Ovariectomy Alters Gene Expression of the Hippocampal Formation in Middle-Aged Rats

Miklós Sárvári[1], Imre Kalló[1,2], Erik Hrabovszky[1], Norbert Solymosi[3] and Zsolt Liposits[1,2*]

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

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

[3]Department of Animal Hygiene, Herd-Health and Veterinary Ethology, University of Veterinary Medicine, Budapest, Hungary.

Abbreviated title: OVX-regulated hippocampal gene expression in rats

Key terms: hippocampus, rat, middle-age, ovariectomy, microarray, PCR, pathway analysis

Word count: 3,268

Number of figures and tables: 7

Corresponding author and person to whom reprint requests should be addressed

Miklós Sárvári, PhD

Institute of Experimental Medicine, Hungarian Academy of Sciences,

Szigony u. 43, 1083 Budapest, Hungary

e-mail: sarvari.miklos@koki.mta.hu

Disclosure Statement: The authors have nothing to disclose

Abstract

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

Introduction

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

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

Material and Methods

Experimental animals

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

Affymetrix Rat Genome 230PM Strip Arrays

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

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

Quantitative real-time PCR

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

Data analysis

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

Results

Modification of hippocampal transcriptome in response to long-term OVX

Microarray study

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

Quantitative real-time PCR study

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

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

KEGG pathway analysis of OVX-regulated genes

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

Predicted networking of hippocampal proteins encoded by OVX-regulated genes

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

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

Discussion

OVX modulates neurotransmission

Modulation of peptidergic signaling

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

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

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

Disturbances in neurotransmitter signaling

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

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

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

Modifications of potassium channels

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

OVX interferes with mechanisms involved in neurogenesis

Influence on growth/troph hormone signaling

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

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

Impact on transcriptional regulation

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

OVX affects defense mechanisms

Modulation of the immune system

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

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

Effects on neuroprotective mechanisms

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

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

References

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://wwwemaeuropaeu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003348pdf 2014;

12. Administration UFaD. Estrogen and estrogen with progestin therapies for postmenopausal women. wwwfdagov/Drugs/DrugSafety/InformationbyDrugClass/ucm135318htm 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

neurotransmission and immune surveillance in the frontal cortex of middle-aged, ovariectomized rats. Endocrinology 2010; 151:3847-3862

19. Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 2006; 147:607-614

20. Bimonte-Nelson HA, Francis KR, Umphlet CD, Granholm AC. Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. Eur J Neurosci 2006; 24:229-242

21. Foster TC. Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging. Hippocampus 2012; 22:656-669

22. Aenlle KK, Kumar A, Cui L, Jackson TC, Foster TC. Estrogen effects on cognition and hippocampal transcription in middle-aged mice. Neurobiol Aging 2009; 30:932-945

23. Fernandez SM, Frick KM. Chronic oral estrogen affects memory and neurochemistry in middle-aged female mice. Behav Neurosci 2004; 118:1340-1351

24. Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Liposits Z. Ovariectomy and subsequent treatment with estrogen receptor agonists tune the innate immune system of the hippocampus in middle-aged female rats. PLoS One 2014; 9:e88540

25. Sarvari M, Kalló I, Hrabovszky E, Solymosi N, Rodolosse A, Liposits Z. Long-Term Estrogen Receptor Beta Agonist Treatment Modifies the Hippocampal Transcriptome in Middle-Aged Ovariectomized Rats

Frontiers in Cellular Neuroscience 2016;

26. Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Rodolosse A, Vastagh C, Auer H, Liposits Z. Hippocampal Gene Expression Is Highly Responsive to Estradiol Replacement in Middle-Aged Female Rats. Endocrinology 2015; 156:2632-2645

27. Kumar A, Bean LA, Rani A, Jackson T, Foster TC. Contribution of estrogen receptor subtypes, ERalpha, ERbeta, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. Hippocampus 2015; 25:1556-1566

28. Bean LA, Ianov L, Foster TC. Estrogen receptors, the hippocampus, and memory. Neuroscientist 2014; 20:534-545

29. Han X, Aenlle KK, Bean LA, Rani A, Semple-Rowland SL, Kumar A, Foster TC. Role of estrogen receptor alpha and beta in preserving hippocampal function during aging. J Neurosci 2013; 33:2671-2683

30. Tanapat P, Hastings NB, Gould E. Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J Comp Neurol 2005; 481:252-265

31. Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci 1999; 19:5792-5801

32. Etgen AM, Jover-Mengual T, Zukin RS. Neuroprotective actions of estradiol and novel estrogen analogs in ischemia: translational implications. Front Neuroendocrinol 2011; 32:336-352

33. Lebesgue D, Traub M, De Butte-Smith M, Chen C, Zukin RS, Kelly MJ, Etgen AM. Acute administration of non-classical estrogen receptor agonists attenuates ischemia-induced hippocampal neuron loss in middle-aged female rats. PLoS One 2010; 5:e8642

34. Arevalo MA, Azcoitia I, Garcia-Segura LM. The neuroprotective actions of oestradiol and oestrogen receptors. Nat Rev Neurosci 2015; 16:17-29

35. Sarvari M, Hrabovszky E, Kallo I, Galamb O, Solymosi N, Liko I, Molnar B, Tihanyi K, Szombathelyi Z, Liposits Z. Gene expression profiling identifies key estradiol targets in the frontal cortex of the rat. Endocrinology 2010; 151:1161-1176

36. Lu KH, Hopper BR, Vargo TM, Yen SS. Chronological changes in sex steroid, gonadotropin and prolactin secretions in aging female rats displaying different reproductive states. Biol Reprod 1979; 21:193-203

37. Wheeler DW, White CM, Rees CL, Komendantov AO, Hamilton DJ, Ascoli GA. Hippocampome.org: a knowledge base of neuron types in the rodent hippocampus. Elife 2015; 4

