Clusters of peptides serving neuropeptide and neurotransmitter signaling are explicit, similar

188 to protein assemblies regulating transcription. The OVX-regulated genes were sorted into up- and
189 downregulated clusters (**Tables 4 and 5**) and the STRING analysis was implemented for both groups
190 (**Figure 1 and 2**). Thirty-four upregulated, interacting proteins were sorted into 3 functional categories
191 including growth/troph factors/transcription regulation, peptides/transmitters/ion channels/signaling
192 and immune response/defense (**Table 4**). The downregulated group consisted of 47 proteins that were
193 grouped in 4 operative categories such as peptides/transmitters/receptors/ion channels/signaling, solute
194 carriers, growth factors/transcription regulation and immune response/defense (**Table 5**).

195

196 **Discussion**

197 **OVX modulates neurotransmission**

198 *Modulation of peptidergic signaling*

199 Ablation of the ovaries affected interneurons of the hippocampal formation that responded mainly by
200 downregulation of neuropeptide expression and also some of their genuine receptors. Cholecystokinin
201 (Cck), neurotensin (Nts), vasoactive intestinal peptide (Vip) and preproenkephalin (Penk) are well-
202 established and characterized constituents of certain hippocampal interneurons (37-45). Cck exerts
203 excitatory role on CA1 pyramidal neurons (46,47) via its B type receptor that was also altered after
204 OVX. Vip also increases excitatory transmission to CA1 pyramidal neurons by disinhibition of their
205 dendrites (48). Nts targets interneurons enhancing GABAergic activity by modulating L-type calcium
206 channels (49). OVX influences the opioid peptide signal transduction in the hippocampus as exemplified
207 by decreased expression genes encoding Penk and its cognate receptor Oprd1. Enkephalins are known
208 to modify the activity of hippocampal circuits (50,51). Opioid inhibition of GABA release from terminal
209 boutons of interneurons has previously been reported (52). These changes suggest that OVX modulates
210 interneuron function via downregulation of neuropeptides resulting in declining excitatory transmission
211 in the hippocampus. *Adcyap1* (PACAP) derives from both interneurons and principal neurons of the
212 hippocampal formation. It exerts effects on CA1 pyramidal neurons by inhibiting the slow after-
213 hyperpolarizing current (53). Increased PACAP level may also serve neurogenesis (54,55) and support
214 neuroprotection (56,57). Accordingly, decreased *Adcyap1* expression can affect the function of principal
215 neurons, neurogenesis and protective mechanisms. Marked downregulation of *Adcyap1* can't be restored

216 by estradiol replacement (26). Decreased levels of type 1 and 2 CRH receptors indicate alteration in
217 CRH signaling. Activation of these receptors has been reported to reduce the amplitude of hippocampal
218 population spike and prevent the onset of long-term potentiation (LTP) (58). Downregulation of genes
219 for CRHR subtypes decreases CRH-CRHR signaling and the impact of stress response on LTP. Estradiol
220 replacement significantly increases *Crhr2* expression.

221 Regarding the upregulated category, the peptides CART, transthyretin, klotho and the *Gnrhr* were
222 involved. The increase of CART and klotho seems to be advantageous for the hippocampus because of
223 their renowned pro-cognitive effects (59,60). Increase in activation of *Gnrhr* may result in excitation of
224 pyramidal neurons (61,62) although data on the expression of its ligand GnRH are controversial (63,64).
225 Estradiol replacement (26) and DPN administration (25) do not affect *Cck*, *Nts* and *Vip*, but activate
226 *Crhr2* and suppress *Cart* mRNA expression.

227 *Disturbances in neurotransmitter signaling*

228 In middle-aged rats, OVX affected transmitter signaling via glutamate, acetylcholine, serotonin and
229 histamine in the hippocampus, mainly by regulating the expression of their receptors (65). In case of
230 glutamatergic signaling, *Gria4*, *Grm1* and also *Gls* were upregulated, whereas *Grm8* and *Slc17a6*
231 downregulated. *Grm1* and AMPA receptors occur in the hippocampus enriched in association with
232 postsynaptic densities of neuronal elements communicating via synapses (66-69). Vesicular glutamate
233 transporter 2 was reported to play a crucial role in the proper development of mature pyramidal neuronal
234 architecture and plasticity, and in the processes of cognition (70). Striking decrease of *Slc17a6*
235 expression affects glutamatergic signaling and cognition in the hippocampus after OVX. The signal
236 transduction by serotonin is influenced via the downregulated *Htr2c*. Its activation regulates anxiety and
237 release of acetylcholine in the hippocampus (71,72). Estradiol replacement and DPN administration
238 restore *Htr2c* mRNA expression after OVX. Acetylcholine is a potent modulator of hippocampal circuits
239 and has a pivotal role in cognition (73). Regarding the modulation of fast-synaptic neurotransmission,
240 OVX influenced *Chrna3*. Its expression in the hippocampus has been reported earlier (74) and the
241 present data indicate its downregulation. Similar to that, the expression of *Chrm4* was also
242 downregulated. Suppression of *Chrm4* may affect synaptic transmission via alteration of glutamate
243 release probability. Hippocampus-dependent memory and synaptic plasticity are modulated by the

244 estradiol milieu of the hippocampus (75-78). OVX also downregulated the expression of *Hrh3*.
245 Antagonizing H3 receptor has been shown to increase the release of acetylcholine in the dorsal
246 hippocampus and improve parameters of cognitive disorders (79).

247 Vesicular monoamine transporter 2 (*Vmat2*) showed upregulation after OVX, an event that influences
248 the transport of dopamine, epinephrine, norepinephrine, serotonin and histamine from the cytosol into
249 synaptic vesicles of neurons and plays role in their vesicular release of transmitters by exocytosis. Mice
250 mutant for *Vmat2* display symptoms of depression (80).

251 *Modifications of potassium channels*

252 Effect of OVX was overwhelmingly manifested in altered expression of different potassium channels.
253 The regulatory influence of estradiol has previously been described in case of slow Ca^{2+} -activated K^+
254 current and large-conductance, voltage- and calcium-activated potassium channels (81,82). The single
255 upregulated gene was *Kcnj6* that codes an ATP-sensitive, inwardly rectifying K^+ channel that is
256 regulated by G proteins and closed by the rise of intracellular ATP levels. Downregulated genes included
257 *Kcnj9*, *Kcnk9*, *Kcnma1* and *Kcnc2*. These alterations may lead to decreased synthesis of *Kcnj9* (G
258 protein-activated inward rectifier potassium channel 3) which regulates resting membrane potential and
259 initiation of action potentials, *Kcnk9* (Task 3 potassium channel) whose current is highly sensitive to
260 changes in extracellular pH, *Kcnma1* (BK, large conductance calcium-activated potassium channel) and
261 *Kcnc2* (Shaw-related K^+ channel). Estrogenic regulation of BK (83) and G-protein-gated inwardly
262 rectifying K^+ (GIRK) (84) channels has previously been reported. The present results indicate that
263 ablation of gonadal hormones in middle-aged rats changes the expression of all four functional types of
264 potassium channels in the hippocampus (85) that depending on their cell type and cellular domain
265 specific expression can modulate the excitability of hippocampal neurons. Long-term DPN treatment
266 attenuates the decrease in *Kcnma1* expression.

