Proestrus Differentially Regulates Expression of Ion Channel and Calcium Homeostasis Genes in GnRH Neurons of Mice

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In proestrus, the changing gonadal hormone milieu alters the physiological properties of GnRH neurons and contributes to the development of the GnRH surge. We hypothesized that proestrus also influences the expression of different ion channel genes in mouse GnRH neurons. Therefore, we performed gene expression profiling of GnRH neurons collected from intact, proestrous and metestrous GnRH-GFP transgenic mice, respectively. Proestrus changed the expression of 37 ion channel and 8 calcium homeostasis-regulating genes. Voltage-gated sodium channels responded with upregulation of three alpha subunits (Scn2a1, Scn3a, and Scn9a). Within the voltage-gated potassium channel class, Kcna1, Kcnd3, Kcnh3, and Kcnq2 were upregulated, while others (Kcna4, Kcn3, Kcnd2, and Kcnq1) underwent downregulation. Proestrus also had impact on inwardly rectifying potassium channel subunits manifested in enhanced expression of Kcnj9 and Kcnj10 genes, whereas Kcnj1, Kcnj11, and Kcnj12 subunit genes were downregulated. The two-pore domain potassium channels also showed differential expression with upregulation of Kcnk1 and reduced expression of three subunit genes (Kcnk7, Kcnk12, and Kcnk16). Changes in expression of chloride channels involved both the voltage-gated (Clcn3 and Clcn6) and the intracellular (Clic1) subtypes. Regarding the pore-forming alpha-1 subunits of voltage-gated calcium channels, two (Cacna1b and Cacna1h) were upregulated, while Cacna1g showed downregulation. The ancillary subunits were also differentially regulated (Cacna2d1, Cacna2d2, Cacnb1, Cacnb3, Cacnb4, Cacng5, Cacng6, and Cacng8). In addition, ryanodine receptor 1 (Ryr1) gene was downregulated, while a transient receptor potential cation channel (Trpm3) gene showed enhanced expression. Genes encoding proteins regulating the intracellular calcium homeostasis were also influenced (Calb1, Hpcal1, Hpcal4, Cebp7, Cab39l, and Cib2). The differential expression of genes coding for ion channel proteins in GnRH neurons at late proestrus indicates that the altering hormone milieu contributes to remodeling of different kinds of ion channels of GnRH neurons, which might be a prerequisite of enhanced cellular activity of GnRH neurons and the subsequent surge release of the neurohormone.

Keywords: GnRH, ion channels, gene expression, mouse, neurons, proestrus, transcriptome
INTRODUCTION

The hypothalamo-pituitary-gonadal (HPG) neuroendocrine axis has a pivotal role in regulation of reproduction (Knobil, 1988) and maintenance of trophic gonadal hormone supply for different hormone sensitive cellular constituents of the body (Heldring et al., 2007). At the hypothalamic level of regulation, gonadotropin-releasing hormone (GnRH)-synthesizing neurons are in charge of initiating the reproductive hormone cascade events (Carmel et al., 1976; Merchenthaler et al., 1980). They also receive information about the functional performance of the controlled units via hormone feedback mechanisms (Sarkar and Fink, 1980; Herbison, 1998). Estrogen hormones are powerful regulators of the HPG axis acting via nuclear and membrane receptors. In female rodents, estradiol (E2) exerts both negative and positive feedback effects on GnRH neurons and their neuronal afferents including the kisspeptin system (Sarkar and Fink, 1980; Herbison, 1998; Moenter et al., 2003b; Radovick et al., 2012; Adams et al., 2018). Estrogen receptor beta (ERβ) (Hrabovszky et al., 2000, 2001) and GPR 30 (Noel et al., 2009), both produced in GnRH neurons, can sense the gonadal cycle-dependent changes of circulating E2 and adjust accordingly the cellular activity of GnRH neurons (Wang et al., 1995; Gore and Roberts, 1997; Finn et al., 1998; Christian et al., 2005; Farkas et al., 2013). The changing molecular and cellular events determine, among others, the production rate, axonal transport and release pattern of GnRH (Wang et al., 1995; Gore and Roberts, 1997; Finn et al., 1998; Christian et al., 2005; Farkas et al., 2013). The pulsatile secretion of GnRH and its pre-ovulatory surge release are characteristic features of the system (Moenter et al., 2003a). Disturbances in pulsatile and surge release mechanisms result in anovulatory syndromes and infertility (Henderson et al., 1976; Schally et al., 1976).

