Short title (Running head)	Sex-Dependent Gene Expression in GnRH Neurons
Section title	Original Paper
Issue ID	000
Citation line	Neuroendocrinology
DOI: 10.1159/000	430818
Article title	Differential Gene Expression in Gonadotropin-Releasing Hormone Neurons of Male and Metestrous Female Mice
Subtitle	

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Received	Received: January 14, 2015
Accepted	Accepted after revision: April 20, 2015
Revised	
Published Online	Published online: April 29, 2015

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Key Words	Gonadotropin-releasing hormone Dimorphism Pico profiling Gene expression Microarray Laser capture microdissection Single-cell qPCR Real-time PCR

Abbreviations	

Abstract

Background: Gonadotropin-releasing hormone (GnRH) neurons play a pivotal role in the regulation of the hypothalamic-pituitary gonadal axis in a sex-specific manner. We hypothesized that the differences seen in reproductive functions of males and females are associated with a sexually dimorphic gene expression profile of GnRH neurons. **Methods and Results:** We compared the transcriptome of GnRH neurons obtained from intact metestrous female and male GnRH-green fluorescent protein transgenic mice. About 1,500 individual GnRH neurons from each sex were sampled with laser capture microdissection followed by whole-transcriptome amplification for gene expression profiling. Under stringent selection criteria (fold change >1.6, adjusted p value 0.01), Affymetrix Mouse Genome 430 PM array analysis identified 543 differentially expressed genes. Sexual dimorphism was most apparent in gene clusters associated with synaptic communication, signal transduction, cell adhesion, vesicular transport and cell metabolism. To validate microarray results, 57 genes were selected, and 91% of their differential expression was confirmed by real-time PCR. Similarly, 88% of microarray results were confirmed with PCR from independent samples obtained by patch pipette harvesting and pooling of 30 GnRH neurons from each sex. We found significant differences in the expression of genes involved in vesicle priming and docking *(Syt1, Cplx1)*, GABAergic *(Gabra3, Gabrb3, Gabrg2)* and glutamatergic *(Gria1, Grin1, Slc17a6)* neurotransmission, peptide signaling *(Sstr3, Npr2, Cxcr4)* and the regulation of intracellular ion homeostasis *(Cacna1, Cacnb1, Cacng5, Kcnq2, Kcnc1)*. **Conclusion:** The striking sexual dimorphism of the GnRH neuron transcriptome we report here contributes to a better understanding of the differences in cellular mechanisms of GnRH

neurons in the two sexes.

Body

Introduction

The gonadotropin-releasing hormone (GnRH) neuronal system of the brain has a fundamental role in maintenance of reproductive physiology [1]. GnRH neurons originate from the olfactory placode and migrate to the preoptic-septal region where they settle down and integrate into the limbic system [2--4]. GnRH axon projections to the hypothalamic median eminence (ME) [5] feed the pituitary-gonadal axis by episodic GnRH secretion into the portal circulation [6, 7], whereas GnRH axonal outflows to different parts of the limbic brain [8] are responsible for orchestrating the extrahypothalamic functions of the GnRH system [9, 10]. GnRH neurons have been shown to receive neuronal information from several basic neurotransmitter systems of the brain [11, 12]. They also communicate with microglia and astroglia [13--16] and process remote metabolic signals originating from different peripheral tissues/organs of the body [17--19]. Events that interfere with the pulsatile secretion of GnRH [20] inhibit reproduction [21--24].

Recent evidence also indicates that GnRH neurons may feed back to their neuronal afferent system [25]. Furthermore, they sense hormones secreted by the gonads. Gonadal hormones are capable of acting on GnRH neurons directly via nongenomic [26, 27] and longer-lasting, genomic mechanisms [28], the latter mediated by gonadal steroid receptors for female and male sex hormones [11, 29--33]. In addition to influencing GnRH neurons directly, gonadal hormones exert feedback action on steroid receptor-expressing interneuron populations that are wired to GnRH neurons via communicating synapses [34--36]. Gonadal hormones exert negative and positive feedback effects on the neuronal machinery controlling reproduction [37]. In male rodents, androgen hormones exert an inhibitory, negative feedback effect on the hypothalamic regulatory network. In females, estrogens also suppress the system almost throughout the ovarian cycle. The exception is the period of proestrus when the positive feedback action of rising estradiol level triggers the development of the hypothalamic GnRH and luteinizing hormone (LH) surge followed by ovulation [38].

The physiology of GnRH neurons differs between sexes, partly because of the distinct gonadal hormone milieu. Changing levels of estradiol across the reproductive cycle in females cause cyclic variations in the electric and secretory activity of GnRH neurons. During critical developmental periods, gonadal steroids also exert long-lasting and sex-specific organizational effects on the neuronal network impinging on GnRH cells and the limbic system [39]. Besides the regulation of gonadal functions, GnRH peptide can also facilitate sex-specific behavior in rats [10, 40].

In this study, we hypothesized that functional differences in the reproductive regulation of males and females are associated with a sexually dimorphic gene expression profile of GnRH neurons. To test this hypothesis, we compared the gene expression profiles of GnRH neurons obtained from intact metestrous female and male GnRH-green fluorescent protein (GFP) transgenic mice under a similar physiological condition characterized by the negative gonadal hormone feedback.

First, whole-transcriptome analysis (Affymetrix Mouse Genome 430 PM array) of male and female GnRH neurons was carried out from RNA samples collected with laser capture microdissection (LCM) of GnRH-GFP neurons. Then, the results were validated with quantitative real-time PCR (qPCR). Finally, PCR control studies were replicated using independent samples obtained by patch pipette harvesting and pooling of 30 GnRH neurons from each model.

The achieved results reveal differential expression of over 500 genes by sex in GnRH neurons that are involved in synaptic signaling, protein homeostasis, signal transduction, axonal transport, vesicular functions and cellular morphogenesis. The sex-dependent differential gene expression of hypothalamic GnRH neurons may account for differences seen in the function of the GnRH neuronal system between the sexes.

Materials and Methods

Ethics Statement

All studies were carried out with the permission from the Animal Welfare Committee of the Institute of Experimental Medicine (IEM) of the Hungarian Academy of Sciences (permission No. A5769-01) and in accordance with legal requirements of the European Community (Decree86/609/EEC). All animal experimentation described was conducted in accordance with accepted standards of humane animal care, and all efforts were made to minimize suffering.

Animals

Adult, gonadally intact female and male mice were used from local colonies bred at the Medical Gene Technology Unit of the IEM. They were housed in a light (12:12 light-dark cycle, lights on at 6:00 h)- and temperature (22 ± 2°C)-controlled environment, with free access to standard food and tap water. GnRH-GFP transgenic mice bred on a C57BL/6J genetic background were used for harvesting the cytoplasm of GnRH neurons. In this animal model, a GnRH promoter segment drives selective GFP expression in the majority of GnRH neurons [41]. In order to avoid possible circadian effects, both male and female animals were sacrificed at the same period of the day, between 15:00 and 17:00 h. To minimize variations in females caused by cyclic changes in serum estradiol, female mice were sacrificed in metestrus when serum estradiol, LH [42, 43] and negative feedback levels are low. The estrous cycle was monitored daily between 9 and 10 a.m. by microscopic evaluation of vaginal cytology [44, 45]. Female mice with at least two consecutive 4- to 6-day-long, regular estrous cycles were used in their metestrous phase.