267 **OVX interferes with mechanisms involved in neurogenesis**

268 *Influence on growth/troph hormone signaling*

269 OVX substantially influenced growth factor signaling within the hippocampus of middle-aged rats by
270 dominantly upregulating the expression certain growth factors and their receptors. In the nerve growth
271 factor family, *Ntf3* and *Ngf* showed enhanced expression after OVX. In addition, two neurotrophin

272 receptor genes, *Ngfr* (coding for p75) and *Ntrk1* (coding for TrkA) showed a similar, upregulated state.
273 Neurotrophin signaling (86) has a key importance in neurogenesis and synaptic plasticity of the
274 hippocampus (87-90). Two components of IGF signaling mechanisms were altered by OVX, the
275 upregulated *Igf2* and the downregulated *Igfbp4*. *Igf2* is a potent regulator of neurogenesis (91) and it
276 also controls memory consolidation and enhancement (92). The differentially expressed *Fgf1* has been
277 shown to support neuroprotective mechanisms (93) and facilitate LTP (94). *Mdk*, the retinoic acid-
278 responsive, heparin-binding growth factor gene also showed higher expression after gonadectomy. *Mdk*
279 was reported to block kainic acid-induced seizure and concomitant cell death (95). We found slight
280 upregulation of neurotrophin and growth factor genes after OVX. In previous studies we demonstrated
281 that estradiol and DPN increase further mRNA expression of many growth factor genes in the
282 hippocampus of ovariectomized rats which may contribute to the enhanced neurogenesis after
283 replacement (96).

284 *Impact on transcriptional regulation*

285 Orthodenticle homeobox 2 (*Otx2*) and hypoxia inducible factor 1 alpha subunit (*Hif1a*) were both
286 upregulated by OVX. *Otx2* shows altered expression in the hippocampus after tricyclic antidepressant
287 treatment (97), whereas *Hif1a* responds to global ischemia (98). Aryl hydrocarbon receptor nuclear
288 translocator 2 (*Arnt2*) was downregulated. Its encoded protein complexes with *Hif1a* and the complex
289 regulates oxygen-responsive genes. Members of the nuclear receptor superfamily were also influenced
290 by OVX resulting in upregulation of *Esr1* and *Nr4a3* (neuron-derived orphan receptor 1).
291 Downregulation characterized the expression of *Thrb* and *Rxra*. The changes suggest that ablation of
292 ovarian hormone supply to the hippocampus interferes with estrogen, thyroid hormone and retinoic acid
293 signaling mechanisms that are basic transcriptional regulators of diverse hippocampal functions
294 (28,99,100).

295 **OVX affects defense mechanisms**

296 *Modulation of the immune system*

297 We have previously reported the impact of OVX and treatment with ER α and ER β specific agonists on
298 the innate immune system of the hippocampal formation in middle aged rats (24). In accordance with
299 that PCR study, here we confirm the differential expression of macrophage markers (*Aif1*, *RT1-EC2*),

300 phagocytic receptors (*CD11b*, *Fcgr3a*), recognition receptors (*Tlr3*, *Tlr4*), complement system (*C3*, *Cfh*)
301 proinflammatory cytokine IL-1 β (*Il1b*) and an IL3 receptor subunit (*Il3ra*). These upregulated genes
302 reflect the sensitization of microglia and increased level of complement components leading to an
303 increased proinflammatory stage in the absence of gonadal hormone signaling to the hippocampus.
304 Chronic estradiol and DPN administrations attenuate OVX-dependent upregulation of microglia-related
305 genes (24). In a recent study, the role NLRP3 inflammasome activation was shown in development of
306 estrogen deficiency-related affective disorders (101).

307 *Effects on neuroprotective mechanisms*

308 Long-term OVX decreased the expression of genes encoding Bcl-2 (*Bcl2*) and superoxide dismutase
309 (*Sod1*). These events are known to lead to increased apoptotic activity and enhanced level of the reactive
310 superoxide radical, O²⁻. The neuroprotective role of Bcl-2 and Sod1 in the hippocampal circuits has been
311 widely explored (102-107). Decreased synthesis of synuclein beta after OVX may promote alpha
312 synuclein accumulation and trigger neurodegeneration (108,109). Estradiol replacement (26) and long-
313 term DPN treatment (25) activate transcription of genes involved in protection against oxidative stress
314 and detoxification such as *Sod3*, *Gpx1*, *Gstm2*, *Gsta4*, but do not increase *Bcl2* and *Sod1* expression in
315 the hippocampus.

316

317 To sum up, the present study demonstrated extensive transcriptional changes in the hippocampus after
318 OVX in middle-aged rats. Ablation of the ovarian hormone supply influences the machinery of
319 transcription, growth factor signaling, channels of synaptic communication, immune and
320 neuroprotective mechanisms modulating neurogenesis, synaptic plasticity and immunomodulation.
321 Some but not all changes can be restored by estradiol replacement. Regarding the translational value,
322 the results suggest the careful consideration and risk evaluation of the effects of oophorectomy (and
323 menopause) on basic neuronal operation and cognitive performance of the hippocampus in middle-aged
324 individuals (110-112).

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326 **References**

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1. McCarthy MM, Schwarz JM, Wright CL, Dean SL. Mechanisms mediating oestradiol modulation of the developing brain. *J Neuroendocrinol* 2008; 20:777-783
2. McEwen BS. Steroid hormones and the brain: cellular mechanisms underlying neural and behavioral plasticity. *Psychoneuroendocrinology* 1980; 5:1-11
3. McEwen BS. Steroid hormones and the chemistry of behavior. *Adv Behav Biol* 1972; 4:41-59
4. Knobil E. The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res* 1980; 36:53-88
5. Brann DW, Mahesh VB. The aging reproductive neuroendocrine axis. *Steroids* 2005; 70:273-283
6. Nelson JF, Bergman MD, Karelus K, Felicio LS. Aging of the hypothalamo-pituitary-ovarian axis: hormonal influences and cellular mechanisms. *J Steroid Biochem* 1987; 27:699-705
7. Boulware MI, Kent BA, Frick KM. The impact of age-related ovarian hormone loss on cognitive and neural function. *Curr Top Behav Neurosci* 2012; 10:165-184
8. Burger HG, Dudley EC, Robertson DM, Dennerstein L. Hormonal changes in the menopause transition. *Recent Prog Horm Res* 2002; 57:257-275
9. Langer RD, Manson JE, Allison MA. Have we come full circle - or moved forward? The Women's Health Initiative 10 years on. *Climacteric* 2012; 15:206-212
10. Gompel A, Santen RJ. Hormone therapy and breast cancer risk 10 years after the WHI. *Climacteric* 2012; 15:241-249
11. Agency EM. Guidelines on clinical investigation of medicinal products for hormone replacement therapy of oestrogen deficiency symptoms in postmenopausal women. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003348.pdf 2014;
12. Administration UFaD. Estrogen and estrogen with progestin therapies for postmenopausal women. www.fda.gov/Drugs/DrugSafety/InformationbyDrugClass/ucm135318.htm 2014;
13. Santen RJ, Allred DC, Ardoin SP, Archer DF, Boyd N, Braunstein GD, Burger HG, Colditz GA, Davis SR, Gambacciani M, Gower BA, Henderson VW, Jarjour WN, Karas RH, Kleerekoper M, Lobo RA, Manson JE, Marsden J, Martin KA, Martin L, Pinkerton JV, Rubinow DR, Teede H, Thiboutot DM, Utian WH, Endocrine S. Postmenopausal hormone therapy: an Endocrine Society scientific statement. *J Clin Endocrinol Metab* 2010; 95:s1-s66
14. Bove R, Secor E, Chibnik LB, Barnes LL, Schneider JA, Bennett DA, De Jager PL. Age at surgical menopause influences cognitive decline and Alzheimer pathology in older women. *Neurology* 2014; 82:222-229
15. Bean LA, Kumar A, Rani A, Guidi M, Rosario AM, Cruz PE, Golde TE, Foster TC. Re-Opening the Critical Window for Estrogen Therapy. *J Neurosci* 2015; 35:16077-16093
16. Voytko ML, Murray R, Higgs CJ. Executive function and attention are preserved in older surgically menopausal monkeys receiving estrogen or estrogen plus progesterone. *J Neurosci* 2009; 29:10362-10370
17. Tinkler GP, Voytko ML. Estrogen modulates cognitive and cholinergic processes in surgically menopausal monkeys. *Prog Neuropsychopharmacol Biol Psychiatry* 2005; 29:423-431
18. Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Toth K, Liko I, Molnar B, Tihanyi K, Liposits Z. Estradiol replacement alters expression of genes related to