In proestrus, the changing gonadal hormone milieu contributes to the development of the pre-ovulatory surge release of GnRH (Clarke and Cummins, 1985; Levine, 1997; Christian and Moenter, 2010; Radovick et al., 2012). It has recently been shown that GnRH neurons exhibit burst-type firing pattern in late proestrus (Farkas et al., 2013; Silveira et al., 2017) and E2 modulates the oscillations and increases the firing (Chu et al., 2012). Proestrus also causes the remodeling of different classical neurotransmitter and neuropeptide receptors of both ionotropic and metabotropic types in GnRH neurons (Vastagh et al., 2016). These events reflect that several neuronal regulators of the GnRH system are also subject of the positive E2 feedback regulation (Gore, 2010; Wang et al., 2016) and the effects of estrogen are relayed to GnRH neurons via altered patterns of neurotransmission. The study has been focused solely on GnRH neurons that do not express estrogen receptor (ER) alpha in mice. Regarding the positive estrogen feedback-targeted kisspeptin neurons that express both isotypes of ER, we have recently shown that proestrus heavily upregulates Kiss1 in the medial preoptic area of mice (Vastagh and Liposits, 2017).

The electrophysiological properties and activity of GnRH neurons highly depend on the actual functional state of their different ion channels. The contribution of different sodium, potassium, calcium and chloride channel activities to the resting state and activation of GnRH neurons has comprehensively been studied (Van Goor et al., 1999; Costantin and Charles, 2001; Bosch et al., 2002, 2013; DeFazio and Moenter, 2002; Kelly et al., 2003; Nunemaker et al., 2003; Toba et al., 2005; Chu and Moenter, 2006; Kato et al., 2006, 2009; Farkas et al., 2007; Spergel, 2007; Zhang et al., 2007, 2009, 2013; Hirazumi et al., 2008; Liu and Herbison, 2008; Xu et al., 2008; Krismanovic et al., 2010; Lee et al., 2010; Moenter, 2010; Ronnekleiv et al., 2010, 2012, 2015; Arroyo et al., 2011; Pielecka-Fortuna et al., 2011; Iremonger and Herbison, 2012; Zhang and Spergel, 2012; Norberg et al., 2013). The expression of certain ion channel genes and particular ion currents generated in GnRH neurons have been found estrogen sensitive (DeFazio and Moenter, 2002; Moenter et al., 2003a; Zhang et al., 2007, 2009; Ronnekleiv et al., 2010, 2015; Wang et al., 2010; Pielecka-Fortuna et al., 2011; Bosch et al., 2013). The majority of the studies has been carried out in ovariectomized, E2-replaced animals that only partially mimic the physiological events occurring during the natural ovarian cycle.

Therefore, the present study was aimed at deciphering the biological effects of proestrus exerted upon expression of ion channels genes of GnRH neurons collected from intact, regularly cycling GnRH-GFP mice shortly before the onset of GnRH surge. The explored differential expression of various ion channel genes gives a better insight into the specific types of ion channels responding to proestrus-related hormonal events and also their reasonable contribution to development of the preovulatory GnRH surge.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with legal requirements of the European Community (Directive 2010/63/EU). The protocol was approved by the Animal Welfare Committee of the Institute of Experimental Medicine Hungarian Academy of Sciences, Budapest, Hungary (Permission Number: A5769-01). The animal experimentations were conducted in accordance with accepted standards of humane animal care and all efforts were made to minimize suffering.

Animals

Adult, gonadally intact female mice were used from local colonies bred at the Medical Gene Technology Unit of the Institute of Experimental Medicine (IEM). They were housed in light (12:12 light-dark cycle, lights on at 06:00 h) – and temperature (22 ± 2°C) controlled environment, with free access to standard food and tap water. GnRH-green-fluorescent protein (GnRH-GFP) transgenic mice (Suter et al., 2000) bred on a C57BL/6J genetic background were used. In this animal model, a GnRH promoter segment drives selective GFP expression in the majority of GnRH neurons. The estrous cycle was monitored daily between 9 and 10 am by microscopic evaluation of vaginal cytology (Byers et al., 2012). Proestrous (n = 6) and metestrous (n = 6) female mice with at least two consecutive, regular estrous cycles were used. In order to avoid the possible circadian effect, animals were sacrificed at the same period.
of the day, between 16:00 and 18:00 h. Those animals were considered to be in the proestrous stage that fulfilled the following criteria: (1) vaginal smear staining with predominance of nucleated epithelial cells (Byers et al., 2012); (2) LH serum concentrations > 5 ng/ml (15.11 ± 3.4 ng/ml); (3) uterus wet weights > 0.15 g (0.19 ± 0.01 g). Accordingly, the following criteria were applied for the metestrous cycle phase: (1) vaginal smears consisting of the three cell types: leukocytes, cornified, and nucleated epithelial cells (Byers et al., 2012); (2) serum LH levels < 0.5 ng/ml (0.35 ± 0.02 ng/ml); (3) uterus wet weights < 0.1 g (0.08 ± 0.01 g).

**Serum LH Measurements**

Blood samples were collected from the heart of deeply anesthetized mice immediately before the brain fixation step. The samples were chilled on ice, centrifuged at 1,300 g for 3 min at 4°C. Plasma was aspirated then frozen and stored at −80°C until further use. Serum LH concentrations were measured with a rodent LH ELISA kit #ERK R7010 from Endocrine Technologies Inc. (Newark, CA, United States) according to manufacturer’s instructions.