Processing of Animals for LCM of GnRH Neurons

Fixation

Brain fixation, preparation of sections for the subsequent LCM and RNA isolation were performed as reported elsewhere [46] with minor modifications. Briefly, male (n = 6) and metestrous female (n = 6) mice were deeply anesthetized with ketamine/xylazine (100 and 10 mg/kg body weight, respectively) and perfused transcardially at a flow rate of 8 ml/min with 80 ml 0.5% paraformaldehyde in DEPC-treated PBS (pH 7.4), followed by 20% sucrose in DEPC-PBS. The brains were removed from the skull, and then frozen quickly on dry ice and stored at --80°C. This protocol minimized mRNA degradation, while preserving sufficiently GFP fluorescence in GnRH neurons.

Section Preparation

For microdissection, 7-µm-thick coronal brain sections were cut at --20°C using a CM3050S cryostat (Leica, Wetzlar, Germany). Sections were mounted on PEN-membrane slides (Zeiss, Jena, Germany), briefly thawed and dried, then dehydrated in 50% ethanol (20 s) and n-butanol (90 s) followed by xylene substitution (60 s). Sections were air-dried and desiccated in a vacuum chamber and processed further for laser microdissection.

Sampling of GnRH Neurons

Uniform and representative sampling of the entire GnRH neuronal population (fig. 1a1, a2) was performed using LCM performed on a PALM Microbeam system (Carl Zeiss Microimaging GmbH, Jena, Germany) which was equipped with an epifluorescent setup. GFP-expressing GnRH neurons of the median preoptic area were identified by their fluorescent activity at 470-nm illumination and characteristic fusiform shape (fig. 1a1, a2). Outlines for the laser cut were carefully adjusted over the image of GnRH-GFP neurons by the PALM software (fig. 1b1) to minimize sample contamination with non-GnRH tissues. Then, the microdissection of GnRH neurons was carried out with a laser beam (fig. 1b2). Cells were then pressure-catapulted with a single laser pulse from the object plane into 0.2-ml collecting tube caps (Carl Zeiss Microimaging) using a ×40 objective lens. 250 GFP-positive neurons were dissected and pooled from 80 to 100 consecutive sections of each brain (fig. 1c).

Tissue Processing for Patch Pipette Harvesting of GnRH Neurons

Brain Slice Preparation

Brain slices were prepared as described earlier [17], with slight modifications. Male and metestrous female mice were deeply anesthetized using isoflurane inhalation. The brain was removed rapidly and immersed in ice-cold sodium-free artificial cerebrospinal fluid (Na-free aCSF) bubbled with a mixture of 95% O_2 and 5% CO_2. The solution contained the following (in mM): saccharose 205, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 5, NaH_2PO_4 1.25, $CaCl_2$ 1, glucose 10. Hypothalamic blocks were dissected, and 100-µm-thick coronal slices were prepared from the medial septum/preoptic area with a VT1000S vibratome (Leica). Slices between bregma levels 1.42--0.6 mm [47] were collected in the ice-cold oxygenated Na-free aCSF. The slices were then equilibrated for 1 h in normal aCSF saturated with O_2/CO_2 and containing the following (in mM): NaCl 130, KCl 3.5, $NaHCO_3$ 26, $MgSO_4$ 1.2, NaH_2PO_4 1.25, $CaCl_2$ 2.5, glucose 10. The initial temperature of aCSF was 33°C, and during equilibration it was allowed to cool to room temperature.

Axopatch-200B patch-clamp amplifier, Digidata-1322A data acquisition system and pCLAMP 9.2 software (Molecular Devices Co., Silicon Valley, Calif., USA) were used. Cells were visualized with a BX51WI IR-DIC microscope (Olympus Co., Tokyo, Japan). The patch electrodes (OD = 1.5 mm, thin wall, Hilgenberg GmbH, Malsfeld, Germany) were pulled with a Flaming-Brown P-97 puller (Sutter Instrument Co., Novato, Calif., USA) and polished with an MF-830 microforge (Narishige Inc., Tokyo, Japan).

GnRH-GFP neurons were identified by brief illumination at 470 nm using an epifluorescent filter set, based on their green fluorescence, typical fusiform shape and topographic location in the preoptic area [41].

Harvesting of the Cytoplasm from GnRH Neurons

To harvest the cytoplasm of GnRH-GFP neurons for the independent PCR experiment, patch pipettes were pulled from capillaries sterilized at 180°C for 6 h. The pipettes were filled with autoclaved intracellular pipette solution containing (in mM) HEPES 10, KCl 140, EGTA 5 and $CaCl_2$ 0.1 (pH 7.3 with NaOH). The resistance of the patch electrodes was 2--3 MΩ. Harvesting was carried out in oxygenated aCSF at 33°C as described earlier [17]. Briefly, after establishing the whole-cell patch configuration, the cytoplasm was harvested by applying gentle negative pressure under visual control (fig. 1d1, d2) [48, 49]. Cytoplasmic samples of 10 cells from each mouse (males n = 3 and females n = 3) were collected by breaking the pipette tip into PCR tubes kept on dry ice.

Pico Profiling of GnRH Neurons

RNA Isolation

GnRH cell samples collected with LCM were incubated in 200 µl lysis buffer (Tris-HCl, pH 8.0, 10 mM; EDTA 50 mM; NaCl 200 mM; SDS 2.2%; anti-RNase 1,000 U/ml; Prot-K 1 mg/ml) at 56°C for 3 h. RNA was isolated from the lysate by an optimized proteinase K/acid phenol method as described earlier [46]. RNA was purified using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany). Total RNA was eluted with 14 µl of ribonuclease-free water and then stored at --80°C. The quality of RNA was measured with Bioanalyzer.

Whole-Transcriptome Amplification

Library preparation and amplification were performed according to the manufacturer's (Sigma-Aldrich) instructions for the WTA2 kit. To monitor amplification yield, SYBR Green (Sigma-Aldrich) was added to the amplification reaction, which was performed in a ViiA 7 real-time PCR instrument (Life Technologies, Carlsbad, Calif., USA). When the SYBR Green signal reached a plateau, the reaction was stopped. The amplified double-stranded cDNA was purified and quantified on a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, Waltham, Mass., USA).

Mouse Genome 430 PM Arrays

A total of 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, Calif., USA). Hybridization, washing, staining and scanning of Affymetrix Mouse Genome 430 PM Strip arrays were performed following the manufacturer's recommendations.

Data Analysis of Mouse Genome 430 PM Arrays

The Mouse Genome 430 PM Strip array allows the analysis of 34,325 well-annotated genes using 45,123 distinct probe sets. Scanned images (DAT files) were transformed into intensities (CEL files) using the AGCC software (Affymetrix). RMA analysis to obtain probe set level expression estimates was performed by means of the Partek Genomics Suite (Partek Inc., St. Louis, Mo., USA).

Bioinformatics, Statistical Analysis

Quality assessment of microarrays was performed using affyQCReport [50]. Raw microarray data were preprocessed for analysis using RMA [51]. Fold change (FC) was calculated from normalized and log2-transformed gene expression microarray data for each probe set.

In microarray data evaluation, FC estimation and difference analysis of gene expression were based on linear models combined with Bayesian methods [52]. Obtained p values were adjusted by the FDR-based method [53]. All statistical and data mining work was performed in R-environment [54] with Bioconductor packages [55].