- 377 neurotransmission and immune surveillance in the frontal cortex of middle-aged,
378 ovariectomized rats. *Endocrinology* 2010; 151:3847-3862
- 379 **19.** Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in
380 middle-aged rats when initiated immediately after ovariectomy but not after a long-term
381 period of ovarian hormone deprivation. *Endocrinology* 2006; 147:607-614
- 382 **20.** Bimonte-Nelson HA, Francis KR, Umphlet CD, Granholm AC. Progesterone reverses
383 the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in
384 middle-aged ovariectomized female rats. *Eur J Neurosci* 2006; 24:229-242
- 385 **21.** Foster TC. Role of estrogen receptor alpha and beta expression and signaling on
386 cognitive function during aging. *Hippocampus* 2012; 22:656-669
- 387 **22.** Aenlle KK, Kumar A, Cui L, Jackson TC, Foster TC. Estrogen effects on cognition and
388 hippocampal transcription in middle-aged mice. *Neurobiol Aging* 2009; 30:932-945
- 389 **23.** Fernandez SM, Frick KM. Chronic oral estrogen affects memory and neurochemistry
390 in middle-aged female mice. *Behav Neurosci* 2004; 118:1340-1351
- 391 **24.** Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Liposits Z. Ovariectomy and subsequent
392 treatment with estrogen receptor agonists tune the innate immune system of the
393 hippocampus in middle-aged female rats. *PLoS One* 2014; 9:e88540
- 394 **25.** Sarvari M, Kalló I, Hrabovszky E, Solymosi N, Rodolosse A, Liposits Z. Long-Term
395 Estrogen Receptor Beta Agonist Treatment Modifies the Hippocampal Transcriptome
396 in Middle-Aged Ovariectomized Rats
397 *Frontiers in Cellular Neuroscience* 2016;
- 398 **26.** Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Rodolosse A, Vastagh C, Auer H,
399 Liposits Z. Hippocampal Gene Expression Is Highly Responsive to Estradiol
400 Replacement in Middle-Aged Female Rats. *Endocrinology* 2015; 156:2632-2645
- 401 **27.** Kumar A, Bean LA, Rani A, Jackson T, Foster TC. Contribution of estrogen receptor
402 subtypes, ERalpha, ERbeta, and GPER1 in rapid estradiol-mediated enhancement of
403 hippocampal synaptic transmission in mice. *Hippocampus* 2015; 25:1556-1566
- 404 **28.** Bean LA, Ianov L, Foster TC. Estrogen receptors, the hippocampus, and memory.
405 *Neuroscientist* 2014; 20:534-545
- 406 **29.** Han X, Aenlle KK, Bean LA, Rani A, Semple-Rowland SL, Kumar A, Foster TC. Role
407 of estrogen receptor alpha and beta in preserving hippocampal function during aging. *J*
408 *Neurosci* 2013; 33:2671-2683
- 409 **30.** Tanapat P, Hastings NB, Gould E. Ovarian steroids influence cell proliferation in the
410 dentate gyrus of the adult female rat in a dose- and time-dependent manner. *J Comp*
411 *Neurol* 2005; 481:252-265
- 412 **31.** Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase
413 in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci*
414 1999; 19:5792-5801
- 415 **32.** Etgen AM, Jover-Mengual T, Zukin RS. Neuroprotective actions of estradiol and novel
416 estrogen analogs in ischemia: translational implications. *Front Neuroendocrinol* 2011;
417 32:336-352
- 418 **33.** Lebesgue D, Traub M, De Butte-Smith M, Chen C, Zukin RS, Kelly MJ, Etgen AM.
419 Acute administration of non-classical estrogen receptor agonists attenuates ischemia-
420 induced hippocampal neuron loss in middle-aged female rats. *PLoS One* 2010; 5:e8642
- 421 **34.** Arevalo MA, Azcoitia I, Garcia-Segura LM. The neuroprotective actions of oestradiol
422 and oestrogen receptors. *Nat Rev Neurosci* 2015; 16:17-29
- 423 **35.** Sarvari M, Hrabovszky E, Kallo I, Galamb O, Solymosi N, Liko I, Molnar B, Tihanyi
424 K, Szombathelyi Z, Liposits Z. Gene expression profiling identifies key estradiol targets
425 in the frontal cortex of the rat. *Endocrinology* 2010; 151:1161-1176

- 426 **36.** Lu KH, Hopper BR, Vargo TM, Yen SS. Chronological changes in sex steroid,
427 gonadotropin and prolactin secretions in aging female rats displaying different
428 reproductive states. *Biol Reprod* 1979; 21:193-203
- 429 **37.** Wheeler DW, White CM, Rees CL, Komendantov AO, Hamilton DJ, Ascoli GA.
430 Hippocampome.org: a knowledge base of neuron types in the rodent hippocampus. *Elife*
431 2015; 4
- 432 **38.** Freund TF, Buzsaki G. Interneurons of the hippocampus. *Hippocampus* 1996; 6:347-
433 470
- 434 **39.** Buckmaster PS, Soltesz I. Neurobiology of hippocampal interneurons: a workshop
435 review. *Hippocampus* 1996; 6:330-339
- 436 **40.** Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of
437 hippocampal circuit operations. *Science* 2008; 321:53-57
- 438 **41.** Roux L, Stark E, Sjulson L, Buzsaki G. In vivo optogenetic identification and
439 manipulation of GABAergic interneuron subtypes. *Curr Opin Neurobiol* 2014; 26:88-
440 95
- 441 **42.** Fuzik J, Zeisel A, Mate Z, Calvigioni D, Yanagawa Y, Szabo G, Linnarsson S, Harkany
442 T. Integration of electrophysiological recordings with single-cell RNA-seq data
443 identifies neuronal subtypes. *Nat Biotechnol* 2016; 34:175-183
- 444 **43.** Chamberland S, Topolnik L. Inhibitory control of hippocampal inhibitory neurons.
445 *Front Neurosci* 2012; 6:165
- 446 **44.** Bezaire MJ, Soltesz I. Quantitative assessment of CA1 local circuits: knowledge base
447 for interneuron-pyramidal cell connectivity. *Hippocampus* 2013; 23:751-785
- 448 **45.** Krook-Magnuson E, Varga C, Lee SH, Soltesz I. New dimensions of interneuronal
449 specialization unmasked by principal cell heterogeneity. *Trends Neurosci* 2012; 35:175-
450 184
- 451 **46.** Dauge V, Bohme GA, Crawley JN, Durieux C, Stutzmann JM, Feger J, Blanchard JC,
452 Roques BP. Investigation of behavioral and electrophysiological responses induced by
453 selective stimulation of CCKB receptors by using a new highly potent CCK analog, BC
454 264. *Synapse* 1990; 6:73-80
- 455 **47.** Bohme GA, Stutzmann JM, Blanchard JC. Excitatory effects of cholecystokinin in rat
456 hippocampus: pharmacological response compatible with 'central'- or B-type CCK
457 receptors. *Brain Res* 1988; 451:309-318
- 458 **48.** Cunha-Reis D, Sebastiao AM, Wirkner K, Illes P, Ribeiro JA. VIP enhances both pre-
459 and postsynaptic GABAergic transmission to hippocampal interneurons leading to
460 increased excitatory synaptic transmission to CA1 pyramidal cells. *Br J Pharmacol*
461 2004; 143:733-744
- 462 **49.** Li S, Geiger JD, Lei S. Neurotensin enhances GABAergic activity in rat hippocampus
463 CA1 region by modulating L-type calcium channels. *J Neurophysiol* 2008; 99:2134-
464 2143
- 465 **50.** Lee HK, Dunwiddie T, Hoffer B. Electrophysiological interactions of enkephalins with
466 neuronal circuitry in the rat hippocampus. II. Effects on interneuron excitability. *Brain*
467 *Res* 1980; 184:331-342
- 468 **51.** Dunwiddie T, Mueller A, Palmer M, Stewart J, Hoffer B. Electrophysiological
469 interactions of enkephalins with neuronal circuitry in the rat hippocampus. I. Effects on
470 pyramidal cell activity. *Brain Res* 1980; 184:311-330
- 471 **52.** Cohen GA, Doze VA, Madison DV. Opioid inhibition of GABA release from
472 presynaptic terminals of rat hippocampal interneurons. *Neuron* 1992; 9:325-335
- 473 **53.** Taylor RD, Madsen MG, Krause M, Sampredo-Castaneda M, Stocker M, Pedarzani P.
474 Pituitary adenylate cyclase-activating polypeptide (PACAP) inhibits the slow
475 afterhyperpolarizing current sIAHP in CA1 pyramidal neurons by activating multiple
476 signaling pathways. *Hippocampus* 2014; 24:32-43