**Laser Capture Microdissection, RNA Isolation and Whole Transcriptome Amplification (WTA)**

Brain fixation, preparation of sections for the subsequent laser capture microdissection (LCM) and microarray profiling were performed as reported elsewhere (Khodosevich et al., 2007; Vastagh et al., 2015). Briefly, metestrous (n = 6) and proestrous female (n = 6) mice were deeply anesthetized with ketamine/xylazine (100 and 10 mg/kg body weight, respectively) and perfused transcardially with 80 ml 0.5% paraformaldehyde followed by 20% sucrose. For microdissection, 7 μm thick coronal brain sections were cut using a CM3050S cryostat (Leica, Wetzlar, Germany). Sections were mounted on PEN-membrane slides (Zeiss, Jena, Germany), processed further for laser microdissection. Uniform and representative sampling of the entire GnRH neuronal population was performed using LCM performed on a PALM Microbeam system (Carl Zeiss Microimaging GmbH, Jena, Germany) which was equipped with an epifluorescent setup. 250 GFP-positive neurons were dissected and pooled from 80 to 100 consecutive sections of each brain.

GnRH cell samples collected with LCM were incubated in 200 μl lysis buffer at 56°C for 3 h. RNA was isolated from the lysate by proteinase K/acid phenol method. RNA was purified using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany). Total RNA was eluted with 14 μl of ribonuclease-free water. The quality of RNA was measured with Bioanalyzer. The integrity of the isolated RNA (RIN values: 6.8–7.2) was proven sufficient for the subsequent amplification steps.

Library preparation and amplification were performed according to the manufacturer’s (Sigma-Aldrich) instructions for the WTA2 kit. When the SYBR Green signal reached a plateau, the reaction was stopped. The yielded cDNA (fragment length: 100–1,000 bp, amount: 7–8 microgram) met the criteria of Pico profiling of low cell numbers (Gonzalez-Roca et al., 2010). The amplified double-stranded cDNA was purified and quantified on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

**Mouse Genome 430 PM Arrays**

Eight 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, United States). Hybridization, washing, staining and scanning of Affymetrix Mouse Genome 430 PM Strip arrays were performed following the manufacturer’s recommendations. 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 was performed by means of the statistical analysis software Partek Genomics Suite (Partek Inc., St. Louis, MO, United States) to obtain probe set level expression estimates.

**Bioinformatics, Data Analysis**

All statistical and data mining works were performed in R-environment (R Core Team, 2018) with Bioconductor packages (Huber et al., 2015). Quality assessment of microarrays (n = 12) was performed using affyQCReport. Raw microarray data were pre-processed for analysis using RMA (Robust Multi-Array Average) (Irizarry et al., 2003). Fold change (FC) estimation and difference analysis of gene expression were based on linear models combined with Bayesian methods. FC was calculated from normalized and log2 transformed gene expression microarray data for each probe sets. The obtained p-values were adjusted by the FDR-based method. The following cut-off criteria were applied on the differentially expressed genes: fold change > 1.5; and adjusted p-value (p_{adj}) < 0.05.

The differentially regulated genes were displayed in heat map. KEGG pathway analysis was used to reveal the main gene ontology (GO) pathways associated with molecular functions linked to the differentially expressed genes (DEGs). The putative interactions among proteins encoded by DEGs were analyzed by the web-based STRING v10.5 program (Szklarczyk et al., 2015).

**Validation of Microarray Data With Quantitative Real-Time PCR Studies**

For quantitative real-time PCR (qPCR) investigations of LCM-derived GnRH samples (proestrous females n = 6, metestrous n = 5) RNA isolation and WTA were performed as described in the previous section. Amplified and column-purified cDNA was used as template for qPCR. Whole transcriptome-amplified cDNA from LCM samples were diluted in 0.1× TE buffer for qPCR investigation. Invented TaqMan assays were used to confirm microarray results by qPCR. Each assay consisted of a FAM dye-labeled 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 ViiA 7 real-time PCR platform (Life Technologies Inc., St. Louis, MO, United States) to obtain probe set level expression estimates.

1. [http://www.genome.jp/kegg/](http://www.genome.jp/kegg/)

2. [https://string-db.org](https://string-db.org)
In this work, we examined the influence of proestrus on the expression of genes encoding various ion channel proteins and regulators of intracellular calcium homeostasis in GnRH neurons dissected from intact, metestrous and proestrous GnRH-GFP transgenic mice brains, respectively.

The used Mouse Genome 430 PM Strip Array allowed the analysis of 20563 genes. The total number of differentially regulated genes was 5791. Kegg pathway analysis and hand-picking were used for forming the “ion channel proteins and regulators of calcium homeostasis” group with 85 members. At the used cut-off values [fold change > 1.5; and adjusted p-value ($p_{adj}$) < 0.05], the final group numbered 45 genes. Twenty-five of them were upregulated (Table 1). The differential expression of individual genes was displayed in heat map (Figure 1). The top gene ontology (GO) “molecular function” pathways linked to the differentially expressed genes are summarized in Table 2. The predicted interactions among proteins encoded by the DEGs in GnRH neurons of late proestrous mice are depicted in Figures 2, 3, as up- and downregulated clusters, respectively.