38. Freund TF, Buzsaki G. Interneurons of the hippocampus. Hippocampus 1996; 6:347-470

39. Buckmaster PS, Soltesz I. Neurobiology of hippocampal interneurons: a workshop review. Hippocampus 1996; 6:330-339

40. Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 2008; 321:53-57

41. Roux L, Stark E, Sjulson L, Buzsaki G. In vivo optogenetic identification and manipulation of GABAergic interneuron subtypes. Curr Opin Neurobiol 2014; 26:88-95

42. Fuzik J, Zeisel A, Mate Z, Calvigioni D, Yanagawa Y, Szabo G, Linnarsson S, Harkany T. Integration of electrophysiological recordings with single-cell RNA-seq data identifies neuronal subtypes. Nat Biotechnol 2016; 34:175-183

43. Chamberland S, Topolnik L. Inhibitory control of hippocampal inhibitory neurons. Front Neurosci 2012; 6:165

44. Bezaire MJ, Soltesz I. Quantitative assessment of CA1 local circuits: knowledge base for interneuron-pyramidal cell connectivity. Hippocampus 2013; 23:751-785

45. Krook-Magnuson E, Varga C, Lee SH, Soltesz I. New dimensions of interneuronal specialization unmasked by principal cell heterogeneity. Trends Neurosci 2012; 35:175-184

46. Dauge V, Bohme GA, Crawley JN, Durieux C, Stutzmann JM, Feger J, Blanchard JC, Roques BP. Investigation of behavioral and electrophysiological responses induced by selective stimulation of CCKB receptors by using a new highly potent CCK analog, BC 264. Synapse 1990; 6:73-80

47. Bohme GA, Stutzmann JM, Blanchard JC. Excitatory effects of cholecystokinin in rat hippocampus: pharmacological response compatible with 'central'- or B-type CCK receptors. Brain Res 1988; 451:309-318

48. Cunha-Reis D, Sebastiao AM, Wirkner K, Illes P, Ribeiro JA. VIP enhances both pre- and postsynaptic GABAergic transmission to hippocampal interneurones leading to increased excitatory synaptic transmission to CA1 pyramidal cells. Br J Pharmacol 2004; 143:733-744

49. Li S, Geiger JD, Lei S. Neurotensin enhances GABAergic activity in rat hippocampus CA1 region by modulating L-type calcium channels. J Neurophysiol 2008; 99:2134-2143

50. Lee HK, Dunwiddie T, Hoffer B. Electrophysiological interactions of enkephalins with neuronal circuitry in the rat hippocampus. II. Effects on interneuron excitability. Brain Res 1980; 184:331-342

51. Dunwiddie T, Mueller A, Palmer M, Stewart J, Hoffer B. Electrophysiological interactions of enkephalins with neuronal circuitry in the rat hippocampus. I. Effects on pyramidal cell activity. Brain Res 1980; 184:311-330

52. Cohen GA, Doze VA, Madison DV. Opioid inhibition of GABA release from presynaptic terminals of rat hippocampal interneurons. Neuron 1992; 9:325-335

53. Taylor RD, Madsen MG, Krause M, Sampedro-Castaneda M, Stocker M, Pedarzani P. Pituitary adenylate cyclase-activating polypeptide (PACAP) inhibits the slow afterhyperpolarizing current sIAHP in CA1 pyramidal neurons by activating multiple signaling pathways. Hippocampus 2014; 24:32-43

54. Ago Y, Yoneyama M, Ishihama T, Kataoka S, Kawada K, Tanaka T, Ogita K, Shintani N, Hashimoto H, Baba A, Takuma K, Matsuda T. Role of endogenous pituitary adenylate cyclase-activating polypeptide in adult hippocampal neurogenesis. Neuroscience 2011; 172:554-561

55. Mercer A, Ronnholm H, Holmberg J, Lundh H, Heidrich J, Zachrisson O, Ossoinak A, Frisen J, Patrone C. PACAP promotes neural stem cell proliferation in adult mouse brain. J Neurosci Res 2004; 76:205-215

56. Riek-Burchardt M, Kolodziej A, Henrich-Noack P, Reymann KG, Hollt V, Stumm R. Differential regulation of CXCL12 and PACAP mRNA expression after focal and global ischemia. Neuropharmacology 2010; 58:199-207

57. Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. Brain Res 1996; 736:280-286

58. Rebaudo R, Melani R, Balestrino M, Izvarina N. Electrophysiological effects of sustained delivery of CRF and its receptor agonists in hippocampal slices. Brain Res 2001; 922:112-117

59. Bharne AP, Borkar CD, Bodakuntla S, Lahiri M, Subhedar NK, Kokare DM. Pro-cognitive action of CART is mediated via ERK in the hippocampus. Hippocampus 2016;

60. Abraham CR, Mullen PC, Tucker-Zhou T, Chen CD, Zeldich E. Klotho Is a Neuroprotective and Cognition-Enhancing Protein. Vitam Horm 2016; 101:215-238

61. Yang SN, Lu F, Wu JN, Liu DD, Hsieh WY. Activation of gonadotropin-releasing hormone receptors induces a long-term enhancement of excitatory postsynaptic currents mediated by ionotropic glutamate receptors in the rat hippocampus. Neurosci Lett 1999; 260:33-36

62. Lu F, Yang JM, Wu JN, Chen YC, Kao YH, Tung CS, Yang SN. Activation of gonadotropin-releasing hormone receptors produces neuronal excitation in the rat hippocampus. Chin J Physiol 1999; 42:67-71

63. Abel TW, Rance NE. Stereologic study of the hypothalamic infundibular nucleus in young and older women. J Comp Neurol 2000; 424:679-688

64. Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, Yin Y, Li B, Liu G, Cai D. Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH. Nature 2013; 497:211-216

65. Vizi ES, Kiss JP. Neurochemistry and pharmacology of the major hippocampal transmitter systems: synaptic and nonsynaptic interactions. Hippocampus 1998; 8:566-607

66. Lujan R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 1996; 8:1488-1500

67. Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P. The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 1993; 11:771-787

68. Baude A, Nusser Z, Molnar E, McIlhinney RA, Somogyi P. High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. Neuroscience 1995; 69:1031-1055

69. Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P. Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron 1998; 21:545-559

70. He H, Mahnke AH, Doyle S, Fan N, Wang CC, Hall BJ, Tang YP, Inglis FM, Chen C, Erickson JD. Neurodevelopmental role for VGLUT2 in pyramidal neuron plasticity, dendritic refinement, and in spatial learning. J Neurosci 2012; 32:15886-15901

71. Alves SH, Pinheiro G, Motta V, Landeira-Fernandez J, Cruz AP. Anxiogenic effects in the rat elevated plus-maze of 5-HT(2C) agonists into ventral but not dorsal hippocampus. Behav Pharmacol 2004; 15:37-43

72. Nair SG, Gudelsky GA. Activation of 5-HT2 receptors enhances the release of acetylcholine in the prefrontal cortex and hippocampus of the rat. Synapse 2004; 53:202-207

73. Kenney JW, Gould TJ. Modulation of hippocampus-dependent learning and synaptic plasticity by nicotine. Mol Neurobiol 2008; 38:101-121

74. Lobron C, Wevers A, Damgen K, Jeske A, Rontal D, Birtsch C, Heinemann S, Reinhardt S, Maelicke A, Schroder H. Cellular distribution in the rat telencephalon of mRNAs encoding for the alpha 3 and alpha 4 subunits of the nicotinic acetylcholine receptor. Brain Res Mol Brain Res 1995; 30:70-76

75. Stelly CE, Cronin J, Daniel JM, Schrader LA. Long-term oestradiol treatment enhances hippocampal synaptic plasticity that is dependent on muscarinic acetylcholine receptors in ovariectomised female rats. J Neuroendocrinol 2012; 24:887-896

76. Davis DM, Jacobson TK, Aliakbari S, Mizumori SJ. Differential effects of estrogen on hippocampal- and striatal-dependent learning. Neurobiol Learn Mem 2005; 84:132-137

77. Daniel JM, Witty CF, Rodgers SP. Long-term consequences of estrogens administered in midlife on female cognitive aging. Horm Behav 2015; 74:77-85

78. Rodgers SP, Bohacek J, Daniel JM. Transient estradiol exposure during middle age in ovariectomized rats exerts lasting effects on cognitive function and the hippocampus. Endocrinology 2010; 151:1194-1203

79. Medhurst AD, Atkins AR, Beresford IJ, Brackenborough K, Briggs MA, Calver AR, Cilia J, Cluderay JE, Crook B, Davis JB, Davis RK, Davis RP, Dawson LA, Foley AG, Gartlon J, Gonzalez MI, Heslop T, Hirst WD, Jennings C, Jones DN, Lacroix LP, Martyn A, Ociepka S, Ray A, Regan CM, Roberts JC, Schogger J, Southam E, Stean TO, Trail BK, Upton N, Wadsworth G, Wald JA, White T, Witherington J, Woolley ML, Worby A, Wilson DM. GSK189254, a novel H3 receptor antagonist that binds to histamine H3 receptors in Alzheimer's disease brain and improves cognitive performance in preclinical models. J Pharmacol Exp Ther 2007; 321:1032-1045

80. Fukui M, Rodriguiz RM, Zhou J, Jiang SX, Phillips LE, Caron MG, Wetsel WC. Vmat2 heterozygous mutant mice display a depressive-like phenotype. J Neurosci 2007; 27:10520-10529

81. Carrer HF, Araque A, Buno W. Estradiol regulates the slow Ca2+-activated K+ current in hippocampal pyramidal neurons. J Neurosci 2003; 23:6338-6344

82. Jamali K, Naylor BR, Kelly MJ, Ronnekleiv OK. Effect of 17beta-estradiol on mRNA expression of large- conductance, voltage-dependent, and calcium-activated potassium channel alpha and beta subunits in guinea pig. Endocrine 2003; 20:227-237

83. Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R. Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. Science 1999; 285:1929-1931

84. Kelly MJ, Qiu J, Ronnekleiv OK. Estrogen modulation of G-protein-coupled receptor activation of potassium channels in the central nervous system. Ann N Y Acad Sci 2003; 1007:6-16

85. Humphries ES, Dart C. Neuronal and Cardiovascular Potassium Channels as Therapeutic Drug Targets: Promise and Pitfalls. J Biomol Screen 2015; 20:1055-1073

86. Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 2000; 14:2919-2937

87. Gomez-Palacio-Schjetnan A, Escobar ML. Neurotrophins and synaptic plasticity. Curr Top Behav Neurosci 2013; 15:117-136

88. Ramos-Languren LE, Escobar ML. Plasticity and metaplasticity of adult rat hippocampal mossy fibers induced by neurotrophin-3. Eur J Neurosci 2013; 37:1248-1259

89. Gage FH, Buzsaki G, Armstrong DM. NGF-dependent sprouting and regeneration in the hippocampus. Prog Brain Res 1990; 83:357-370

90. Hennigan A, O'Callaghan RM, Kelly AM. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochem Soc Trans 2007; 35:424-427

91. Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD, Jr., Suh H, Couillard-Despres S, Aigner L, Gage FH, Jessberger S. Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. J Neurosci 2012; 32:3376-3387

92. Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D, Blitzer RD, Alberini CM. A critical role for IGF-II in memory consolidation and enhancement. Nature 2011; 469:491-497

93. Sasaki K, Oomura Y, Suzuki K, Hanai K, Yagi H. Acidic fibroblast growth factor prevents death of hippocampal CA1 pyramidal cells following ischemia. Neurochem Int 1992; 21:397-402

94. Sasaki K, Oomura Y, Figurov A, Yagi H. Acidic fibroblast growth factor facilitates generation of long-term potentiation in rat hippocampal slices. Brain Res Bull 1994; 33:505-511

95. Kim YB, Ryu JK, Lee HJ, Lim IJ, Park D, Lee MC, Kim SU. Midkine, heparin-binding growth factor, blocks kainic acid-induced seizure and neuronal cell death in mouse hippocampus. BMC Neurosci 2010; 11:42

96. Duarte-Guterman P, Yagi S, Chow C, Galea LA. Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. Horm Behav 2015; 74:37-52

97. Lisowski P, Juszczak GR, Goscik J, Stankiewicz AM, Wieczorek M, Zwierzchowski L, Swiergiel AH. Stress susceptibility-specific phenotype associated with different hippocampal transcriptomic responses to chronic tricyclic antidepressant treatment in mice. BMC Neurosci 2013; 14:144

98. Jin KL, Mao XO, Nagayama T, Goldsmith PC, Greenberg DA. Induction of vascular endothelial growth factor and hypoxia-inducible factor-1alpha by global ischemia in rat brain. Neuroscience 2000; 99:577-585

99. McCaffery P, Zhang J, Crandall JE. Retinoic acid signaling and function in the adult hippocampus. J Neurobiol 2006; 66:780-791

100. Remaud S, Gothie JD, Morvan-Dubois G, Demeneix BA. Thyroid hormone signaling and adult neurogenesis in mammals. Front Endocrinol (Lausanne) 2014; 5:62

101. Xu Y, Sheng H, Bao Q, Wang Y, Lu J, Ni X. NLRP3 inflammasome activation mediates estrogen deficiency-induced depression- and anxiety-like behavior and hippocampal inflammation in mice. Brain Behav Immun 2016;

102. Chan PH, Kawase M, Murakami K, Chen SF, Li Y, Calagui B, Reola L, Carlson E, Epstein CJ. Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. J Neurosci 1998; 18:8292-8299

103. Nilsen J, Diaz Brinton R. Mechanism of estrogen-mediated neuroprotection: regulation of mitochondrial calcium and Bcl-2 expression. Proc Natl Acad Sci U S A 2003; 100:2842-2847

104. Sales S, Ureshino RP, Pereira RT, Luna MS, Pires de Oliveira M, Yamanouye N, Godinho RO, Smaili SS, Porto CS, Abdalla FM. Effects of 17beta-estradiol replacement on the apoptotic effects caused by ovariectomy in the rat hippocampus. Life Sci 2010; 86:832-838

105. Sharma K, Mehra RD. Long-term administration of estrogen or tamoxifen to ovariectomized rats affords neuroprotection to hippocampal neurons by modulating the expression of Bcl-2 and Bax. Brain Res 2008; 1204:1-15

106. Sugawara T, Noshita N, Lewen A, Gasche Y, Ferrand-Drake M, Fujimura M, Morita-Fujimura Y, Chan PH. Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. J Neurosci 2002; 22:209-217

107. Zhao L, Wu TW, Brinton RD. Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. Brain Res 2004; 1010:22-34

108. Hashimoto M, Bar-On P, Ho G, Takenouchi T, Rockenstein E, Crews L, Masliah E. Beta-synuclein regulates Akt activity in neuronal cells. A possible mechanism for neuroprotection in Parkinson's disease. J Biol Chem 2004; 279:23622-23629

109. Hashimoto M, Rockenstein E, Mante M, Crews L, Bar-On P, Gage FH, Marr R, Masliah E. An antiaggregation gene therapy strategy for Lewy body disease utilizing beta-synuclein lentivirus in a transgenic model. Gene Ther 2004; 11:1713-1723

110. Au A, Feher A, McPhee L, Jessa A, Oh S, Einstein G. Estrogens, inflammation and cognition. Front Neuroendocrinol 2016; 40:87-100

111. Davis SR, Lambrinoudaki I, Lumsden M, Mishra GD, Pal L, Rees M, Santoro N, Simoncini T. Menopause. Nat Rev Dis Primers 2015; 1:15004

112. Stuenkel CA, Davis SR, Gompel A, Lumsden MA, Murad MH, Pinkerton JV, Santen RJ. Treatment of Symptoms of the Menopause: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2015; 100:3975-4011