- 477 **54.** Ago Y, Yoneyama M, Ishihama T, Kataoka S, Kawada K, Tanaka T, Ogita K, Shintani
478 N, Hashimoto H, Baba A, Takuma K, Matsuda T. Role of endogenous pituitary
479 adenylate cyclase-activating polypeptide in adult hippocampal neurogenesis.
480 *Neuroscience* 2011; 172:554-561
- 481 **55.** Mercer A, Ronnholm H, Holmberg J, Lundh H, Heidrich J, Zachrisson O, Ossoinak A,
482 Frisen J, Patrone C. PACAP promotes neural stem cell proliferation in adult mouse
483 brain. *J Neurosci Res* 2004; 76:205-215
- 484 **56.** Riek-Burchardt M, Kolodziej A, Henrich-Noack P, Reymann KG, Holtt V, Stumm R.
485 Differential regulation of CXCL12 and PACAP mRNA expression after focal and
486 global ischemia. *Neuropharmacology* 2010; 58:199-207
- 487 **57.** Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA. Prevention of
488 ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase
489 activating polypeptide. *Brain Res* 1996; 736:280-286
- 490 **58.** Rebaudo R, Melani R, Balestrino M, Izvarina N. Electrophysiological effects of
491 sustained delivery of CRF and its receptor agonists in hippocampal slices. *Brain Res*
492 2001; 922:112-117
- 493 **59.** Bharne AP, Borkar CD, Bodakuntla S, Lahiri M, Subhedar NK, Kokare DM. Pro-
494 cognitive action of CART is mediated via ERK in the hippocampus. *Hippocampus*
495 2016;
- 496 **60.** Abraham CR, Mullen PC, Tucker-Zhou T, Chen CD, Zeldich E. Klotho Is a
497 Neuroprotective and Cognition-Enhancing Protein. *Vitam Horm* 2016; 101:215-238
- 498 **61.** Yang SN, Lu F, Wu JN, Liu DD, Hsieh WY. Activation of gonadotropin-releasing
499 hormone receptors induces a long-term enhancement of excitatory postsynaptic currents
500 mediated by ionotropic glutamate receptors in the rat hippocampus. *Neurosci Lett* 1999;
501 260:33-36
- 502 **62.** Lu F, Yang JM, Wu JN, Chen YC, Kao YH, Tung CS, Yang SN. Activation of
503 gonadotropin-releasing hormone receptors produces neuronal excitation in the rat
504 hippocampus. *Chin J Physiol* 1999; 42:67-71
- 505 **63.** Abel TW, Rance NE. Stereologic study of the hypothalamic infundibular nucleus in
506 young and older women. *J Comp Neurol* 2000; 424:679-688
- 507 **64.** Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, Yin Y, Li B, Liu G, Cai D.
508 Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and
509 GnRH. *Nature* 2013; 497:211-216
- 510 **65.** Vizi ES, Kiss JP. Neurochemistry and pharmacology of the major hippocampal
511 transmitter systems: synaptic and nonsynaptic interactions. *Hippocampus* 1998; 8:566-
512 607
- 513 **66.** Lujan R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P. Perisynaptic location of
514 metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic
515 spines in the rat hippocampus. *Eur J Neurosci* 1996; 8:1488-1500
- 516 **67.** Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P. The
517 metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic
518 membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron*
519 1993; 11:771-787
- 520 **68.** Baude A, Nusser Z, Molnar E, McIlhinney RA, Somogyi P. High-resolution
521 immunogold localization of AMPA type glutamate receptor subunits at synaptic and
522 non-synaptic sites in rat hippocampus. *Neuroscience* 1995; 69:1031-1055
- 523 **69.** Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P. Cell type and pathway
524 dependence of synaptic AMPA receptor number and variability in the hippocampus.
525 *Neuron* 1998; 21:545-559

- 526 **70.** He H, Mahnke AH, Doyle S, Fan N, Wang CC, Hall BJ, Tang YP, Inglis FM, Chen C,
527 Erickson JD. Neurodevelopmental role for VGLUT2 in pyramidal neuron plasticity,
528 dendritic refinement, and in spatial learning. *J Neurosci* 2012; 32:15886-15901
- 529 **71.** Alves SH, Pinheiro G, Motta V, Landeira-Fernandez J, Cruz AP. Anxiogenic effects in
530 the rat elevated plus-maze of 5-HT(2C) agonists into ventral but not dorsal
531 hippocampus. *Behav Pharmacol* 2004; 15:37-43
- 532 **72.** Nair SG, Gudelsky GA. Activation of 5-HT2 receptors enhances the release of
533 acetylcholine in the prefrontal cortex and hippocampus of the rat. *Synapse* 2004;
534 53:202-207
- 535 **73.** Kenney JW, Gould TJ. Modulation of hippocampus-dependent learning and synaptic
536 plasticity by nicotine. *Mol Neurobiol* 2008; 38:101-121
- 537 **74.** Lobron C, Wevers A, Damgen K, Jeske A, Rontal D, Birtsch C, Heinemann S,
538 Reinhardt S, Maelicke A, Schroder H. Cellular distribution in the rat telencephalon of
539 mRNAs encoding for the alpha 3 and alpha 4 subunits of the nicotinic acetylcholine
540 receptor. *Brain Res Mol Brain Res* 1995; 30:70-76
- 541 **75.** Stelly CE, Cronin J, Daniel JM, Schrader LA. Long-term oestradiol treatment enhances
542 hippocampal synaptic plasticity that is dependent on muscarinic acetylcholine receptors
543 in ovariectomised female rats. *J Neuroendocrinol* 2012; 24:887-896
- 544 **76.** Davis DM, Jacobson TK, Aliakbari S, Mizumori SJ. Differential effects of estrogen on
545 hippocampal- and striatal-dependent learning. *Neurobiol Learn Mem* 2005; 84:132-137
- 546 **77.** Daniel JM, Witty CF, Rodgers SP. Long-term consequences of estrogens administered
547 in midlife on female cognitive aging. *Horm Behav* 2015; 74:77-85
- 548 **78.** Rodgers SP, Bohacek J, Daniel JM. Transient estradiol exposure during middle age in
549 ovariectomized rats exerts lasting effects on cognitive function and the hippocampus.
550 *Endocrinology* 2010; 151:1194-1203
- 551 **79.** Medhurst AD, Atkins AR, Beresford IJ, Brackenborough K, Briggs MA, Calver AR,
552 Cilia J, Cluderay JE, Crook B, Davis JB, Davis RK, Davis RP, Dawson LA, Foley AG,
553 Gartlon J, Gonzalez MI, Heslop T, Hirst WD, Jennings C, Jones DN, Lacroix LP,
554 Martyn A, Ociecka S, Ray A, Regan CM, Roberts JC, Schogger J, Southam E, Stean
555 TO, Trail BK, Upton N, Wadsworth G, Wald JA, White T, Witherington J, Woolley
556 ML, Worby A, Wilson DM. GSK189254, a novel H3 receptor antagonist that binds to
557 histamine H3 receptors in Alzheimer's disease brain and improves cognitive
558 performance in preclinical models. *J Pharmacol Exp Ther* 2007; 321:1032-1045
- 559 **80.** Fukui M, Rodriguiz RM, Zhou J, Jiang SX, Phillips LE, Caron MG, Wetsel WC. Vmat2
560 heterozygous mutant mice display a depressive-like phenotype. *J Neurosci* 2007;
561 27:10520-10529
- 562 **81.** Carrer HF, Araque A, Buno W. Estradiol regulates the slow Ca²⁺-activated K⁺ current
563 in hippocampal pyramidal neurons. *J Neurosci* 2003; 23:6338-6344
- 564 **82.** Jamali K, Naylor BR, Kelly MJ, Ronnekleiv OK. Effect of 17beta-estradiol on mRNA
565 expression of large- conductance, voltage-dependent, and calcium-activated potassium
566 channel alpha and beta subunits in guinea pig. *Endocrine* 2003; 20:227-237
- 567 **83.** Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE,
568 Vergara C, Latorre R. Acute activation of Maxi-K channels (hSlo) by estradiol binding
569 to the beta subunit. *Science* 1999; 285:1929-1931
- 570 **84.** Kelly MJ, Qiu J, Ronnekleiv OK. Estrogen modulation of G-protein-coupled receptor
571 activation of potassium channels in the central nervous system. *Ann N Y Acad Sci* 2003;
572 1007:6-16
- 573 **85.** Humphries ES, Dart C. Neuronal and Cardiovascular Potassium Channels as
574 Therapeutic Drug Targets: Promise and Pitfalls. *J Biomol Screen* 2015; 20:1055-1073
- 575 **86.** Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the
576 vertebrate nervous system. *Genes Dev* 2000; 14:2919-2937