**Differential Expression of Genes Encoding Voltage-Gated Sodium Channels**

Analysis of microarray data revealed differentially expressed genes encoding sodium channels. Three different ion-pore forming alpha 1 subunits of the voltage-gated sodium channels (Scn2a1, Scn3a, and Scn9a) were affected. All of them showed upregulation (Table 1 and Figure 1).

**Influence of Proestrus on the Expression of Potassium Channel Genes**

Similar to the sodium channel-coding genes, proestrus altered heavily the expression of potassium channel genes (Table 1 and Figure 1). Altogether 18 genes displayed differential expression. Within the voltage-gated potassium channel class, 8 genes were influenced. Kcna1, Kcnd3, Kcnh3, and Kcnj2 were upregulated, while Kcnj4, Kcnj3, Kcnj2, and Kcnj1 responded with downregulation. Proestrus also had impact on inwardly rectifying potassium channel subunits manifested in increased expression of Kcnj9 and Kcnj10 genes, whereas Kcnj1, Kcnj11, and Kcnj12 subunit genes showed reduced expression. The two-pore domain potassium channel subfamily also displayed differential expression with upregulation of Kcnk1 and diminished expression of 3 subunit genes (Kcnk7, Kcnk12, and Kcnk16). The BK group of calcium-activated potassium channel class *per se* was not affected, only the non-coding strand of the beta 4 subunit (Kcnmb4os) showed downregulation in proestrus.

**Proestrus Evoked-Changes in Expression of Chloride Channel Genes**

Proestrus influenced the expression of chloride channel genes (Table 1 and Figure 1). Three of them demonstrated differential expression. Two voltage-gated chloride channel genes were upregulated (Clcn3 and Clcn6), while the expression of the intracellular type of chloride channel gene (Clic1) showed an opposite trend.

**Differential Expression of Calcium Channel-Related Genes**

Altogether 13 calcium channel-related genes were differentially regulated by proestrus, 8 exhibited upregulation (Table 1 and Figure 1). From the ion pore-forming alpha 1 subunits of the voltage-dependent calcium channel, two were upregulated (Cacna1b and Cacna1h), while Cacna1g showed downregulation, indicating that both N- and T-types of voltage dependent calcium channel are influenced by proestrus. Genes coding for the auxiliary subunits of the channel were also differentially regulated. Two genes belonging to the alpha2 delta subunit were altered, Cacna2d1 showed upregulation, while expression of Cacna2d2 was decreased. Three auxiliary beta subunits displayed marked upregulation (Cacnb1, Cacnb3, and Cacnb4). Out of the three regulated gamma subunits, Cacng5 increased its expression, whereas Cacng6 and Cacng8 were downregulated. In addition, the intracellular calcium channel coding gene, ryanodine receptor 1 (Ryr1) was downregulated. A transient receptor potential cation channel (Trpm3) gene showed enhanced expression.

**Effects of Proestrus on Regulators of Intracellular Calcium Homeostasis**

In this functional category, genes coding for calbindin 1 (Calb1), hippocalcin (Hpca), hippocalcin-like 1 (Hpcal1), hippocalcin-like 4 (Hpcal4) and the calcium/calmodulin-regulated enzyme, inositol 1,4,5-trisphosphate 3-kinase B gene (Itpkb) were all upregulated. In contrast, other calcium binding protein genes showed downregulation (Cabp7, Cab39l, and Cib2).

**Validation of the Microarray Data**

TaQMan real-time PCR was used for validation of microarray data (Figure 4). The expression of seven arbitrarily chosen genes was confirmed including upregulated (Kcnj10, Trpm3, Calb1, Clcn6, Kcnq2, and Cacna1b) and downregulated (Kcnj3) representatives of the proestrus-regulated ion channels. The coefficient of determination ($r^2 = 0.7349$) indicates the rate of explained variation and predictable association between the log2 transformed FC (microarray) and RQ (qPCR) data. The Pearson product-moment statistics showed significant linear correlation between microarray and PCR data (Pearson’s $r = 0.8685$, df = 5, $p = 0.0137$, CI = 0.95).
TABLE 1 | Differentially expressed genes encoding ion channels and regulators of calcium homeostasis in GnRH neurons of proestrous mice.