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	*Spock3*	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 3
1368585_PM_at	1.188	2.278	0.141	*Cartpt*	CART prepropeptide
1391387_PM_s_at	1.158	2.232	0.479	*Slbp*	stem-loop binding protein
1391208_PM_at	1.087	2.124	0.178	*Pcdh20*	protocadherin 20
1380805_PM_at	1.060	2.084	0.310	*Pvrl3*	poliovirus receptor-related 3
1393247_PM_at	0.974	1.965	0.462	*Zfp26*	zinc finger protein 26
1378679_PM_at	0.963	1.949	0.446	*Usp25*	ubiquitin specific peptidase 25
1390812_PM_a_at	0.961	1.947	0.159	*Rerg*	RAS-like, estrogen-regulated, growth-inhibitor
1398355_PM_at	0.950	1.931	0.456	*Trpm7*	transient receptor potential cation channel, subfamily M, 7
1380279_PM_at	0.934	1.911	0.410	*Prcp*	prolylcarboxypeptidase (angiotensinase C)
1383396_PM_at	0.929	1.904	0.460	*Fndc3a*	fibronectin type III domain containing 3a
1375676_PM_at	0.915	1.886	0.460	*Lin7c*	lin-7 homolog C (*C. elegans*)
1374959_PM_at	0.894	1.858	0.457	*Nqo2*	NAD(P)H dehydrogenase, quinone 2
1389479_PM_at	0.890	1.853	0.419	*Klf3*	Kruppel-like factor 3 (basic)
1395744_PM_at	0.886	1.849	0.477	*Sppl2a*	signal peptide peptidase-like 2A
1389362_PM_at	0.856	1.811	0.246	*Ptpn3*	protein tyrosine phosphatase, non-receptor type 3
1382171_PM_at	0.851	1.804	0.515	*Tsc22d2*	TSC22 domain family, member 2
1387076_PM_at	0.850	1.802	0.460	*Hif1a*	hypoxia-inducible factor 1, alpha subunit
1375538_PM_at	0.835	1.784	0.519	*Vcl*	vinculin
1376319_PM_at	0.834	1.783	0.061	*Sema3c*	semaphorin 3C
1382390_PM_at	0.809	1.752	0.469	*Fubp3*	far upstream element (FUSE) binding protein 3
1367598_PM_at	0.793	1.733	0.616	*Ttr*	transthyretin
1372905_PM_at	0.782	1.720	0.530	*Vcl*	vinculin
1377651_PM_at	0.774	1.710	0.445	*Trio*	triple functional domain (PTPRF interacting)
Downregulated Genes					
1370472_PM_a_at	-0.802	0.574	0.445	*Kcnma1*	K large conductance Ca-activated channel, subfamily M, α 1
1391074_PM_at	-0.807	0.572	0.440	*Crabp1*	cellular retinoic acid binding protein 1
1367768_PM_at	-0.807	0.572	0.222	*Lxn*	latexin
1383386_PM_a_at	-0.814	0.569	0.463	*Sec3l1*	SEC3-like 1 (*S. cerevisiae*)
1391075_PM_at	-0.817	0.568	0.341	*Rgs17*	regulator of G-protein signaling 17
1392823_PM_at	-0.818	0.567	0.419	*Snhg11*	small nucleolar RNA host gene 11 (non-protein coding)
1369418_PM_at	-0.828	0.563	0.419	*Kcnj9*	potassium inwardly-rectifying channel, subfamily J, member 9
1369280_PM_at	-0.833	0.561	0.326	*Kcnk9*	potassium channel, subfamily K, member 9
1387291_PM_at	-0.861	0.551	0.123	*Itih3*	inter-alpha trypsin inhibitor, heavy chain 3
1379481_PM_at	-0.863	0.550	0.460	*Pabpn1*	poly(A) binding protein, nuclear 1
1370248_PM_at	-0.873	0.546	0.159	*Fxyd6*	FXYD domain-containing ion transport regulator 6
1371132_PM_a_at	-0.875	0.545	0.459	*Ank3*	ankyrin 3, node of Ranvier
1389089_PM_at	-0.887	0.541	0.479	*Slc39a7*	solute carrier family 39 (zinc transporter), member 7
1386940_PM_at	-0.911	0.532	0.419	*Timp2*	TIMP metallopeptidase inhibitor 2
1379046_PM_at	-0.920	0.528	0.479	*Mlec*	malectin
1398303_PM_s_at	-0.948	0.518	0.459	*Tpm3*	tropomyosin 3, gamma
1368883_PM_at	-0.969	0.511	0.184	*Nov*	nephroblastoma overexpressed gene
1376980_PM_at	-0.973	0.510	0.222	*Htr2c*	5-hydroxytryptamine (serotonin) receptor 2C
1392555_PM_at	-1.029	0.490	0.166	*Etv1*	ets variant 1
1397513_PM_at	-1.045	0.485	0.431	*Ralyl*	RALY RNA binding protein-like
1371462_PM_at	-1.054	0.482	0.103	*Igfbp4*	insulin-like growth factor binding protein 4
1373257_PM_at	-1.068	0.477	0.419	*Arpp21*	cAMP-regulated phosphoprotein 21
1389155_PM_at	-1.081	0.473	0.393	*Dos*	downstream of Stk11
1368381_PM_at	-1.095	0.468	0.439	*Crtac1*	cartilage acidic protein 1
1384809_PM_at	-1.119	0.460	0.026	*Gpr123*	G protein-coupled receptor 123
1390865_PM_at	-1.121	0.460	0.026	*Cadps2*	Ca++-dependent secretion activator 2
1385491_PM_at	-1.143	0.453	0.299	*Pnmal2*	PNMA-like 2
1370602_PM_at	-1.301	0.406	0.159	*Atp2b4*	ATPase, Ca++ transporting, plasma membrane 4
1385788_PM_at	-1.479	0.359	0.419	*Ephb3*	Eph receptor B3
1368564_PM_at	-1.482	0.358	0.133	*Slc17a6*	sodium-dependent inorganic phosphate cotransporter
1368892_PM_at	-1.654	0.318	0.026	*Adcyap1*	adenylate cyclase activating polypeptide 1
1368505_PM_at	-1.868	0.274	0.032	*Rgs4*	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