- 577 **87.** Gomez-Palacio-Schjetnan A, Escobar ML. Neurotrophins and synaptic plasticity. *Curr*
578 *Top Behav Neurosci* 2013; 15:117-136
- 579 **88.** Ramos-Languren LE, Escobar ML. Plasticity and metaplasticity of adult rat
580 hippocampal mossy fibers induced by neurotrophin-3. *Eur J Neurosci* 2013; 37:1248-
581 1259
- 582 **89.** Gage FH, Buzsaki G, Armstrong DM. NGF-dependent sprouting and regeneration in
583 the hippocampus. *Prog Brain Res* 1990; 83:357-370
- 584 **90.** Hennigan A, O'Callaghan RM, Kelly AM. Neurotrophins and their receptors: roles in
585 plasticity, neurodegeneration and neuroprotection. *Biochem Soc Trans* 2007; 35:424-
586 427
- 587 **91.** Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD, Jr., Suh
588 H, Couillard-Despres S, Aigner L, Gage FH, Jessberger S. Gene expression profiling of
589 neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult
590 hippocampal neurogenesis. *J Neurosci* 2012; 32:3376-3387
- 591 **92.** Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D,
592 Blitzer RD, Alberini CM. A critical role for IGF-II in memory consolidation and
593 enhancement. *Nature* 2011; 469:491-497
- 594 **93.** Sasaki K, Oomura Y, Suzuki K, Hanai K, Yagi H. Acidic fibroblast growth factor
595 prevents death of hippocampal CA1 pyramidal cells following ischemia. *Neurochem*
596 *Int* 1992; 21:397-402
- 597 **94.** Sasaki K, Oomura Y, Figurov A, Yagi H. Acidic fibroblast growth factor facilitates
598 generation of long-term potentiation in rat hippocampal slices. *Brain Res Bull* 1994;
599 33:505-511
- 600 **95.** Kim YB, Ryu JK, Lee HJ, Lim IJ, Park D, Lee MC, Kim SU. Midkine, heparin-binding
601 growth factor, blocks kainic acid-induced seizure and neuronal cell death in mouse
602 hippocampus. *BMC Neurosci* 2010; 11:42
- 603 **96.** Duarte-Guterman P, Yagi S, Chow C, Galea LA. Hippocampal learning, memory, and
604 neurogenesis: Effects of sex and estrogens across the lifespan in adults. *Horm Behav*
605 2015; 74:37-52
- 606 **97.** Lisowski P, Juszczak GR, Goscik J, Stankiewicz AM, Wieczorek M, Zwierzchowski L,
607 Swiergiel AH. Stress susceptibility-specific phenotype associated with different
608 hippocampal transcriptomic responses to chronic tricyclic antidepressant treatment in
609 mice. *BMC Neurosci* 2013; 14:144
- 610 **98.** Jin KL, Mao XO, Nagayama T, Goldsmith PC, Greenberg DA. Induction of vascular
611 endothelial growth factor and hypoxia-inducible factor-1alpha by global ischemia in rat
612 brain. *Neuroscience* 2000; 99:577-585
- 613 **99.** McCaffery P, Zhang J, Crandall JE. Retinoic acid signaling and function in the adult
614 hippocampus. *J Neurobiol* 2006; 66:780-791
- 615 **100.** Remaud S, Gothie JD, Morvan-Dubois G, Demeneix BA. Thyroid hormone signaling
616 and adult neurogenesis in mammals. *Front Endocrinol (Lausanne)* 2014; 5:62
- 617 **101.** Xu Y, Sheng H, Bao Q, Wang Y, Lu J, Ni X. NLRP3 inflammasome activation mediates
618 estrogen deficiency-induced depression- and anxiety-like behavior and hippocampal
619 inflammation in mice. *Brain Behav Immun* 2016;
- 620 **102.** Chan PH, Kawase M, Murakami K, Chen SF, Li Y, Calagui B, Reola L, Carlson E,
621 Epstein CJ. Overexpression of SOD1 in transgenic rats protects vulnerable neurons
622 against ischemic damage after global cerebral ischemia and reperfusion. *J Neurosci*
623 1998; 18:8292-8299
- 624 **103.** Nilsen J, Diaz Brinton R. Mechanism of estrogen-mediated neuroprotection: regulation
625 of mitochondrial calcium and Bcl-2 expression. *Proc Natl Acad Sci U S A* 2003;
626 100:2842-2847

- 627 **104.** Sales S, Ureshino RP, Pereira RT, Luna MS, Pires de Oliveira M, Yamanouye N,
628 Godinho RO, Smaili SS, Porto CS, Abdalla FM. Effects of 17beta-estradiol replacement
629 on the apoptotic effects caused by ovariectomy in the rat hippocampus. *Life Sci* 2010;
630 86:832-838
- 631 **105.** Sharma K, Mehra RD. Long-term administration of estrogen or tamoxifen to
632 ovariectomized rats affords neuroprotection to hippocampal neurons by modulating the
633 expression of Bcl-2 and Bax. *Brain Res* 2008; 1204:1-15
- 634 **106.** Sugawara T, Noshita N, Lewen A, Gasche Y, Ferrand-Drake M, Fujimura M, Morita-
635 Fujimura Y, Chan PH. Overexpression of copper/zinc superoxide dismutase in
636 transgenic rats protects vulnerable neurons against ischemic damage by blocking the
637 mitochondrial pathway of caspase activation. *J Neurosci* 2002; 22:209-217
- 638 **107.** Zhao L, Wu TW, Brinton RD. Estrogen receptor subtypes alpha and beta contribute to
639 neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. *Brain*
640 *Res* 2004; 1010:22-34
- 641 **108.** Hashimoto M, Bar-On P, Ho G, Takenouchi T, Rockenstein E, Crews L, Masliah E.
642 Beta-synuclein regulates Akt activity in neuronal cells. A possible mechanism for
643 neuroprotection in Parkinson's disease. *J Biol Chem* 2004; 279:23622-23629
- 644 **109.** Hashimoto M, Rockenstein E, Mante M, Crews L, Bar-On P, Gage FH, Marr R, Masliah
645 E. An antiaggregation gene therapy strategy for Lewy body disease utilizing beta-
646 synuclein lentivirus in a transgenic model. *Gene Ther* 2004; 11:1713-1723
- 647 **110.** Au A, Feher A, McPhee L, Jessa A, Oh S, Einstein G. Estrogens, inflammation and
648 cognition. *Front Neuroendocrinol* 2016; 40:87-100
- 649 **111.** Davis SR, Lambrinoudaki I, Lumsden M, Mishra GD, Pal L, Rees M, Santoro N,
650 Simoncini T. Menopause. *Nat Rev Dis Primers* 2015; 1:15004
- 651 **112.** Stuenkel CA, Davis SR, Gompel A, Lumsden MA, Murad MH, Pinkerton JV, Santen
652 RJ. Treatment of Symptoms of the Menopause: An Endocrine Society Clinical Practice
653 Guideline. *J Clin Endocrinol Metab* 2015; 100:3975-4011
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Table 1. List of top hippocampal genes regulated by long-term OVX in middle-aged rats