The following cutoff criteria were applied on the differentially expressed genes: (1) FC >1.6; (2) adjusted p value (p_{adj}) <0.01; (3) normalized raw expression value >6.0. To highlight the most relevant gene annotations associated with the gene list, a functional annotation tool, DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov), was used [56, 57] at its default setting. As the result of the analysis, annotation clusters of terms were ranked hierarchically by their score number calculated from the modified Fisher's exact p value of each term.

qPCR Studies

For qPCR investigations of LCM-derived GnRH samples (males n = 4, females n = 6), RNA isolation and whole-transcriptome amplification were performed as described in the previous section. Amplified and column-purified cDNA was used as a template for

qPCR. The patch pipette-harvested cytoplasm contents were directly reverse transcribed in 20-µl reactions using the ViLO SuperScript III cDNA reverse transcription kit (Life Technologies). The cDNA product of the reverse transcription reaction served as a template for the subsequent preamplification using the Preamp Master Mix kit (Life Technologies) according to the manufacturer's protocol. The preamplification products were diluted 1:10 with 0.1× Tris-EDTA buffer before use in qPCR. Whole transcriptome-amplified cDNA from LCM samples were diluted 1:50 in 0.1× Tris-EDTA buffer for qPCR investigation. TaqMan low-density array was designed to confirm microarray results by qPCR. The microfluidic card (Life Technologies) was preloaded by the manufacturer with selected inventoried gene expression assays for the targets of our interest and two housekeeping genes including 18S rRNA and GAPDH. Each assay consisted of a FAM dye-labelled TaqMan MGB probe and two PCR primers. Thermal cycling conditions of the qPCR were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C using the ViiA 7 real-time PCR platform (Life Technologies).

Results

RNA Sample Collection with LCM Is a Suitable Approach for Reliable Transcriptome Profiling of GnRH Neurons

GnRH-GFP neurons were sampled by LCM for RNA isolation (fig. 1a--c). The integrity of the isolated RNA (RIN values: 6.8--7.2) was proven to be sufficient for subsequent amplification steps. Transcriptome amplification steps yielded cDNA (fragment length: 100--1,000 bp; amount: 7--8 µg) that meets the criteria of Pico profiling of low cell numbers [58]. An advantage of the technique is its higher throughput sampling capacity and the systematic sampling of the entire GnRH neuron population in both its rostrocaudal and mediolateral dimensions.

Microarray Analysis Identifies 543 Genes Expressed Differentially between Male and Female GnRH Neurons

Microarray analysis revealed 543 differentially expressed genes between male and metestrous female mice at the following cutoff criteria: FC was >1.6 for 482 genes (transcripts enriched in male GnRH) and <0.63 for 61 genes (transcripts enriched in female GnRH); $p_{adj} < 0.01$; normalized expression value >7 in the female GnRH (online suppl. table 1; for all online suppl. material, see

www.karger.com/doi/10.1159/000430817). In order to analyze putative interactions among the differentially expressed genes, we used web-based public resources. All of the 543 differentially expressed genes were analyzed using the functional gene annotation tool DAVID [56, 57]. With this tool, a ranked list of clusters was generated by grouping gene annotation terms based on their similarity (online suppl. table 2). Ranking of annotation clusters was done by their enrichment score (ES), a factor calculated from the p_{adj} of terms. Individual genes within each cluster were then analyzed, and predicted protein-protein interactions were also graphically illustrated by the web-based resource STRING [59] (fig. 2). Top annotation clusters were arbitrarily named and listed by their ES from the higher to the lower values. The most significant cluster 'synaptic signaling' (cluster 1, ES: 4.98) involved the term synapse (p_{adj} value: 1.1E-05) with 34 genes including *Syt1* (synaptotagmin 1), *Cplx1* (complexin 1), *Dlg4* (disks large homolog 4, coding for postsynaptic density protein 95) and *Gria1* [glutamate receptor, ionotropic, α-amino-3-hydro-5-methyl-4-isoxazole-propionic acid 1 (AMPA 1)]. The cluster of 'protein homeostasis' (cluster 2, ES: 4.66) was enriched in genes of ubiquitination process-related proteins that may participate in ubiquitin-like conjugation (p_{adj} value: 1.1E-3) and isopeptide bond (p_{adj} value: 2.1E-3), i.e. *Ubb* (ubiquitin), *Ubc* (polyubiquitin-c) and *Rps27a* (ribosomal protein S27A). The group of 'vesicle functions' (cluster 3, ES: 4.46) incorporated synaptic, vesicular trafficking- and exocytosis-related genes (i.e. synaptotagmins). The cluster name 'neuron projection' (cluster 4, ES: 4.32) covered a large number of genes coding for proteins in axonal transport and structure *(Rac1, Akt1)*. The category of 'protein transport' (cluster 5, ES: 3.94) collected numerous genes of protein transport and trafficking. The enrichment of GTP-binding protein genes under 'signal transduction' (cluster 6, ES: 2.85) reflected the differential expression of genes in G-protein-mediated signaling *(Gnaq, Gnas)*. The 'neuron development' (cluster 7, ES: 2.59) incorporated genes of microtubule-associated *(Dcx)* and neuronal cell adhesion molecules *(L1cam, Ctnnb1)*. Genes involved in the glucose catabolic process (enolase 1, *Eno1*; cytochrome C, *Cycs*) were enriched in the 'glucose metabolism' cluster (cluster 8, ES: 2.52).

qPCR Confirms Sex-Specific Regulation of the GnRH Neuron Transcriptome

Microarray hybridizations have revealed significant differences in gene expression between male and metestrous female GnRH neurons. The differentially regulated 543 genes (online suppl. table 1) were clustered into biological functions and molecular

processes (fig. 2). Annotation clusters were more representative in the male compared to the female GnRH neurons. For the validation by qPCR, target genes hitting the highest ranked clusters (synaptic communication, signal transduction, axon and vesicle function and cell metabolism) and other differentially regulated genes were selected. The microarray-based differential expression of the selected target genes was depicted in a heat map (fig. 3).

Two independent sampling approaches were used for the validation: (1) the whole transcriptome of the laser-microdissected GnRH neurons was amplified according to the protocol for pico profiling (see Materials and Methods) and used as a template for qPCR (LCM-qPCR); (2) the cytoplasm of GnRH-GFP neurons was harvested by patch electrode from acute brain slices (fig. 1d1, d2) for preamplification and the subsequent qPCR (PCH-qPCR; see Materials and Methods). Out of the 57 targets, differential expression of 46 genes was confirmed by PCR (FC >1.5) from both LCM and PCH samples; thus, the overlapping confirmation rate was 79% (fig. 4; online suppl. table 3). By the PCH-qPCR method alone, differential expression of 50 genes (88% of the targets) was validated (fig. 4; online suppl. table 3).

Evaluation of the interrelation between microarray and qPCR data revealed strong, negative correlation (Pearson's $R^2 = 0.64$) between normalized log2-transformed microarray expression and the cycle threshold (Ct) values from qPCR data (online suppl. fig. 1).

Sexually Dimorphic Transcripts of GnRH Neurons Include mRNAs Coding for Neurotransmitter and Hormone Receptors, Ion Channels and Vesicular Transport

Differentially expressed target genes validated by qPCR were grouped into functional categories as follows: synaptic signaling, ion channels, membrane receptors, cell adhesion molecules, transcription factor binding, protein sorting and enzymes (fig. 4; suppl. table 3). Within the synaptic signaling group, differential expression of all but one gene was validated by both LCM-qPCR and the independent PCH-qPCR in male versus metestrous female GnRH neurons. Notably, they include: $GABA_A$ receptor subunits *(Gabra3, Gabrg2, Gabrb3)* and the $GABA_B$ receptor 2 *(Gabbr1)*; AMPA and N-methyl-D-aspartate (NMDA) receptor subunits *(Grin1, Gria2, Gria4, Gria1)*, glutaminase *(Gls)* and calcium/calmodulin-dependent protein kinase II *(CamkII)* for glutamatergic signaling; genes that are essential for vesicle docking and priming *(Cplx1, Syt1 Stxbp1, Rab3a)*, and the vesicular glutamate transporter 2 *(Slc17a6)*. The fatty acid amide hydrolase *(Faah)*

and the cannabinoid receptor type 1 *(Cnr1)* are involved in the retrograde endocannabinoid (ECB) signalization.