| Probe ID     | Gene symbol | Gene synonym | Description                        | FC   | adj. p-val |
|--------------|-------------|--------------|------------------------------------|------|-----------|
| Sodium channel | Scn3a       | Nav1.3     | Sodium channel, voltage-gated, type III, alpha | 3.33 | 6.23E-03  |
|              | Scn9a       | Nav1.7     | Sodium channel, voltage-gated, type IX, alpha | 2.46 | 1.10E-02  |
|              | Scn2a1      | Nav1.2     | Sodium channel, voltage-gated, type II, alpha 1 | 1.98 | 3.71E-03  |
| Potassium channel | Kcnq2    | Kv7.2     | Potassium voltage-gated channel, subfamily Q, member 2 | 2.68 | 2.56E-03  |
|              | Kcnq10      | Kr4.1     | Potassium inwardly rectifying channel, subfamily J, member 10 | 2.59 | 1.46E-02  |
|              | Kcnk1       | K2p1.1    | Potassium channel, subfamily K, member 1 | 2.06 | 2.20E-02  |
|              | Kcnld3      | Kv4.3     | Potassium voltage-gated channel, Shal-related family, member 3 | 2.02 | 6.94E-04  |
|              | Kcnq9       | K3.3      | Potassium inwardly rectifying channel, subfamily J, member 9 | 1.84 | 2.87E-03  |
|              | Kcn585      | Kv1.1     | Potassium voltage-gated channel, shaker-related subfamily, member 1 | 1.80 | 4.80E-02  |
|              | Kcn3        | Kv2.1     | Potassium voltage-gated channel, subfamily H (eag-related), member 3 | 1.59 | 3.23E-02  |
|              | Kcn16       | K2p16.1   | Potassium channel, subfamily K, member 16 | 0.66 | 5.95E-03  |
|              | Kcn7        | K2p7.1    | Potassium channel, subfamily K, member 7 | 0.66 | 1.19E-02  |
|              | Kcn12       | K2r.2     | Potassium inwardly rectifying channel, subfamily J, member 12 | 0.65 | 1.46E-02  |
|              | Kcn4        | Kv1.4     | Potassium voltage-gated channel, shaker-related subfamily, member 4 | 0.65 | 5.98E-03  |
|              | Kcn3        | Kv3.3     | Potassium voltage-gated channel, Shaw-related subfamily, member 3 | 0.61 | 1.25E-03  |
|              | Kcn1        | K1.1      | Potassium inwardly rectifying channel, subfamily J, member 1 | 0.60 | 7.91E-03  |
|              | Kcn12       | K2p12.1   | Potassium channel, subfamily K, member 12 | 0.59 | 1.14E-02  |
|              | Kcnmb4os1   | Kcnmb4os1 | Potassium large conductance calcium-activated channel, subfamily M, beta member 4, opposite strand 1 | 0.56 | 3.72E-03  |
| Chloride channel | Ocn3      | Ocn3       | Chloride channel 3 | 4.53 | 2.42E-04  |
|              | Ocn6        | Ocn6       | Chloride channel 6 | 2.27 | 2.81E-04  |
|              | Clic1       | Clic1      | Chloride intracellular channel 1 | 0.61 | 1.79E-02  |
| Calcium channel | Cacn1      | Cacn1      | Calcium channel, voltage-dependent, beta 1 subunit | 2.11 | 4.13E-02  |
|              | Cacn3       | Cacn3      | Calcium channel, voltage-dependent, beta 3 subunit | 2.08 | 1.04E-02  |
|              | Cacn2d1     | Cacn2d1   | Calcium channel, voltage-dependent, alpha2/delta subunit 1 | 1.99 | 7.15E-03  |
|              | Cacn4       | Cacn4      | Calcium channel, voltage-dependent, beta 4 subunit | 1.96 | 2.76E-02  |
|              | Trpm3       | Trpm3     | Transient receptor potential cation channel, subfamily M, member 3 | 1.72 | 7.28E-04  |
|              | Cacng5      | Cacng5    | Calcium channel, voltage-dependent, gamma subunit 5 | 1.70 | 6.23E-03  |
|              | Cacng6      | Cacng6    | Calcium channel, voltage-dependent, N type, alpha 1B subunit | 1.68 | 9.04E-03  |
|              | Cacna2d2    | Cacna2d2  | Calcium channel, voltage-dependent, alpha 2/delta subunit 2 | 0.64 | 1.74E-02  |
|              | Cacna1g     | Cacna1g   | Calcium channel, voltage-dependent, T type, alpha 1G subunit | 0.63 | 6.43E-03  |
|              | Cacng8      | Cacng8    | Calcium channel, voltage-dependent, gamma subunit 8 | 0.39 | 5.34E-05  |
| Calcium homeostasis | Calb1    | Calb1      | Calbindin 1 | 3.49 | 2.65E-05  |
|              | Hpcion      | Hpcion    | Hippocalcin | 3.41 | 2.98E-02  |
|              | Itpkb       | Itpkb     | Inositol 1,4,5-trisphosphate 3-kinase B | 1.88 | 1.79E-02  |
|              | Hpcal1      | Hpcal1    | Hippocalcin-like 1 | 1.76 | 9.06E-03  |
|              | Hpcal4      | Hpcal4    | Hippocalcin-like 4 | 1.75 | 3.25E-02  |
|              | Calc39      | Calc39    | Calcium binding protein 39-like | 0.62 | 1.12E-02  |
|              | Calb7       | Calb7     | Calcium binding protein 7 | 0.45 | 8.49E-03  |
|              | Calb2       | Calb2     | Calcium and integrin binding family member 2 | 0.22 | 2.32E-03  |