Probeset ID	logFC	FC	adj. P	Symbol	Description
Upregulated Genes					
1394252_PM_at	1.194	2.288	0.356	<i>Spock3</i>	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 3
1368585_PM_at	1.188	2.278	0.141	<i>Cartpt</i>	CART prepropeptide
1391387_PM_s_at	1.158	2.232	0.479	<i>Slbp</i>	stem-loop binding protein
1391208_PM_at	1.087	2.124	0.178	<i>Pcdh20</i>	protocadherin 20
1380805_PM_at	1.060	2.084	0.310	<i>Pvrl3</i>	poliovirus receptor-related 3
1393247_PM_at	0.974	1.965	0.462	<i>Zfp26</i>	zinc finger protein 26
1378679_PM_at	0.963	1.949	0.446	<i>Usp25</i>	ubiquitin specific peptidase 25
1390812_PM_a_at	0.961	1.947	0.159	<i>Rerg</i>	RAS-like, estrogen-regulated, growth-inhibitor
1398355_PM_at	0.950	1.931	0.456	<i>Trpm7</i>	transient receptor potential cation channel, subfamily M, 7
1380279_PM_at	0.934	1.911	0.410	<i>Prpc</i>	prolylcarboxypeptidase (angiotensinase C)
1383396_PM_at	0.929	1.904	0.460	<i>Fndc3a</i>	fibronectin type III domain containing 3a
1375676_PM_at	0.915	1.886	0.460	<i>Lin7c</i>	lin-7 homolog C (<i>C. elegans</i>)
1374959_PM_at	0.894	1.858	0.457	<i>Nqo2</i>	NAD(P)H dehydrogenase, quinone 2
1389479_PM_at	0.890	1.853	0.419	<i>Klf3</i>	Kruppel-like factor 3 (basic)
1395744_PM_at	0.886	1.849	0.477	<i>Sppl2a</i>	signal peptide peptidase-like 2A
1389362_PM_at	0.856	1.811	0.246	<i>Ptpn3</i>	protein tyrosine phosphatase, non-receptor type 3
1382171_PM_at	0.851	1.804	0.515	<i>Tsc22d2</i>	TSC22 domain family, member 2
1387076_PM_at	0.850	1.802	0.460	<i>Hif1a</i>	hypoxia-inducible factor 1, alpha subunit
1375538_PM_at	0.835	1.784	0.519	<i>Vcl</i>	vinculin
1376319_PM_at	0.834	1.783	0.061	<i>Sema3c</i>	semaphorin 3C
1382390_PM_at	0.809	1.752	0.469	<i>Fubp3</i>	far upstream element (FUSE) binding protein 3
1367598_PM_at	0.793	1.733	0.616	<i>Ttr</i>	transthyretin
1372905_PM_at	0.782	1.720	0.530	<i>Vcl</i>	vinculin
1377651_PM_at	0.774	1.710	0.445	<i>Trio</i>	triple functional domain (PTPRF interacting)
Downregulated Genes					
1370472_PM_a_at	-0.802	0.574	0.445	<i>Kcna1</i>	K large conductance Ca-activated channel, subfamily M, α 1
1391074_PM_at	-0.807	0.572	0.440	<i>Crabp1</i>	cellular retinoic acid binding protein 1
1367768_PM_at	-0.807	0.572	0.222	<i>Lxn</i>	latexin
1383386_PM_a_at	-0.814	0.569	0.463	<i>Sec31</i>	SEC3-like 1 (<i>S. cerevisiae</i>)
1391075_PM_at	-0.817	0.568	0.341	<i>Rgs17</i>	regulator of G-protein signaling 17
1392823_PM_at	-0.818	0.567	0.419	<i>Shhg11</i>	small nucleolar RNA host gene 11 (non-protein coding)
1369418_PM_at	-0.828	0.563	0.419	<i>Kcnj9</i>	potassium inwardly-rectifying channel, subfamily J, member 9
1369280_PM_at	-0.833	0.561	0.326	<i>Kcnk9</i>	potassium channel, subfamily K, member 9
1387291_PM_at	-0.861	0.551	0.123	<i>Itih3</i>	inter-alpha trypsin inhibitor, heavy chain 3
1379481_PM_at	-0.863	0.550	0.460	<i>Pabpn1</i>	poly(A) binding protein, nuclear 1
1370248_PM_at	-0.873	0.546	0.159	<i>Fxyd6</i>	FXD domain-containing ion transport regulator 6
1371132_PM_a_at	-0.875	0.545	0.459	<i>Ank3</i>	ankyrin 3, node of Ranvier
1389089_PM_at	-0.887	0.541	0.479	<i>Slc39a7</i>	solute carrier family 39 (zinc transporter), member 7
1386940_PM_at	-0.911	0.532	0.419	<i>Timp2</i>	TIMP metalloproteinase inhibitor 2
1379046_PM_at	-0.920	0.528	0.479	<i>Mlec</i>	malectin
1398303_PM_s_at	-0.948	0.518	0.459	<i>Tpm3</i>	tropomyosin 3, gamma
1368883_PM_at	-0.969	0.511	0.184	<i>Nov</i>	nephroblastoma overexpressed gene
1376980_PM_at	-0.973	0.510	0.222	<i>Htr2c</i>	5-hydroxytryptamine (serotonin) receptor 2C
1392555_PM_at	-1.029	0.490	0.166	<i>Etv1</i>	ets variant 1
1397513_PM_at	-1.045	0.485	0.431	<i>Raly1</i>	RALY RNA binding protein-like
1371462_PM_at	-1.054	0.482	0.103	<i>Igfbp4</i>	insulin-like growth factor binding protein 4
1373257_PM_at	-1.068	0.477	0.419	<i>Arpp21</i>	cAMP-regulated phosphoprotein 21
1389155_PM_at	-1.081	0.473	0.393	<i>Dos</i>	downstream of Stk11
1368381_PM_at	-1.095	0.468	0.439	<i>Crtac1</i>	cartilage acidic protein 1
1384809_PM_at	-1.119	0.460	0.026	<i>Gpr123</i>	G protein-coupled receptor 123
1390865_PM_at	-1.121	0.460	0.026	<i>Cadps2</i>	Ca ⁺⁺ -dependent secretion activator 2
1385491_PM_at	-1.143	0.453	0.299	<i>Pnmal2</i>	PNMA-like 2
1370602_PM_at	-1.301	0.406	0.159	<i>Atp2b4</i>	ATPase, Ca ⁺⁺ transporting, plasma membrane 4
1385788_PM_at	-1.479	0.359	0.419	<i>Ephb3</i>	Eph receptor B3
1368564_PM_at	-1.482	0.358	0.133	<i>Slc17a6</i>	sodium-dependent inorganic phosphate cotransporter
1368892_PM_at	-1.654	0.318	0.026	<i>Adcyap1</i>	adenylate cyclase activating polypeptide 1
1368505_PM_at	-1.868	0.274	0.032	<i>Rgs4</i>	regulator of G-protein signaling 4

660 Expression profiling by Affymetrix oligonucleotide microarray revealed that long-term OVX intensely
661 regulates the hippocampal transcriptome in middle-aged rats. Using the $FC > 1.7$ selection criterion, the
662 top list contains 24 up- and 32 downregulated genes.