Similarly to the GABA and glutamate receptor functions, the ion transport-related genes coding for voltage-dependent Ca^{2+} and K^{+} channel subunits *(Cacng5, Cacnb1, Cacna1e, Kcnq2, Kcnc1)* and chloride ion channel *(Clcn3)* were also expressed at a higher level in the male neurons.

Transcripts of membrane receptor genes, like the nicotinic acetylcholine receptor beta 4 subunit *(Chrnb4)*, immunoglobulin-like domain containing receptor 2 *(Ildr2)*, natriuretic peptide receptor 2 *(Npr2)* and transforming growth factor beta receptor III *(Tgfbr3)*, were enriched in the male GnRH neurons. Differential expression of chemokine receptor 4 *(Cxcr4)*, delta/notch-like EGF-related receptor (*Dner*) and the progesterone receptor membrane component 1 *(Pgrmc1)* was validated by PCH-qPCR [relative quantities (RQs) according to LCM-qPCR were <1.5], revealing upregulation of transcripts in the male GnRH neurons, whereas the expression of G protein-coupled receptor 56 *(Gpr56)* could not be evaluated by PCH-qPCR.

Female-enriched expression of somatostatin receptor 3 *(Sstr3)* was validated by qPCR using cytoplasm-harvested GnRH samples.

Messenger RNAs that code for cell adhesion molecules (beta catenin, *Ctnnb1*; neural cell adhesion molecule 1, *Ncam1*) exhibit male-specific upregulation in GnRH neurons.

Two genes associated with transcription factor binding functions also showed male-enriched expression, namely, the thyroid hormone receptor alpha *(Thra)* and the nuclear receptor co-repressor *(Ncor1)*.

Genes that play a role in receptor trafficking and sorting *(Gabarapl1, Ssr1, Sort1, Slc25a4)* and in vesicle transport and release, like *Syt11*, *Rab14* and *Mapk8ip3*, are upregulated in the male GnRH transcriptome.

Several enzymes involved in various cell signaling processes (fat mass and obesity associated, *Fto*; glutamic acid decarboxylase, *Gad1*; prostaglandin D_2 synthase, *Ptgds*; uncoupling protein 2, *Ucp2*) also exhibit a male-specific regulation in GnRH neurons.

Discussion

Novel Sampling Strategy for Genomic Analysis of Preoptic GnRH Neurons

Sampling Strategy for an Unbiased Representation of the Whole GnRH Cell Population

To analyze and characterize the transcriptome profile of GnRH neurons of male and metestrous female mice, we introduced a novel approach to reveal sex-specific differences at the mRNA level. As previously shown by Khodosevich et al. [46], the laser microdissection approach was suitable for the isolation of 3,000 GFP-expressing striatal medium spiny neurons followed by high-throughput microarray analysis. In this study, we used this approach to isolate total RNA of 250 GnRH cells from each brain. The microdissection provides the advantage of the uniform sampling of the total GnRH neuronal population dispersed in both rostrocaudal and mediolateral directions within the preoptic region of the mouse brain, thus ensuring an unbiased representation of the GnRH mRNA pool for microarray analysis.

Expression Profiling of GnRH Neurons

In order to achieve this goal, we implemented a novel approach, the pico profiling [58], which is based on the amplification of the whole transcriptome and provides expression profiles from total RNA of 10 cells. To our knowledge, this study is the first to combine the GFP expression-based LCM and RNA isolation method [46] with pico profiling. This technique was especially useful in our hands considering that the GnRH system consists of a limited number of neurons.

Stabilization of mRNA Profiles ‘in situ’

An important consideration of the LCM method has been the preservation of mRNA integrity and profile by perfusion fixation of the brains which eliminated the likely disadvantages (stress reaction, immediate-early gene response, etc.) of in vivo-type tissue processing. Results from the microarray analysis were confirmed by qPCR both from identical (LCM) and independent (PCH) sources of the RNA/cDNA template at a high level of confidence (91 and 88%, respectively), indicating that the LCM is a reliable approach to collect RNA for gene expression profiling experiments.

Differentially Expressed Genes Subserving Fundamental Regulatory Mechanisms

Microarray analysis of male and female GnRH transcriptome resulted in 543 differentially expressed genes (FC >1.6 and <0.63, FDR <0.01; online suppl. table 1) where the log2-transformed normalized gene expression was also implemented as threshold (values >6). The rationale for the expression cutoff threshold was that only gene targets with Ct values under 28 were considered to be expressed in GnRH neurons: transcripts with expression values higher than 6 were found to be validated by qPCR, and an inverse correlation between Ct and normalized gene expression values of microarray hybridization was shown. Genes following these criteria were then analyzed with functional annotation clustering tools (DAVID Bioinformatics, STRING) to reveal direct or predicted interaction between the targets. The majority of the differentially expressed genes and the most significant clusters were characteristic of the male GnRH neurons and were associated with synaptic signaling, morphogenesis, cell metabolism, protein and vesicle transport cell and morphogenesis. It is widely accepted that GABAergic and glutamatergic inputs are the most important afferent regulators of the GnRH system [60, 61]. In the present study, it has been shown that numerous components of the GABA and glutamate neurotransmitter signaling are upregulated in male GnRH neurons.

Regulation of Cell Signaling in GnRH Neurons

Neurotransmitter Receptors

GABA Receptors. GABA and glutamate are dominant neurotransmitters in the hypothalamus [62, 63], playing fundamental roles in the afferent regulation of GnRH neurons [60, 61, 64]. GnRH neurons express GABA [65--68] as well as AMPA and NMDA [69--72] receptors that are synaptically activated. Electrophysiological studies have suggested that activation of $GABA_A$ receptors via local GABA application can depolarize and potentially excite GnRH neurons in mice and rats [73--75], but timing of GABA administration and glutamatergic input or activation of voltage-sensitive Ca channels may all have major roles in shaping $GABA_A$ receptor-dependent responses [74, 76]. It was shown earlier that sex differences exist in the $GABA_A$ receptor subunit mRNAs expressed by GnRH neurons [77], but the RQ of the $GABA_A$ receptor mRNAs remains unknown. Here, we showed that several $GABA_A$ receptor subunits are regulated differently: the mRNAs of *Gabra3*, *Gabrb3* and *Gabrg2* subunits are more abundant in the male GnRH

neurons as compared to metestrous females. In $GABA_A$ receptor subunit gamma-2 *(Gabrg2)* knockout mice, 70--90% reductions in the normal levels of $GABA_A$ receptor activity were shown with altered response to gonadectomy between the sexes [78], indicating the importance of $GABA_A$ receptor subunit composition in GnRH function during the negative feedback period of gonadal hormones.

In addition to ionotropic $GABA_A$, the functional metabotropic $GABA_B$ receptor is also present in the GnRH neurons and plays an important role in controlling excitability and in modulating the activity of these cells [79]. Liu and Herbison [79] found that in male mice fewer GnRH neurons responded to $GABA_B$ receptor stimuli compared to diestrous females. In this study, we found that the GABBR1 receptor is expressed differentially between males and metestrous females, further supporting the sex- and estrous cycle-dependent GnRH regulation via the $GABA_B$ receptor.