664 **Table 2. PCR results**

Assay ID	Symbol	Target name	RQ	P value
Neurotransmitter and neuropeptide signaling/ Synaptic plasticity				
Rn01645174_m1	*Cartpt*	CART propeptide	2.598	0.003
Rn00562124_m1	*Ttr*	transthyretin	2.251	0.097
Rn00564688_m1	*Slc18a2*	vesicular monoamine transporter 2	1.645	0.001
Rn00578981_m1	*Gnrhr*	gonadotropin releasing hormone receptor	1.631	0.004
Rn01454304_m1	*Dagla*	diacylglycerol lipase alpha	1.387	0.013
Rn00582505_m1	*Slc12a2*	Na-K-Cl cotransporter	1.386	0.094
Rn03993699_s1	*Cnr2*	cannabinoid receptor 2	1.250	0.070
Rn01505088_m1	*Enpp2*	ectonucleotide phosphodiesterase 2	1.221	0.015
Rn01234233_m1	*Kcnc2*	voltage-gated potassium channel	0.861	0.058
Rn00563215_m1	*Cck*	cholecystokinin	0.821	0.007
Rn01512605_s1	*Chrm4*	cholinergic receptor, muscarinic 4	0.775	0.031
Rn00578611_m1	*Crhr1*	corticotropin releasing hormone receptor 1	0.766	0.063
Rn00565867_m1	*Cckbr*	cholecystokinin B receptor	0.748	0.111
Rn01430567_m1	*Vip*	vasoactive intestinal peptide	0.733	0.001
Rn00561699_m1	*Oprd1*	delta 1 opioid receptor	0.723	0.051
Rn00585276_m1	*Hrh3*	histamine receptor H3	0.686	0.000
Rn00575617_m1	*Crhr2*	corticotropin releasing hormone receptor 2	0.650	0.016
Rn00567566_m1	*Penk*	preproenkephalin	0.644	0.000
Rn01456072_m1	*Cadps2*	Ca-dependent activator protein for secretion	0.599	0.001
Rn02532012_s1	*Sstr1*	somatostatin receptor 1	0.382	0.001
Rn00562748_m1	*Htr2c*	5HT receptor 2C	0.363	0.000
Rn00573505_m1	*Grm8*	glutamate metabotropic receptor 8	0.357	0.001
Rn00584780_m1	*Slc17a6*	vesicular glutamate transporter 2	0.260	0.000
Rn00583820_m1	*Chrna3*	cholinergic receptor nicotinic alpha 3 subunit	0.214	0.001
Growth and troph factor signaling/ Neurogenesis				
Rn01414596_m1	*Otx2*	orthodenticle homeobox 2	4.934	0.014
Rn00591759_m1	*Folr1*	folate receptor 1	2.360	0.004
Rn00580123_m1	*Kl*	klotho	2.104	0.094
Rn00572130_m1	*Ntrk1*	neurotrophic receptor tyrosine kinase 1	1.870	0.069
Rn01754856_m1	*Ucp2*	uncoupling protein 2	1.548	0.002
Rn00561634_m1	*Ngfr*	nerve growth factor receptor	1.506	0.008
Rn00579280_m1	*Ntf3*	neurotrophin 3	1.491	0.003
Rn01640372_m1	*Esr1*	estrogen receptor alpha	1.422	0.030
Rn01427989_s1	*Cdkn1a*	cyclin-dependent kinase inhibitor 1A	1.404	0.002
Rn00675549_g1	*Mdk*	midkine	1.355	0.054
Rn01533872_m1	*Ngf*	nerve growth factor	1.306	0.023
Rn00689153_m1	*Fgf1*	fibroblast growth factor 1	1.278	0.039
Rn01537468_g1	*Sgk1*	serum- glucocorticoid-regulated kinase 1	1.257	0.018
Rn01454518_m1	*Igf2*	insulin-like growth factor 2	1.160	0.109
Rn00578713_m1	*Adcy2*	adenylate cyclase 2	0.881	0.004
Rn00575368_m1	*Fkbp1b*	FK506 binding protein 1B	0.863	0.017
Rn00441185_m1	*Rxra*	retinoid X receptor alpha	0.852	0.068
Rn00567957_m1	*Map6*	microtubule-associated protein 6	0.814	0.020
Rn99999125_m1	*Bcl2*	B-cell lymphoma 2	0.780	0.001
Rn00562044_m1	*Thrb*	thyroid hormone receptor beta	0.777	0.008
Rn00578390_m1	*Nov*	nephroblastoma overexpressed	0.732	0.001
Rn01464112_m1	*Igfbp4*	insulin-like growth factor binding protein 4	0.480	0.001
Rn00566438_m1	*Adcyap1*	adenylate cyclase activating polypeptide 1	0.411	0.001
Rn01483363_m1	*Atp2b4*	ATPase plasma membrane Ca transporting 4	0.361	0.000
Rn00566938_m1	*Sod1*	superoxide dismutase 1	0.284	0.001
Rn01490867_g1	*Rgs4*	regulator of G protein signaling 4	0.272	0.000
Immune modulation/ Defense				
Rn03034964_u1	*RT1-EC2*	RT1-EC2	2.121	0.012
Rn00580555_m1	*Ccl2*	C-C motif chemokine ligand 2	1.930	0.010
Rn00566466_m1	*C3*	complement C3	1.750	0.001
Rn01488472_g1	*Tlr3*	toll-like receptor 3	1.663	0.006
Rn00709342_m1	*Itgam*	Cd11b	1.639	0.005
Rn00580432_m1	*Il1b*	interleukin 1beta	1.437	0.114
Rn00560589_m1	*A2m*	alpha-2-macroglobulin	1.330	0.001
Rn00564605_m1	*Ptgds*	prostaglandin D2 synthase	1.253	0.039
Rn01483598_m1	*Fcgr3a*	Fc gamma receptor 3a	1.245	0.058
Rn00574125_g1	*Aif1*	allograft inflammatory factor 1	1.207	0.036
Rn00569848_m1	*Tlr4*	toll-like receptor 4	1.197	0.028
Rn00563082_m1	*Mfge8*	milk fat globule-EGF factor 8 protein	0.777	0.001