663

Table 2. PCR results

Assay ID	Symbol	Target name	RQ	P value
Neurotransmitter and neuropeptide signaling/ Synaptic plasticity				
Rn01645174_m1	<i>Cartpt</i>	CART propeptide	2.598	0.003
Rn00562124_m1	<i>Ttr</i>	transthyretin	2.251	0.097
Rn00564688_m1	<i>Slc18a2</i>	vesicular monoamine transporter 2	1.645	0.001
Rn00578981_m1	<i>Gnhr</i>	gonadotropin releasing hormone receptor	1.631	0.004
Rn01454304_m1	<i>Dagla</i>	diacylglycerol lipase alpha	1.387	0.013
Rn00582505_m1	<i>Slc12a2</i>	Na-K-Cl cotransporter	1.386	0.094
Rn03993699_s1	<i>Cnr2</i>	cannabinoid receptor 2	1.250	0.070
Rn01505088_m1	<i>Enpp2</i>	ectonucleotide phosphodiesterase 2	1.221	0.015
Rn01234233_m1	<i>Kcnc2</i>	voltage-gated potassium channel	0.861	0.058
Rn00563215_m1	<i>Cck</i>	cholecystokinin	0.821	0.007
Rn01512605_s1	<i>Chrm4</i>	cholinergic receptor, muscarinic 4	0.775	0.031
Rn00578611_m1	<i>Crhrl</i>	corticotropin releasing hormone receptor 1	0.766	0.063
Rn00565867_m1	<i>Cckbr</i>	cholecystokinin B receptor	0.748	0.111
Rn01430567_m1	<i>Vip</i>	vasoactive intestinal peptide	0.733	0.001
Rn00561699_m1	<i>Oprd1</i>	delta 1 opioid receptor	0.723	0.051
Rn00585276_m1	<i>Hrh3</i>	histamine receptor H3	0.686	0.000
Rn00575617_m1	<i>Crh2</i>	corticotropin releasing hormone receptor 2	0.650	0.016
Rn00567566_m1	<i>Penk</i>	preproenkephalin	0.644	0.000
Rn01456072_m1	<i>Cadps2</i>	Ca-dependent activator protein for secretion	0.599	0.001
Rn02532012_s1	<i>Sstr1</i>	somatostatin receptor 1	0.382	0.001
Rn00562748_m1	<i>Htr2c</i>	5HT receptor 2C	0.363	0.000
Rn00573505_m1	<i>Grm8</i>	glutamate metabotropic receptor 8	0.357	0.001
Rn00584780_m1	<i>Slc17a6</i>	vesicular glutamate transporter 2	0.260	0.000
Rn00583820_m1	<i>Chma3</i>	cholinergic receptor nicotinic alpha 3 subunit	0.214	0.001
Growth and troph factor signaling/ Neurogenesis				
Rn01414596_m1	<i>Otx2</i>	orthodenticle homeobox 2	4.934	0.014
Rn00591759_m1	<i>Folr1</i>	folate receptor 1	2.360	0.004
Rn00580123_m1	<i>Kl</i>	klotho	2.104	0.094
Rn00572130_m1	<i>Ntrk1</i>	neurotrophic receptor tyrosine kinase 1	1.870	0.069
Rn01754856_m1	<i>Ucp2</i>	uncoupling protein 2	1.548	0.002
Rn00561634_m1	<i>Ngfr</i>	nerve growth factor receptor	1.506	0.008
Rn00579280_m1	<i>Ntf3</i>	neurotrophin 3	1.491	0.003
Rn01640372_m1	<i>Esr1</i>	estrogen receptor alpha	1.422	0.030
Rn01427989_s1	<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A	1.404	0.002
Rn00675549_g1	<i>Mdk</i>	midkine	1.355	0.054
Rn01533872_m1	<i>Ngf</i>	nerve growth factor	1.306	0.023
Rn00689153_m1	<i>Fgf1</i>	fibroblast growth factor 1	1.278	0.039
Rn01537468_g1	<i>Sgk1</i>	serum- glucocorticoid-regulated kinase 1	1.257	0.018
Rn01454518_m1	<i>Igf2</i>	insulin-like growth factor 2	1.160	0.109
Rn00578713_m1	<i>Adcy2</i>	adenylate cyclase 2	0.881	0.004
Rn00575368_m1	<i>Fkbp1b</i>	FK506 binding protein 1B	0.863	0.017
Rn00441185_m1	<i>Rxra</i>	retinoid X receptor alpha	0.852	0.068
Rn00567957_m1	<i>Map6</i>	microtubule-associated protein 6	0.814	0.020
Rn99999125_m1	<i>Bcl2</i>	B-cell lymphoma 2	0.780	0.001
Rn00562044_m1	<i>Thrb</i>	thyroid hormone receptor beta	0.777	0.008
Rn00578390_m1	<i>Nov</i>	nephroblastoma overexpressed	0.732	0.001
Rn01464112_m1	<i>Igfbp4</i>	insulin-like growth factor binding protein 4	0.480	0.001
Rn00566438_m1	<i>Adcyap1</i>	adenylate cyclase activating polypeptide 1	0.411	0.001
Rn01483363_m1	<i>Atp2b4</i>	ATPase plasma membrane Ca transporting 4	0.361	0.000
Rn00566938_m1	<i>Sod1</i>	superoxide dismutase 1	0.284	0.001
Rn01490867_g1	<i>Rgs4</i>	regulator of G protein signaling 4	0.272	0.000
Immune modulation/ Defense				
Rn03034964_u1	<i>RT1-EC2</i>	RT1-EC2	2.121	0.012
Rn00580555_m1	<i>Ccl2</i>	C-C motif chemokine ligand 2	1.930	0.010
Rn00566466_m1	<i>C3</i>	complement C3	1.750	0.001
Rn01488472_g1	<i>Tlr3</i>	toll-like receptor 3	1.663	0.006
Rn00709342_m1	<i>Ilgam</i>	Cd11b	1.639	0.005
Rn00580432_m1	<i>Il1b</i>	interleukin 1beta	1.437	0.114
Rn00560589_m1	<i>A2m</i>	alpha-2-macroglobulin	1.330	0.001
Rn00564605_m1	<i>Ptgds</i>	prostaglandin D2 synthase	1.253	0.039
Rn01483598_m1	<i>Fcgr3a</i>	Fc gamma receptor 3a	1.245	0.058
Rn00574125_g1	<i>Alif1</i>	allograft inflammatory factor 1	1.207	0.036
Rn00569848_m1	<i>Tlr4</i>	toll-like receptor 4	1.197	0.028
Rn00563082_m1	<i>Mfge8</i>	milk fat globule-EGF factor 8 protein	0.777	0.001

666 Real-time PCR study revealed transcriptional regulation of 62 genes. Thirty-three of them were
667 upregulated. The OVX-regulated genes were grouped in three functional clusters: neurotransmitter and
668 neuropeptide signaling/ synaptic plasticity, growth and troph factor signaling/ neurogenesis, immune
669 modulation/ defense. RQ, relative quantity.

670

671 **Table 3. Pathway analysis**

KEGG Pathways			
Pathway ID	Pathway description	Count	FDR
4080	Neuroactive ligand-receptor interaction	15	0.0007
4723	Retrograde endocannabinoid signaling	9	0.0007
5202	Transcriptional misregulation in cancer	10	0.0032
4210	Apoptosis	7	0.0057
5205	Proteoglycans in cancer	11	0.0072
4915	Estrogen signaling pathway	7	0.0075
4725	Cholinergic synapse	7	0.0111
4750	Inflammatory mediator regulation of TRP channels	7	0.0111
4724	Glutamatergic synapse	7	0.0134
4919	Thyroid hormone signaling pathway	7	0.0170
5200	Pathways in cancer	12	0.0204
4810	Regulation of actin cytoskeleton	9	0.0328
4261	Adrenergic signaling in cardiomyocytes	7	0.0379
4921	Oxytocin signaling pathway	7	0.0419
4726	Serotonergic synapse	6	0.0480

672

673 Top gene ontology pathways affected by OVX in middle-aged rats. The analysis was performed on the

674 web-based KEGG platform. Terms were ranked based on their FDR values. FDR, false discovery rate.