Glutamate Receptors. Glutamate signaling via synaptic transmission also regulates the activity of GnRH neurons [61, 70, 71, 77] in an estradiol-dependent manner mediated by AMPA/KA and NMDA receptors [80]. In acute slice preparations, about 20 and 65% of the GnRH neurons responded to NMDA and AMPA stimuli, respectively, resulting in a rise of the intracellular Ca^{2+} ion concentration via IP3R-mediated pathway and/or L-type voltage-gated calcium channels [81]. During negative estradiol feedback, glutamate transmission is suppressed from the AVPV, whereas at the onset of the LH surge, the number of dual-phenotype inputs increases in an estradiol-dependent manner [82], suggesting sex-dependent glutamate receptor expression. In this study, we showed that the gene expression of several AMPA (AMPA1, 2, 4) and the NMDA1 receptor subunits is enriched in male GnRH neurons and might result in an altered regulation of GnRH neurons by glutamate.

Acetylcholine Receptor. Nicotinic acetylcholine *(Ach)* receptor activation provoked an increased GnRH release in perifused hypothalamic primary cell cultures and immortalized GnRH producing GT1--7 cells, and induced a rapid $[Ca^{2+}]_i$ elevation simultaneously [83], which can be modulated by 17β-estradiol [84]. In rats, GnRH neurons receive direct cholinergic afferent inputs on their cell bodies and dendrites [85]. Previously, Todman et al. [86] showed that a subpopulation of GnRH neurons express nicotinic acetylcholine receptor subunits *(Chrnb1, Chrnb2* and *Chrng)* at the mRNA level. In the present study, we found that the beta polypeptide 4 subunit of the cholinergic receptor *(Chrnb4)* exhibits male-biased upregulation.

Ion Channels

Calcium Channels. In hypothalamic GnRH neurons, high-voltage-activated Ca currents pass through multiple (L-, N-, P-, Q- and R-type) Ca^{2+} channels [87, 88]. Though L-type calcium channels are essential for the firing activity of GnRH neurons, enhancing $GABA_A$ receptor-mediated signaling [89], the function of R-type Ca^{2+} channel as a major current component is inevitable in Ca^{2+}-dependent GnRH release [90]. We have shown that the expression of voltage-dependent R-type calcium channels is gender specific: the alpha-1e *(Cacna1e)*, beta-1 *(Cacnb1)* and gamma-5 *(Cacng5)* subunits are expressed at higher levels in males. Recently, Bosch et al. [91] have shown that $Ca_V2.3$ (the R-type Ca^{2+} channel) is the most prominent form in mouse GnRH neurons; moreover, its mRNA expression is increased by estradiol, irrespective of time of the day.

Potassium Channel. GnRH neurons express functional Kv7.2, a potassium channel protein coded by the gene *Kcnq2*, a component of the M channel that inhibits excitability of the GnRH neuron [92]. The GnRH peptide activates the M channel via GnRH receptors, suggesting an ultra-short feedback mechanism in GnRH excitability [92]. The functional significance of the observed differential expression of the *Kcnq2* gene requires clarification.

Steroid Receptor Signaling

The effect of progesterone (P_4) is associated with the activity of GnRH pulse generator and the daytime frequency of GnRH release under physiological (i.e. puberty) and pathological conditions [93--95]. In a recent study, the expression of P_4 receptor membrane component 1 (PgRMC1) and its partner SERPINE1 mRNA-binding protein 1 (SERBP1) has been shown in GnRH neurons. They mediate direct and rapid P_4 action, resulting in inhibiting the activity of these neurons [33]. We have shown the differential expression of the *Pgrmc1* and *Serbp1* genes, raising the possibility of altered P_4 signalization and response in male and female GnRH neurons, especially during the negative feedback period.

Peptide Signaling

Somatostatin. A subpopulation of GnRH neurons expresses somatostatin receptor type 3 *(Sstr3)* in rodents [86, 96, 97], where the somatostatinergic appositions exert strong inhibition in mice [96]. Though *Sstr2* was found to be the predominant form in both rats and mice, *Sstr3* expression was detected only in the GnRH neurons of female rodents

[97]. In accordance with these observations, our microarray and PCH-qPCR data have confirmed that the expression level of *Sstr3* mRNA is higher in the female in comparison with the male mice.

Natriuretic Peptide. According to our data, *Npr2* is expressed in GnRH neurons and upregulated in male GnRH neurons. Evidence of the functional natriuretic peptide receptor (NPR2), a membrane-bound guanylate cyclase expressed in GT1--7 cells, was provided by Olcese et al. [98]. Later, it was shown that immortalized GnRH cells synthesize C-type natriuretic peptide [99], suggesting an autocrine regulation via an ultrashort, positive feedback mechanisms on GnRH production and release.

Stromal Cell-Derived Factor 1-CXCR4 Signaling. Signaling via stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 is required for survival and migration of GnRH neurons during embryonic development [100, 101]. It has been shown that simultaneous activation by GABA and SDF-1 acts synergistically to stabilize linear rather than random movement of migratory GnRH neurons [102]. Our data indicate that CXCR4 is also expressed in adult GnRH neurons at the mRNA level and exhibits male-specific upregulation. The expression and role of CXCR4 needs further investigation in settled hypothalamic GnRH neurons in adulthood.

Cannabinoid Signaling. ECBs are known to influence the activity of GnRH neurons [17, 103]. ECBs exert direct inhibitory effect on the secretion of the GnRH peptide [104], mostly in the territory of ME [105]. It has been shown that immortalized GnRH neuronal cells (GT1) also express CB1 and CB2 receptors, produce and secrete 2-AG and degrade ECBs by FAAH [104]. Gammon et al. [104] have also demonstrated that GnRH neurons may express CB1 receptors. Recently, CB1 immunoreactivity (IR) was detected by immunoelectron microscopy over neurosecretory axons in the territory of ME [106] and in the axons of GnRH neurons by double-label confocal microscopy at the external zone of ME [Fekete, pers. commun.]. In this study, we have demonstrated that the expression of both the CB1 receptor and FAAH enzyme is upregulated in male GnRH neurons at the mRNA level. The functional significance of CB1 receptors in GnRH neurons can only be speculated: they may have a role in shaping the autocrine feedback regulation inside the GnRH neuronal network.

Cell Adhesion Markers

NCAM is a glycoprotein expressed on the surface of glia and neurons. NCAM can be posttranslationally modified by the addition of polysialic acid (PSA-NCAM), resulting in a

reduced cell-cell adhesion capacity. Expression of PSA-NCAM was shown on the surface of GnRH neurons in various species, exhibiting photoperiod- and seasonal breeding-dependent IR [107, 108] that suggests a functional role of NCAM in the secretory activity of GnRH neurons. During the estrous cycle of the female rat, PSA-NCAM-IR associated with GnRH neurons was significantly higher in proestrus as compared to diestrus, accompanied with structural reorganization of GnRH axon terminals in the ME [109], indicating an estrous cycle-dependent and, presumably, sexually dimorphic regulation of the *Ncam1* gene. In the present work, we detected upregulation of the *Ncam1* gene in the male GnRH neurons, which in part supports the observed differences in the neuroplastic changes between sexes.

Vesicular Transport and Release

Complexin-1 *(Cplx1)*, synaptotagmin-1 *(Syt1)*, syntaxin-binding protein 1 *(Stxbp1)* and Ras-related protein Rab-3A *(Rab3a)* are of critical importance in docking and priming mechanisms of secretory vesicles [110--112], and were found to be expressed at high levels in mouse GnRH neurons and regulated differentially by sex. SYT1 serves as a Ca^{2+} sensor in the control of vesicle fusion at the time of neurotransmitter release [113]. According to a recent observation in geese, the mRNA expression of *Syt1* exhibits an egg-laying cycle-dependent regulation and is considered as a key molecule in regulating the secretion of hormones in the hypothalamo-hypophyseal area [114]. Differential expression of genes that are essential in exocytotic vesicle priming and release might reflect distinct neurosecretory release mechanisms between sexes also in GnRH neurons of male and metestrous female mice.