665

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

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

676 **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		
Esr1	estrogen receptor alpha	1.422
Fgf1	fibroblast growth factor 1	1.278
Hif1a	hypoxia-inducible factor 1, alpha subunit	*1.802*
Igf2	insulin-like growth factor 2	1.160
Mdk	midkine	1.355
Ngf	nerve growth factor	1.306
Ngfr	nerve growth factor receptor	1.506
Ntrk1	neurotrophic tyrosine kinase, receptor, type 1	*1.520*
Ntf3	neurotrophin 3	1.491
Nr4a3	nuclear receptor subfamily 4, group A, member 3	*1.522*
Otx2	orthodenticle homeobox 2	4.934
Rerg	RAS-like, estrogen-regulated, growth-inhibitor	1.947
Peptides/transmitters/ion channels/signaling		
Cartpt	CART prepropeptide	*2.278*
Gria4	glutamate receptor, ionotrophic, AMPA 4	*1.506*
Grm1	glutamate receptor, metabotropic 1	*1.532*
Gls	glutaminase	*1.580*
Gnrhr	gonadotropin releasing hormone receptor	1.631
Kcnj6	K inwardly-rectifying channel, subfamily J, 6	*1.517*
Kl	klotho	2.104
Slc12a2	Na-K-Cl cotransporter	1.386
Ttr	transthyretin	2.251
Slc18a2	vesicular monoamine transporter 2	1.645
Immune response/defense		
Aif1	allograft inflammatory factor 1	1.207
A2m	alpha-2-macroglobulin	*1.330*
Ccl2	C-C motif chemokine ligand 2	1.930
C3	complement C3	1.750
Itgam	Cd11b	*1.639*
Cfh	complement factor H	*1.551*
Fcgr3a	Fc gamma receptor 3a	1.245
Il1b	interleukin 1beta	1.437
RT1-EC2	RT1-EC2	*2.121*
Sep7	septin 7	1.545
Tlr3	toll-like receptor 3	1.663
Tlr4	toll-like receptor 4	1.197

677

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

682 **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		
Htr2c	5HT receptor 2C	*0.363*
Adcy2	adenylate cyclase 2	*0.881*
Adcyap1	adenylate cyclase activating polypeptide 1	*0.411*
Crebl2	cAMP responsive element binding protein-like 2	0.604
Cbln2	cerebellin 2 precursor	0.613
Cck	cholecystokinin	*0.821*
Cckbr	cholecystokinin B receptor	*0.748*
Chrna3	cholinergic receptor nicotinic alpha 3 subunit	*0.214*
Chrm4	cholinergic receptor, muscarinic 4	*0.775*
Crhr1	corticotropin releasing hormone receptor 1	*0.766*
Crhr2	corticotropin releasing hormone receptor 2	*0.650*
Oprd1	delta 1 opioid receptor	*0.723*
Grm8	glutamate metabotropic receptor 8	*0.357*
Hrh3	histamine receptor H3	*0.686*
Kcnk9	K channel, subfamily K, member 9	0.561
Kcnj9	K inwardly-rectifying channel, subfamily J, 9	0.563
Kcnma1	K large conductance Ca-activated channel, subfamily M	0.574
Nts	neurotensin	0.653
Penk	preproenkephalin	*0.644*
Rgs4	regulator of G protein signaling 4	*0.272*
Rgs17	regulator of G-protein signaling 17	0.568
Sstr1	somatostatin receptor 1	*0.382*
Vip	vasoactive intestinal peptide	*0.733*
Slc17a6	vesicular glutamate transporter 2	*0.260*
Vdac1	voltage-dependent anion channel 1	0.641
Kcnc2	voltage-gated potassium channel	*0.861*
Solute carriers		
Slc1a4	solute carrier family 1, member 4	0.657
Slc10a4	solute carrier family 10, member 4	0.638
Slc17a6	solute carrier family 17, member 6	0.358
Slc25a18	solute carrier family 25, member 18	0.646
Slc39a7	solute carrier family 39, member 7	0.541
Slc4a3	solute carrier family 4, member 3	0.653
Slc6a17	solute carrier family 6, member 17	0.623
Slc9a1	solute carrier family 9, member 1	0.631
Slc9a5	solute carrier family 9, member 5	0.648
Growth factor/transcription regulation		
Arnt2	aryl hydrocarbon receptor nuclear translocator 2	0.657
Crabp1	cellular retinoic acid binding protein 1	0.572
Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	0.646
Igfbp4	insulin-like growth factor binding protein 4	*0.480*
Rxra	retinoid X receptor alpha	*0.852*
Thrb	thyroid hormone receptor beta	*0.777*
Immune response/defense		
Apitd1	apoptosis-inducing, TAF9-like domain 1	0.643
Bcl2	B-cell lymphoma 2	*0.780*
C1ql3	complement component 1, q subcomponent-like 3	0.646
Il3ra	interleukin 3 receptor, alpha	0.661
Sod1	superoxide dismutase 1	*0.284*
Sncb	synuclein, beta	0.614