List of differentially expressed genes (DEGs). FC values indicate the changes of gene expression in proestrous versus metestrous GnRH neurons. Gene symbols and FC values of upregulated genes are in bold. FC, fold change; adj.P.Val, adjusted P-value.
DISCUSSION

The basic types of ion channels have been described in GnRH neurons and correlated with characteristics of different currents generated under various physiological conditions in these neurosecretory cells (Kato et al., 2009; Moenter, 2010; Bosch et al., 2013; Norberg et al., 2013). Due to the fundamental role of E2 in regulation of the HPG axis via negative and positive
abolishes the supply of several other indispensable hormones, O VX – in addition to ceasing the natural E2 signaling – also E2 replacement and preserve a fully functional ovarian system. we aimed to exclude the diverse effects of ovariectomy and as a consequence of proestrus. Accordingly, using this model, expression of ion channels genes in mouse GnRH neurons, has been to gain knowledge about changes occurring in the intact, cycling mice was purposeful in the study. Our intention et al., 2011; Bosch et al., 2013). 

Choosing the preovulatory functional state of GnRH neurons in intact, cycling mice was purposeful in the study. Our intention has been to gain knowledge about changes occurring in the expression of ion channels genes in mouse GnRH neurons, as a consequence of proestrus. Accordingly, using this model, we aimed to exclude the diverse effects of ovariectomy and E2 replacement and preserve a fully functional ovarian system. O VX – in addition to ceasing the natural E2 signaling – also abolishes the supply of several other indispensable hormones, such as progesterone, activin, inhibin and anti-Müllerian hormone to the brain, including GnRH neurons and their afferents. Furthermore, the E2 replacement of O VX mice cannot totally mimic the natural fluctuation of E2. That might explain why the proestrus driven expression pattern of ion channels in GnRH neurons only partially match data published in O VX + E2 mice (Zhang et al., 2007; Bosch et al., 2013). The functional differences between the two rodent models are important and should be further clarified at both molecular and network levels of GnRH neurons. Recent electrophysiological reports are in support of the rational using intact, regularly cycling mice (Farkas et al., 2013, 2018; Adams et al., 2018).

Accordingly, in the present transcriptome study of mouse GnRH neurons, we focused on the impact of proestrus on the expression of genes coding for ion channel and calcium binding proteins. Proestrus resulted in differential expression of 45 genes. A similar robust effect of proestrus on the expression of neurotransmitter receptor genes in GnRH neurons has recently been reported (Vastagh et al., 2016).

### Proestrus Upregulates Voltage-Gated Sodium Channels

Sodium and calcium channels have been found to regulate the LH surge-generating mechanisms in the preoptic area of proestrous rats (Fukushima et al., 2003). GnRH neurons display tetrodotoxin-sensitive voltage-gated sodium channels, with a high density in the initial 150 micrometer segment of their dendrites (Iremonger and Herbison, 2012). The channels are involved in generation of the rapid depolarizing phase of the action potential and formation of slow after depolarization (sADP) (Chu and Moenter, 2006) that contributes to repetitive firing. Proestrus upregulated three alpha subunits [Scn2a1 (Nav1.2), Scn3a (Nav1.3) and Scn9a (Nav 1.7)] of the sodium channels without any effect on the expression of the beta subunit. These changes suggest that proestrus including the characteristic rise and peak of E2 leads to the modulation of sodium conductance in GnRH neurons. Chu and Moenter (2006) have shown that tetrodotoxin (TTX)-sensitive sodium conductance mediates the intrinsically generated sADPs which seem to contribute to repetitive firing of GnRH cells and release of the neurohormone. These events – in addition – to serving the burst firing also prolong the depolarization, change the responsiveness to network influences, alter the frequency of GnRH pulses and synchronize the network for an optimal hormone discharge (Chu and Moenter, 2006), features that also characterize the surging GnRH neurons.

### Voltage-Gated, Inwardly Rectifying and Two-Pore-Domain Potassium Channels Are Targeted by Proestrus

The different potassium conductances and their estrogen sensitivity in GnRH neurons have recently been reviewed (Norberg et al., 2013). Voltage-gated potassium currents (I_A and I_K) are regulated by feedback actions of E2 (DeFazio and Moenter, 2002; Pielecka-Fortuna et al., 2011) and they are known to control the excitability and discharge