Dimorphism in the Camp Response Element-Binding Protein Phosphorylation Pathway at the mRNA Level

An important aspect of differential gene expression between sexes is related to the intracellular signalization pathways. Camp response element-binding protein (CREB) phosphorylation is a critical step in the expression initiation of numerous genes in neurons. Sex- and GABA receptor activity-dependent CREB phosphorylation in the medial preoptic area has been previously demonstrated in the neonatal rat brain [115].

CREB phosphorylation in the GnRH neurons is regulated by estradiol in female mice [116]. The number of GnRH-pCREB neurons in the male is significantly higher compared to the female at a low serum E_2 level [26] indicating sexually dimorphic intracellular

phosphorylation cascades that lead to the activation of CREB [117]. In this study, we showed that mRNA expression of numerous genes (*Gria1, 2, 4, Grin1; Cacng5*, *Cacnb1*, *Cacna1e*, *Gnas*, CamkII*/Camk2d*, *Calm*/predicted gene for CaM, MEK*/Map2k*, PKC*/Prkcb*, PKA*/Prkacb*, *AKT*) involved in the CREB signaling pathway is significantly higher in the male GnRH neurons (fig. 5). This observation might underlie the male-biased CREB phosphorylation in GnRH neurons. Activation of the CREB phosphorylation pathway might explain, in part, the higher representation of upregulated genes in the male GnRH transcriptome.

The observed genomic differences between male and metestrous female GnRH neurons could be due to the different hormonal milieu (especially androgen levels) that might shape gene expression profiles of these cells. We have shown that the number of upregulated genes is higher in males than metestrous females (482 and 61, respectively). In a recent study [118], differential expression of 611 genes was shown in the hypothalamus of juncos *(Junco hyemalis)* by sex, with the higher expression of 483 genes in intact males than females and higher expression of 128 genes in intact females than males, providing another example of a male-biased overrepresentation of genes in a brain subregion, suggesting a possible effect of androgens on differential gene expression.

In conclusion, the data presented here provided further insight into the transcriptome of hypothalamic GnRH neurons that exhibited a robust sex-dependent dimorphism in the negative feedback period of gonadal steroids in mice: the expression of more than 500 genes was found to be different in male and female GnRH neurons. This striking sexual dimorphism is manifested in gene expressional changes associated with fundamental mechanisms like vesicle and protein transport, cell morphogenesis and metabolism. Among these functional clusters, cell signaling was the most significant and involved genes coding for GABA and glutamate receptor subunits, vesicle priming and docking proteins, ion channels and peptide receptors. The observed differences in gene expression may account for the differences seen in the diverse functions of the GnRH neuronal system between the sexes.

Acknowledgments

The authors would like to express their thanks to Dr. Suzanne M. Moenter (Department of Molecular and Integrative Physiology, University of Michigan) who kindly provided us with the transgenic mice used in this study; to Dr. Csaba Fekete [Lendület

Laboratory of Integrative Neurobiology, IEM, Hungarian Academy of Sciences (IEM-HAS)] for personal communication and advice regarding LCM methodology and to Dr. Erik Hrabovszky (Laboratory of Endocrine Neurobiology, IEM-HAS) for the thorough reviewing of the manuscript. This work was supported by grants of the Hungarian Scientific Research Fund (OTKA K100722), the National Development Agency of Hungary (NFUBONUS-HU08/2-2011-0006) and the European Community's Seventh Framework Programme (FP7/2007-2013, No. 245009).

Disclosure Statement

The authors have nothing to disclose.

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Appendix after References (Editorial Comments)

Legend(s)

Fig. 1. Sampling of GnRH-GFP neurons by LCM and patch pipette cytoplasm harvesting in mice. **a1** GnRH-GFP neurons (arrows) located in the median preoptic nucleus (MnPO) and in the vicinity of the vascular organ of the lamina terminalis (asterisk). **a2** Characteristic fusiform GnRH neurons framed in **a1** are shown at higher magnification. **b1** GnRH-GFP perikaryon (arrow) selected for laser microdissection along the yellow line. **b2** Vacant site in the section (yellow line) after pressure

catapulting the dissected GnRH-GFP cell body shown in **b1**. **c** Group of GnRH perikarya collected in the cap of the sampling tube after LCM. **Insert** A GnRH perikaryon with its characteristic shape at higher magnification. **d1**, **d2** Cytoplasm harvesting from GFP-expressing GnRH neurons by patch-pipette shown by fluorescent microscopy (**d1**) and Nomarski optics (**d2**). Arrowheads point toward the tip of the patch-pipette. Scale bars: 50 µm (**a1**), 20 µm (**a2**), 10 µm (**b1**, **b2**), 50 µm (**c**) and 5 µm (**d1**, **d2**).

Fig. 2. Main functional clusters of genes expressed differentially in male and female GnRH neurons. The list of differentially expressed genes was analyzed using a web-based public functional annotation clustering tool in DAVID Bioinformatics Resources [42, 43]. The annotation clusters were numbered and ranked based on their ES as follows: synaptic signaling (cluster 1), protein homeostasis (cluster 2), vesicle functions (cluster 3), neuron projection (cluster 4), protein transport (cluster 5), GTPase-mediated signal transduction (cluster 6), neuron development (cluster 7) and glucose metabolism (cluster 8). A lower cluster number indicates a higher level of enrichment in gene ontology terms. The official gene symbols are given in each cluster. Predicted interactions of the target gene-encoded proteins are shown as revealed by String 9.1 [44]; stronger protein associations are represented by thicker lines.

Fig. 3. Heat map showing hierarchical clustering of the microarray probes differentially expressed in the GnRH neurons. The rows represent the sexually dimorphic probe sets with corresponding gene symbols on the right side of the figure. The expression level of each probe is color coded: for decoding, see the continuous color key. The individual samples are shown as columns. The 6 female and male samples are coded in yellow and violet, respectively. The dendrogram to the left of the heat map illustrates the similarities between sexually dimorphic probes in terms of their expression levels in the different samples. Clustering displayed above the individual samples indicates similarity between them.

Fig. 4. Validation of the microarray data by qPCR. In total, 57 differentially expressed genes were selected (FC >1.6 and <0.63, n = 543; see online suppl. table 1) for qPCR validation. For sampling of GnRH neurons, LCM-qPCR (average RQ ± SEM) and PCH-qPCR (average RQ ± SEM) were applied followed by qPCR investigations. The qPCR confirmed the differential expression of 46 genes in both samples (FC >1.6 and RQ >1.5; see online suppl. table 3 for the data). Genes

were grouped into functional categories, labelled with roman numerals from I to IV. The largest group, synaptic signaling (I), involves GABA receptors (A), glutamatergic synapse (B), vesicle docking and priming (C) and cannabinoid signaling (D). Further categories are ion channels (II), membrane receptors (III) and cell adhesion molecules (IV), transcription factor binding (V), protein sorting and transport (VI) and enzymes (VII). Microarray data are in blue, PCH data in purple and LCM results are in dark red.