683

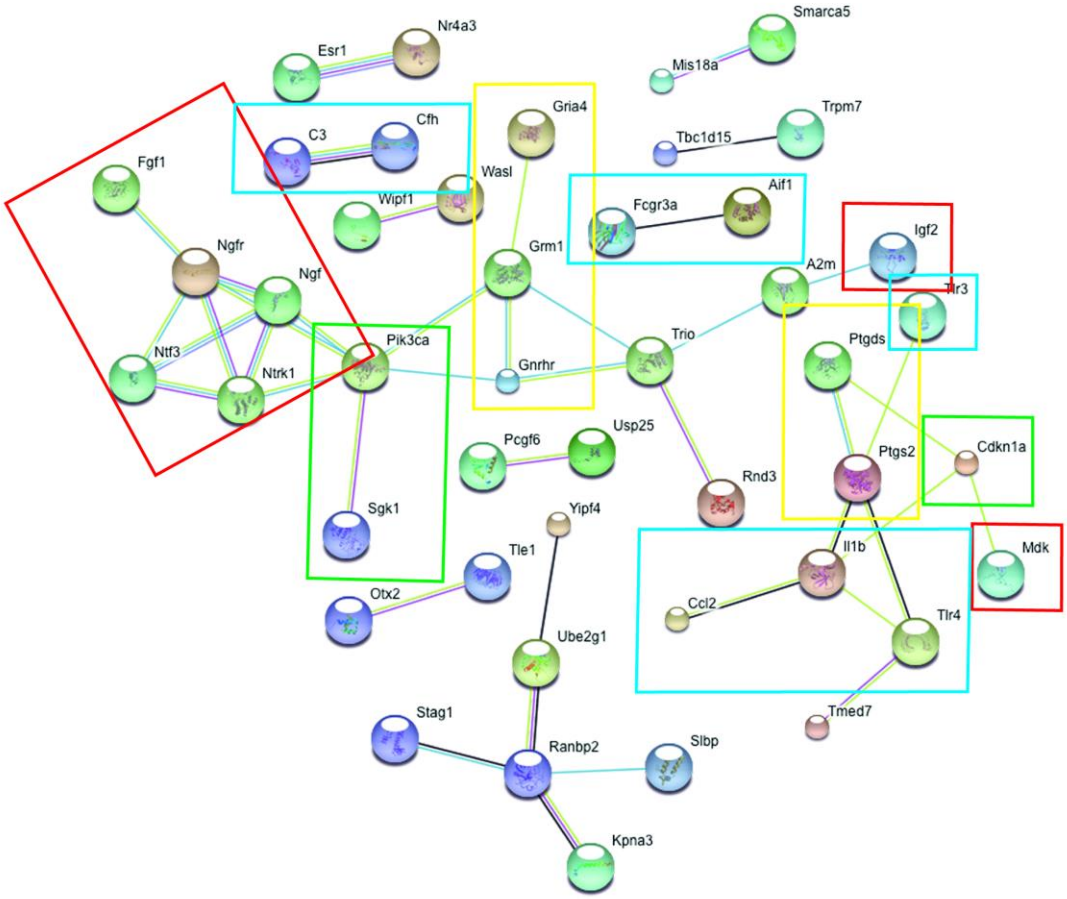
Representative downregulated genes assembled in four functional clusters as peptides/transmitters/receptors/ion channels/signaling, solute carriers, growth factor/transcription regulation and immune response/defense. FC in italics refers to PCR results. FC, fold change.

Figure legends

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

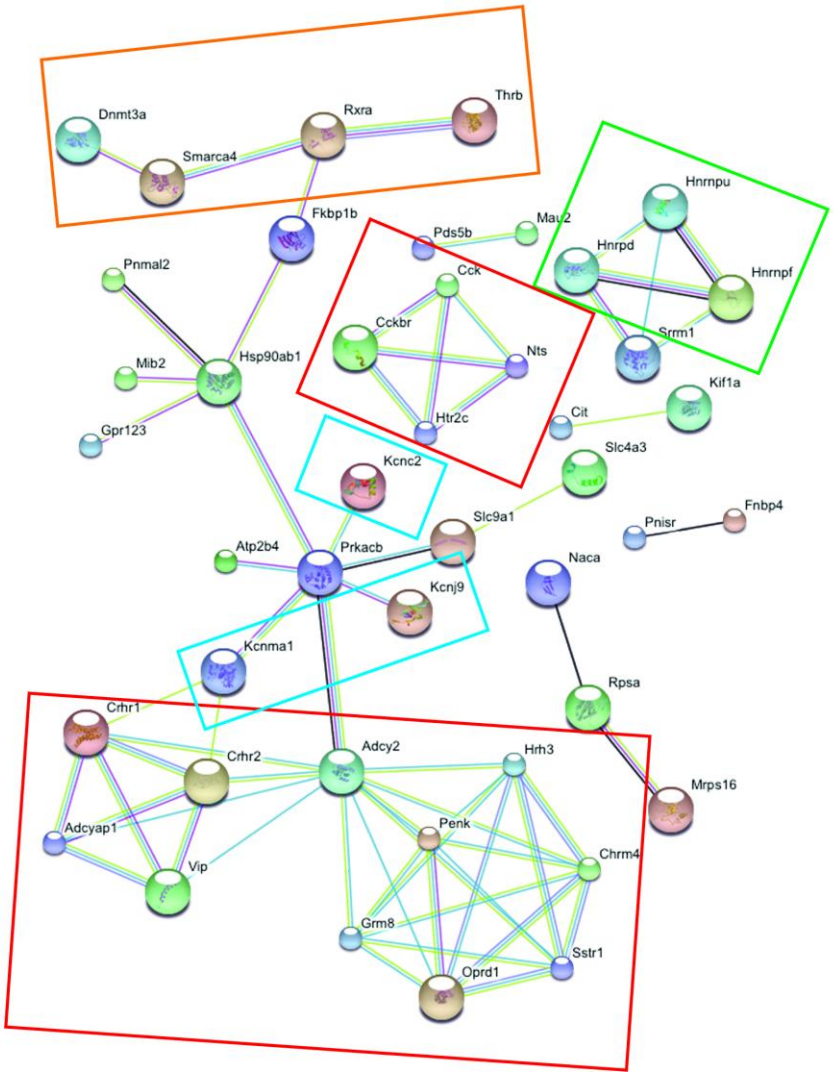
Figure 2. Predicted interactions among proteins encoded by downregulated genes in long-term ovariectomized, middle aged rats. The network is based on combined results of microarray and quantitative real-time PCR studies and was constructed by using the STRING 10 Known and Predicted Protein-Protein Interactions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

705 Figure 1



706

707 Figure 2



708