### Table 2: Gene ontology.

| Pathway ID | Pathway description | Count in gene set |
|------------|---------------------|-------------------|
| GO:0005244 | Voltage-gated ion channel activity | 31 |
| GO:0005261 | Cation channel activity | 28 |
| GO:0005283 | Voltage-gated cation channel activity | 24 |
| GO:0048873 | Metal ion transmembrane transporter activity | 28 |
| GO:0022857 | Transmembrane transporter activity | 32 |
| GO:0005267 | Potassium channel activity | 17 |
| GO:0005249 | Voltage-gated potassium channel activity | 15 |
| GO:0015077 | Monovalent inorganic cation transmembrane transporter activity | 18 |
| GO:0005262 | Calcium channel activity | 10 |
| GO:0005245 | Voltage-gated calcium channel activity | 8 |
| GO:0005242 | Inward rectifier potassium channel activity | 6 |
| GO:0008331 | High voltage-gated calcium channel activity | 4 |
| GO:0015276 | Ligand-gated ion channel activity | 7 |
| GO:0015272 | ATP-activated inward rectifier potassium channel activity | 3 |
| GO:0005251 | Delayed rectifier potassium channel activity | 4 |
| GO:0031420 | Alkaline metal ion binding | 3 |
| GO:0016247 | Channel regulator activity | 5 |
| GO:0005250 | A-type (transient outward) potassium channel activity | 2 |
| GO:0005509 | Calcium channel activity | 8 |
| GO:0044325 | Ion channel binding | 4 |
| GO:0005246 | Calcium channel regulator activity | 3 |
| GO:0039065 | Potassium ion binding | 2 |
| GO:002841 | Potassium ion leak channel activity | 2 |
| GO:0005247 | Voltage-gated chloride channel activity | 2 |
| GO:0005254 | Chloride channel activity | 3 |

List of GO molecular function pathways affected by the 45 differentially regulated ion channel genes in GnRH neurons of proestrous mice. The top 10 pathways are characterized by large count numbers in the gene set. The top 10 pathways are characterized by large count numbers in the gene set (8–32).
activity of GnRH cells. $K_{ATP}$ activity is also modified by E2 (Zhang et al., 2007), similar to the $S_{K}$ channel (Bosch et al., 2002). GnRH neurons also exhibit BK (Hiraizumi et al., 2008) and M (Xu et al., 2008) currents, although they have been reported to lack estrogen sensitivity (Norberg et al., 2013).

**Voltage-Gated Potassium Channel**

Four voltage-gated potassium channels responded to proestrus with upregulation. Kcnq2 (Kv7.2) which contributes to the formation of M-potassium channel was robustly upregulated, while the other two known constituents of M channel in GnRH neurons, Kcnq3 and Kcnq5 (Xu et al., 2008) were not altered. The M-type potassium currents are subthreshold, non-inactivating channels that diminish cell excitability (Norberg et al., 2013). They may contribute to the autoregulation of the GnRH neuronal network via a self-feedback loop (Xu et al., 2008). The enhanced Kcnq2 mRNA expression may reflect the preparation of the GnRH neuron for the forthcoming shutdown of surge-related electrophysiological events and desynchronization of the GnRH network.

The upregulated Kcnid3 (Kv4.3) shows the influence of high E2 on the expression of the Shal-related, fast inactivating A-type potassium channel. This finding is in line with a previous report showing the increased expression of Kv4.3 in GnRH neurons of diestrus-proestrus rats compared to metestrous animals (Arroyo et al., 2011).

The coding gene of a slowly inactivating, Shaker-related, K-type potassium channel, the Kcnal (Kv1.1) also displayed upregulation, together with the Kcnh3 (Kv3.3), the slowly activating channel gene. The shaker-related subfamily of potassium channel exists in mouse GnRH neurons (Liu and Herbsom, 2008). Elevated levels of intracellular calcium lead to its activation and control of the firing dynamism of neurons.

The downregulated category was comprised of mainly A type potassium channel genes, the Kcna4 (Kv1.4), Kcn3 (Kv3.3), and Kcnd2 (Kv4.2). The modifier/silencer channel Kcnng1 (Kv6.1) also showed decreased expression.

These data indicate that the high E2 level at late proestrus alters the expression of several voltage-gated potassium channel subunit genes and modifies $I_A$, $I_K$, and $I_M$ types of potassium channels.
conductances in GnRH neurons that control their excitability and hormone release (DeFazio and Moenter, 2002).

Inwardly Rectifying Potassium Channel

Proestrus exerted influence on the expression of a wide range of genes known to code inwardly rectifying potassium channels. Two members of this potassium channel subfamily displayed upregulation, Kcnj9 (Kir3.3,GIRK3) and Kcnj10 (Kir.4.1). The others were downregulated including Kcnj1 (Kir1.1), Kcnj11 (Kir6.2, K_ATP), and Kcnj12 (Kir2.2, IRK2). Among the inwardly rectifying potassium channel proteins, Kir6.2 has comprehensively been studied in GnRH neurons (Zhang et al., 2007, 2009). Together with sulphonylurea receptor, it forms the ATP sensitive K⁺ (K_ATP) channel. It is expressed in about 50% of GnRH neurons of both sexes. In females, the current carried by the K_ATP channel is controlled by E2, while the expression of Kir6.2 is not regulated by E2 (Zhang et al., 2007). Our present findings indicate that the expression of Kir. 6.2. is downregulated in proestrus.

The hyperpolarization imposed on dendrites and somata of GnRH neurons by K_ATP (Norberg et al., 2013) may be diminished or canceled by the decreased expression of Kir.6.2 in proestrus.

The role of G protein-gated inwardly rectifying potassium channels (GIRKs) in regulation of GnRH neurons has been demonstrated in case of galanin (Constantin and Wray, 2016) and luteinizing hormone (Hu et al., 2006) modulation.