Fig. 5. Differentially regulated genes in the GnRH neurons participating in multiple signal transduction pathways. Schematic representation of Ca-dependent CREB phosphorylation pathways that contribute to CREB Ser133 phosphorylation in response to Ca^{2+} influx. The differentially regulated genes shown in red letters include: ionotropic glutamate receptors (iGluRs: AMPA1, 2, 4, NMDA1); voltage-dependent calcium channel subunits (CaCN: Cacng5, Cacnb1, Cacna1e); guanine nucleotide-binding protein, alpha stimulating, complex locus (Gαs, Gnas); calcium/calmodulin-dependent protein kinase II (CAMKII); calmodulin (CALM), mitogen-activated protein kinase kinase (MEK/Map2k); protein kinase C (PKC/Prkcb), cAMP-dependent protein kinase (PKA/Prkab) and thymoma viral proto-oncogene 1 (AKT/PKB).

Table(s)

Footnote(s)

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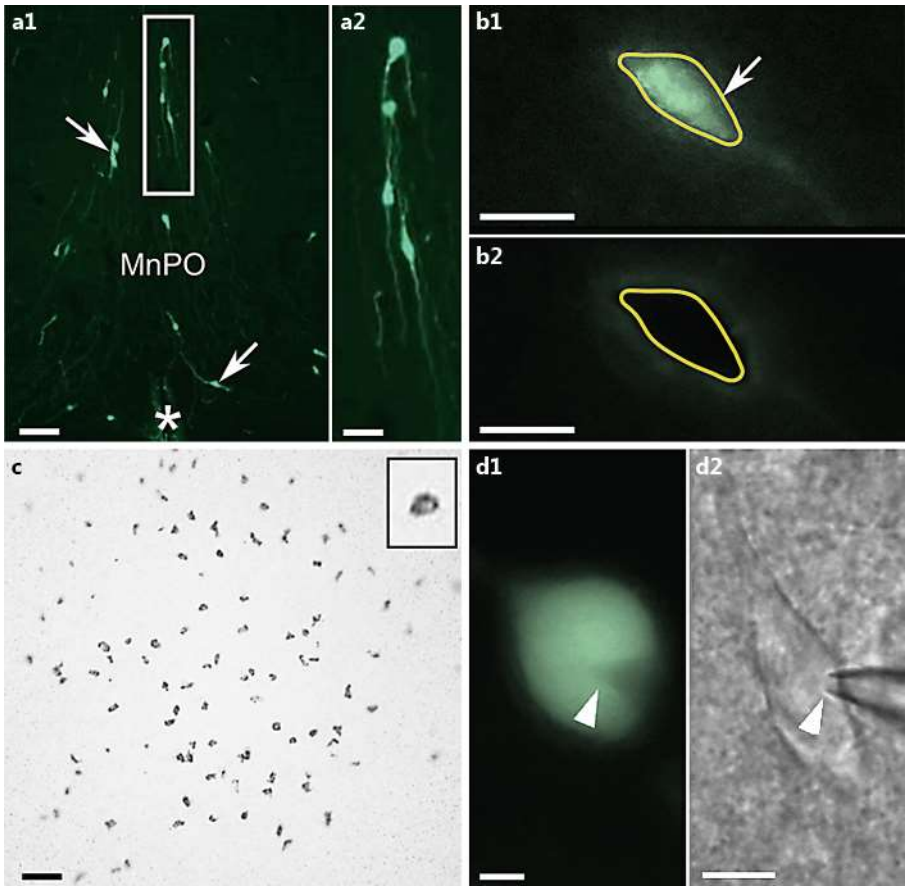

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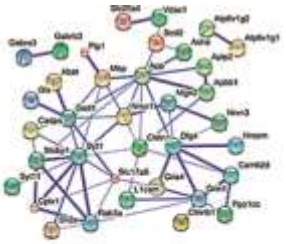

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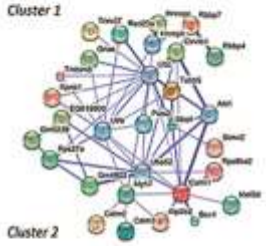

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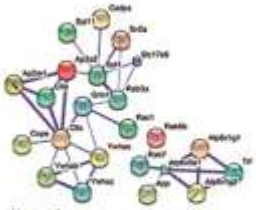

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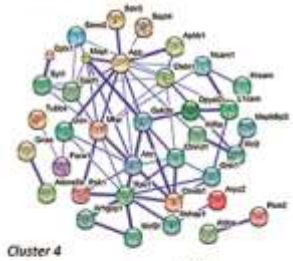

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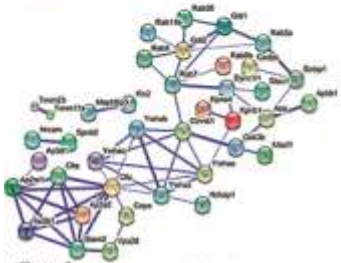
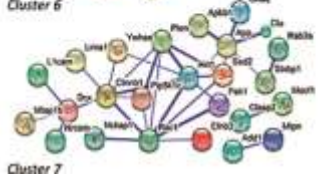


Cluster 5

Cluster 6



Cluster 7

Cluster 1	ES: 4.98	Count	P value
GO:0045202 - synapse		34	3.12E-08
GO:0019226 - transmission of nerve impulse		27	3.82E-08
GO:0007268 - synaptic transmission		23	1.48E-07
GO:0001505 - regulation of neurotransmitter levels		11	4.00E-05
GO:0007267 - cell-cell signaling		23	3.42E-04

Cluster 2	ES: 4.66	Count	P value
Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)		19	5.77E-06
ubl conjugation		36	2.86E-05
isopeptide bond		23	6.27E-05

Cluster 3	ES: 4.46	Count	P value
GO:0016023 - cytoplasmic membrane-bounded vesicle		35	5.31E-06
GO:0031988 - membrane-bound vesicle		35	7.32E-06
GO:0048770 - pigment granule		14	1.09E-05
GO:0042470 - melanosome		14	1.09E-05
GO:0031410 - cytoplasmic vesicle		39	1.32E-05
GO:0031982 - vesicle		39	2.09E-05

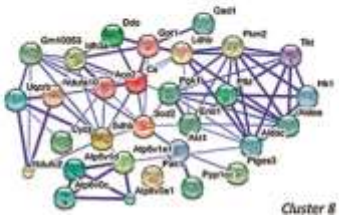
Cluster 4	ES: 4.32	Count	P value
GO:0030424 - axon		17	1.70E-06
GO:0042995 - cell projection		44	1.95E-06
GO:0043005 - neuron projection		26	2.59E-06
GO:0033267 - axon part		6	1.91E-03

Cluster 5	ES: 3.94	Count	P value
GO:0046907 - intracellular transport		34	1.66E-05
GO:0070727 - cellular macromolecule localization		26	5.03E-05
GO:0008104 - protein localization		48	5.99E-05
GO:0006886 - intracellular protein transport		24	9.42E-05
GO:0034613 - cellular protein localization		25	1.21E-04
GO:0015031 - protein transport		42	1.48E-04

Cluster 6	ES: 2.85	Count	P value
nucleotide phosphate-binding region:GTP		26	2.50E-06
nucleotide binding		12	5.96E-06
P-loop		12	6.96E-06
GTP binding		9	1.27E-05
gtp-binding		26	1.86E-05
GO:0007264 - small GTPase mediated signal transduction		24	2.71E-05

Cluster 7	ES: 2.59	Count	P value
GO:0030030 - cell projection organization		26	9.08E-05
GO:0031175 - neuron projection development		20	1.61E-04
GO:0048812 - neuron projection morphogenesis		17	3.39E-04
GO:0048666 - neuron development		22	1.02E-03
GO:0007409 - axonogenesis		15	1.34E-03

Cluster 8	ES: 2.52	Count	P value
GO:0006091 - generation of precursor metabolites and energy		29	2.70E-08
GO:0006006 - glucose metabolic process		15	2.18E-04
glycolysis		8	2.28E-04
GO:0006096 - glycolysis		8	4.28E-04
GO:0044275 - cellular carbohydrate catabolic process		9	6.18E-04