675

Table 4. Functional clusters of upregulated genes in OVX, middle-aged rats

Functional gene clusters upregulated by OVX		
Symbol	Gene name	FC
Growth/troph factors/transcription regulation		
<i>Esr1</i>	estrogen receptor alpha	1.422
<i>Fgf1</i>	fibroblast growth factor 1	1.278
<i>Hif1a</i>	hypoxia-inducible factor 1, alpha subunit	1.802
<i>Igf2</i>	insulin-like growth factor 2	1.160
<i>Mdk</i>	midkine	1.355
<i>Ngf</i>	nerve growth factor	1.306
<i>Ngfr</i>	nerve growth factor receptor	1.506
<i>Ntrk1</i>	neurotrophic tyrosine kinase, receptor, type 1	1.520
<i>Ntf3</i>	neurotrophin 3	1.491
<i>Nr4a3</i>	nuclear receptor subfamily 4, group A, member 3	1.522
<i>Otx2</i>	orthodenticle homeobox 2	4.934
<i>Rerg</i>	RAS-like, estrogen-regulated, growth-inhibitor	1.947
Peptides/transmitters/ion channels/signaling		
<i>Cartpt</i>	CART prepropeptide	2.278
<i>Gria4</i>	glutamate receptor, ionotropic, AMPA 4	1.506
<i>Grm1</i>	glutamate receptor, metabotropic 1	1.532
<i>Gls</i>	glutaminase	1.580
<i>Gnrhr</i>	gonadotropin releasing hormone receptor	1.631
<i>Kcnj6</i>	K inwardly-rectifying channel, subfamily J, 6	1.517
<i>Kl</i>	klotho	2.104
<i>Slc12a2</i>	Na-K-Cl cotransporter	1.386
<i>Ttr</i>	transthyretin	2.251
<i>Slc18a2</i>	vesicular monoamine transporter 2	1.645
Immune response/defense		
<i>Aif1</i>	allograft inflammatory factor 1	1.207
<i>A2m</i>	alpha-2-macroglobulin	1.330
<i>Ccl2</i>	C-C motif chemokine ligand 2	1.930
<i>C3</i>	complement C3	1.750
<i>Itgam</i>	Cd11b	1.639
<i>Cfh</i>	complement factor H	1.551
<i>Fcgr3a</i>	Fc gamma receptor 3a	1.245
<i>Il1b</i>	interleukin 1beta	1.437
<i>RT1-EC2</i>	RT1-EC2	2.121
<i>Sep7</i>	septin 7	1.545
<i>Tlr3</i>	toll-like receptor 3	1.663
<i>Tlr4</i>	toll-like receptor 4	1.197

678 Representative upregulated genes grouped in three functional clusters as growth/troph factors/
679 transcriptional regulation, peptides/transmitters/ion channels/signaling and immune response/ defense.
680 FC in italics refers to PCR results. FC, fold change.
681

Table 5. Functional clusters of downregulated genes in OVX, middle-aged rats

Functional gene clusters downregulated by OVX		
Symbol	Gene name	FC
Peptides/transmitters/receptors/ion channels/signaling		
<i>Htr2c</i>	5HT receptor 2C	0.363
<i>Adcy2</i>	adenylate cyclase 2	0.881
<i>Adcyap1</i>	adenylate cyclase activating polypeptide 1	0.411
<i>Crebl2</i>	cAMP responsive element binding protein-like 2	0.604
<i>Cbln2</i>	cerebellin 2 precursor	0.613
<i>Cck</i>	cholecystokinin	0.821
<i>Cckbr</i>	cholecystokinin B receptor	0.748
<i>Chna3</i>	cholinergic receptor nicotinic alpha 3 subunit	0.214
<i>Chrm4</i>	cholinergic receptor, muscarinic 4	0.775
<i>Cthr1</i>	corticotropin releasing hormone receptor 1	0.766
<i>Cthr2</i>	corticotropin releasing hormone receptor 2	0.650
<i>Oprd1</i>	delta 1 opioid receptor	0.723
<i>Grm8</i>	glutamate metabotropic receptor 8	0.357
<i>Hrh3</i>	histamine receptor H3	0.686
<i>Kcnk9</i>	K channel, subfamily K, member 9	0.561
<i>Kcnj9</i>	K inwardly-rectifying channel, subfamily J, 9	0.563
<i>Kcma1</i>	K large conductance Ca-activated channel, subfamily M	0.574
<i>Nts</i>	neurotensin	0.653
<i>Penk</i>	preproenkephalin	0.644
<i>Rgs4</i>	regulator of G protein signaling 4	0.272
<i>Rgs17</i>	regulator of G-protein signaling 17	0.568
<i>Sstr1</i>	somatostatin receptor 1	0.382
<i>Vip</i>	vasoactive intestinal peptide	0.733
<i>Slc17a6</i>	vesicular glutamate transporter 2	0.260
<i>Vdac1</i>	voltage-dependent anion channel 1	0.641
<i>Kcnc2</i>	voltage-gated potassium channel	0.861
Solute carriers		
<i>Slc1a4</i>	solute carrier family 1, member 4	0.657
<i>Slc10a4</i>	solute carrier family 10, member 4	0.638
<i>Slc17a6</i>	solute carrier family 17, member 6	0.358
<i>Slc25a18</i>	solute carrier family 25, member 18	0.646
<i>Slc39a7</i>	solute carrier family 39, member 7	0.541
<i>Slc4a3</i>	solute carrier family 4, member 3	0.653
<i>Slc6a17</i>	solute carrier family 6, member 17	0.623
<i>Slc9a1</i>	solute carrier family 9, member 1	0.631
<i>Slc9a5</i>	solute carrier family 9, member 5	0.648
Growth factor/transcription regulation		
<i>Amt2</i>	aryl hydrocarbon receptor nuclear translocator 2	0.657
<i>Crabp1</i>	cellular retinoic acid binding protein 1	0.572
<i>Hsp90ab1</i>	heat shock protein 90 alpha (cytosolic), class B member 1	0.646
<i>Igfbp4</i>	insulin-like growth factor binding protein 4	0.480
<i>Rxra</i>	retinoid X receptor alpha	0.852
<i>Thrb</i>	thyroid hormone receptor beta	0.777
Immune response/defense		
<i>Apitd1</i>	apoptosis-inducing, TAF9-like domain 1	0.643
<i>Bcl2</i>	B-cell lymphoma 2	0.780
<i>C1ql3</i>	complement component 1, q subcomponent-like 3	0.646
<i>Il3ra</i>	interleukin 3 receptor, alpha	0.661
<i>Sod1</i>	superoxide dismutase 1	0.284
<i>Snca</i>	synuclein, beta	0.614

684 Representative downregulated genes assembled in four functional clusters as
685 peptides/transmitters/receptors/ion channels/signaling, solute carriers, growth factor/transcription
686 regulation and immune response/defense. FC in italics refers to PCR results. FC, fold change.
687

688 **Figure legends**

689

690 **Figure 1. Predicted interactions among proteins encoded by upregulated genes in long-term**
691 **ovariectomized, middle aged rats.** The network is based on combined results of microarray and
692 quantitative real-time PCR studies and was constructed by using the STRING 10 Known and Predicted
693 Protein-Protein Interactions program (<http://string-db.org/>). Analysis was performed at confidence value
694 0.6 and non-interacting elements were excluded. Selected protein clusters of the network are shown by
695 color frames. The red box marks growth factor signaling, blue identifies immune response, yellow
696 indicates peptide and transmitter signaling, green marks regulation of transcription.

697 **Figure 2. Predicted interactions among proteins encoded by downregulated genes in long-**
698 **term ovariectomized, middle aged rats.** The network is based on combined results of microarray and
699 quantitative real-time PCR studies and was constructed by using the STRING 10 Known and Predicted
700 Protein-Protein Interactions program (<http://string-db.org/>). Analysis was performed at confidence value
701 0.6 and non-interacting elements were excluded. Selected protein clusters of the network are shown by
702 color frames. The red box marks peptide and transmitter signaling, blue indicates potassium channels,
703 orange marks transcriptional regulation, green identifies heterogeneous nuclear ribonucleoproteins.
704