Cluster 8

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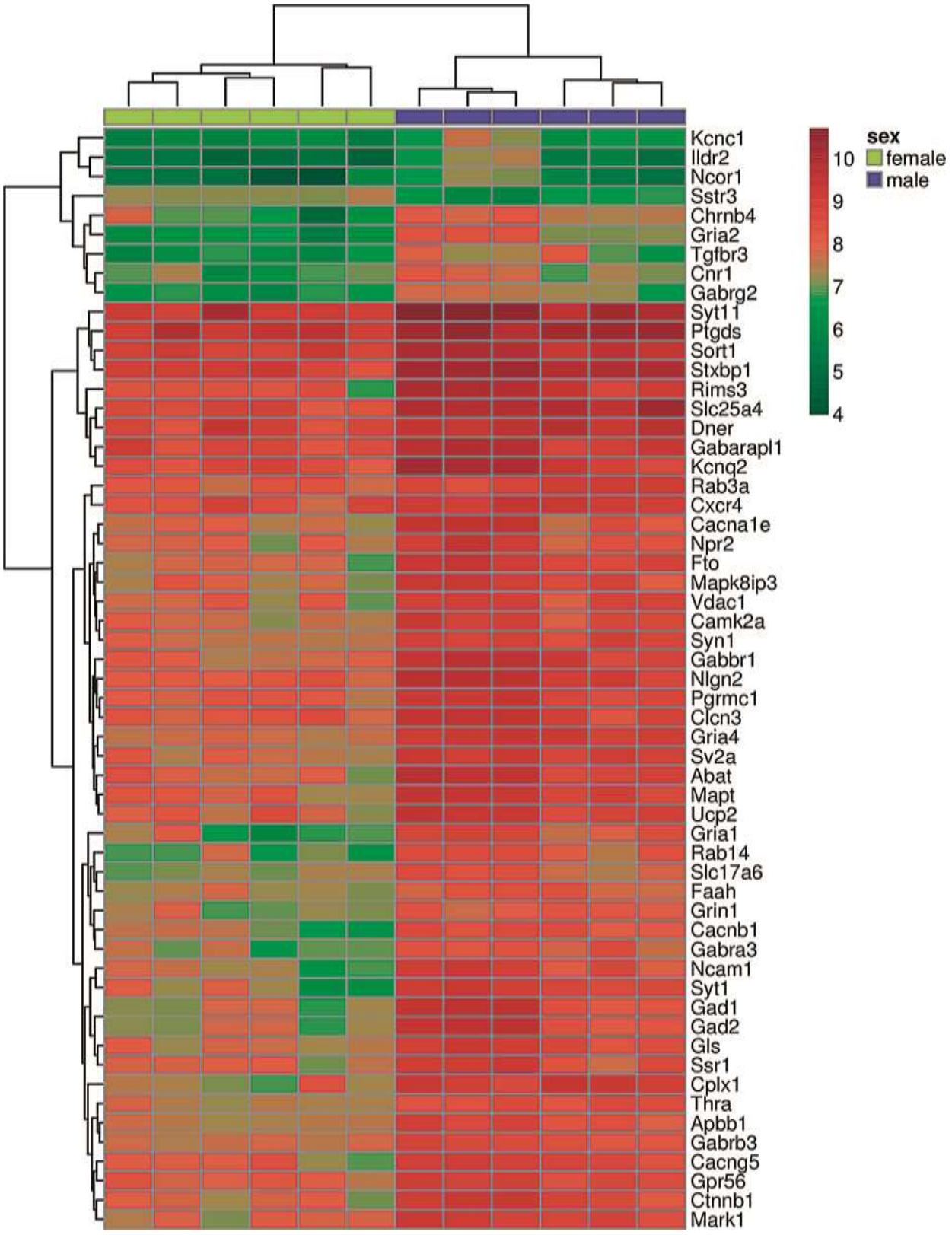

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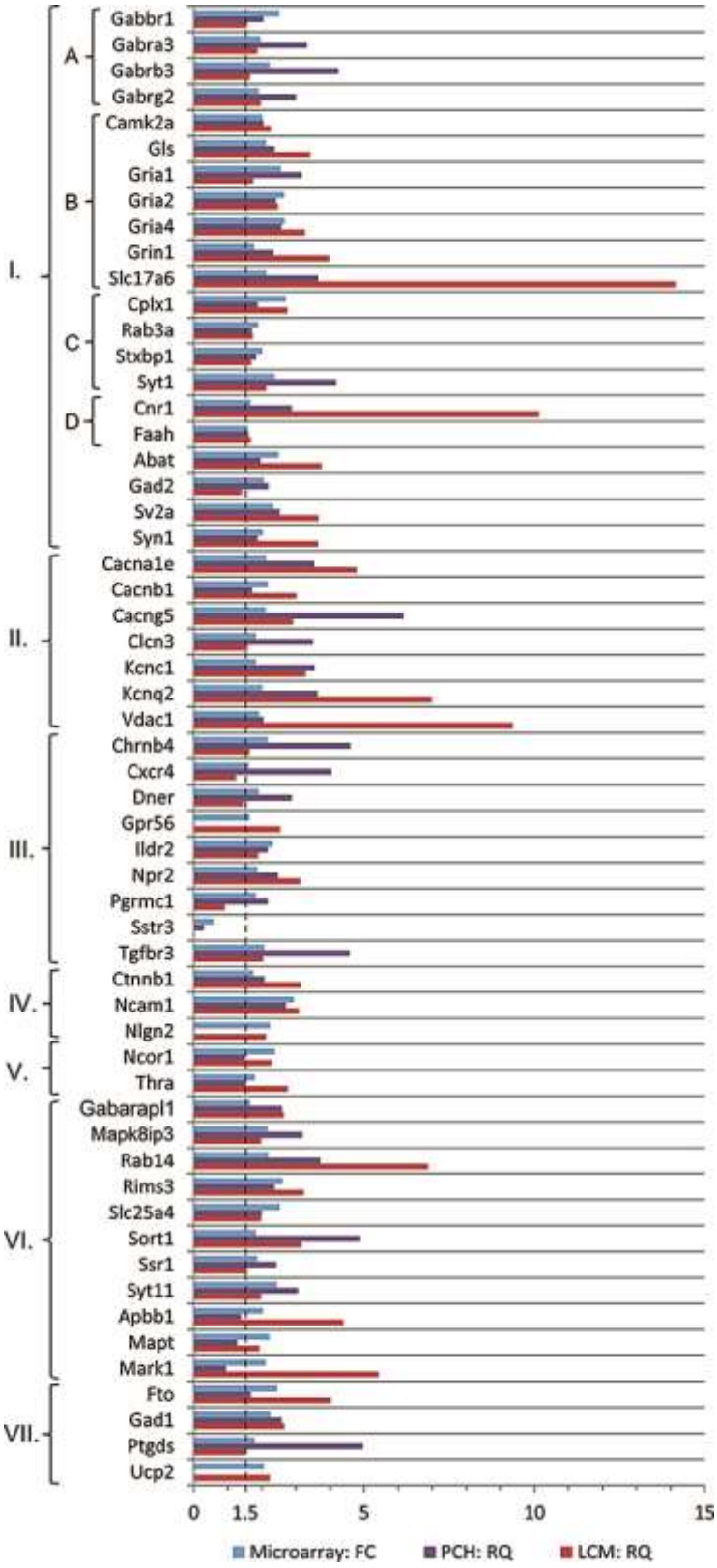

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