Two-Pore-Domain Potassium Channel

This subfamily K of potassium channel participates in coding for proteins of leak potassium channels (Enyedi and Czirjak, 2010) which contribute to the resting potential. In addition, they are also regulated by G protein-coupled receptors (Mathie, 2007). The present results clearly show that proestrus influences the expression of this potassium channel subfamily, evoking the upregulation of Kcnk1, and decreased expression of
Knck7, Knk12, and Knck16. Understanding the exact role of these channels in physiology of GnRH neurons awaits further studies.

**Ca^{2+}-Activated Potassium Channel**

In contrast to the above described three, main potassium channel types altered by proestrus, the subunits of the large conductance calcium-activated potassium channel (BK channel) (Hiraizumi et al., 2008) were not influenced by proestrus. Only the non-coding strand of beta 4 subunit (Kcnmb4os1) showed a downregulatory response in proestrus.

**Influence of Proestrus on Chloride Channel-Coding Genes**

The intracellular concentration of chloride ions in rodent GnRH neurons is maintained by several mechanisms including ligand-gated neurotransmitter receptors (GABA\textsubscript{A} receptor and glycine...
facilitates neurotransmitter release. The skeletal muscle type ryanodine receptor (Ryr1) is widely expressed in the brain, including the hypothalamus (De Crescenzo et al., 2012). We found its expression downregulated in late proestrus. Ryr1 is coupled to L-type calcium channels and controls the voltage-induced calcium release from internal Ca\(^{2+}\) stores. Its significance in the calcium homeostasis of GnRH neurons requires further studies.

Activation of the transient receptor potential melastatin-3 (TRPM3) channel results in a rise of intracellular calcium (Thiel et al., 2017). Proestrus caused a significant upregulation of its coding gene (Trpm3). The participation of TRPM3 channel in the modulation of the intracellular calcium concentration and its putative contribution to development of calcium transients and concurrent burst firing of GnRH neurons (Constantin et al., 2012) await further studies.

**Expression of Calcium Channels Is Regulated by Proestrus**

Proestrus heavily changed the expression of voltage-gated calcium channel genes. Both ion pore-forming, voltage-sensing alpha subunits and the different auxiliary subunits (beta, alpha2-delta, and gamma) were altered. In addition, a transient receptor potential cation channel gene (Trpm3) that encodes a channel for constitutive entry of Ca\(^{2+}\) and a ryanodine receptor-coding gene (Ryr1) were also differentially regulated in proestrus.

**Effects of Proestrus on Voltage-Gated Calcium Channel Expression**

Both high and low voltage-activated calcium channels were altered. Regarding the high voltage class, Cacna1b (Cav2.2) was upregulated, indicating a selective effect upon the N type channel. In the low voltage category, two T-type channels were targeted. Cacna1h (Cav3.2) was upregulated, while Cacna1g (Cav3.1) showed decreased expression. The auxiliary units of the channel also showed a marked response. Three beta subunit genes underwent upregulation (Cacnb1, Cacnb3, and Cacnb4). The alpha2 delta and gamma subunits changed their expression in both directions. Alpha2-delta 1 (Cacna2d1) and gamma subunit five (Cacng5) genes increased their expression in proestrus. In contrast, alpha2-delta 2 (Cacna2d2), gamma subunit six (Cacng6) and gamma subunit 8 (Cacng8) all showed downregulation. GnRH neurons express all four types of high voltage-activated calcium channels (L, N, P/Q, and R) and the T-type channel regulated by low voltage (Bosma, 1993; Kato et al., 2003, 2009; Nunemaker et al., 2003; Tanaka et al., 2010; Bosch et al., 2013). E2 treatment of O VX mice increased the expression of the alpha1 subunits of the T type channel and augmented the density of T calcium currents (Zhang et al., 2009). Similar to the O VX-E2 animal model, these channel events may also contribute to the burst firing of GnRH neurons in late proestrus. In another study, the low voltage-mediated calcium currents were not affected by E2, while the high voltage-activated currents, especially the L-and N-type components, were influenced by E2 with cooperation of estrogen receptor beta and GPR30 activation (Sun et al., 2010).

The skeletal muscle type ryanodine receptor (Ryr1) is widely expressed in the brain, including the hypothalamus (De Crescenzo et al., 2012). We found its expression
of the calcium channel, the response was manifested in 3 subunits
of the voltage-dependent calcium channels. The downregulated
potassium channel proteins belonged to the K-type subfamily and
the voltage-gated potassium channel class. The downregulation
of these potassium channel components may support the
increased activity of GnRH neurons at late proestrus.

CONCLUSION

In this study, we elucidated the expression profile of ion channel
genes in GnRH neurons of regularly cycling mice processed
before the onset of the GnRH surge. The dataset allows insight
into the putative remodeling of the different channels. Although,
E2 is a key hormone in the positive gonadal steroid feedback
acting on GnRH neurons and their afferent neurons, other ovary-
born hormones (inhibin, anti-Mullerian hormone and others)
also have regulatory effects (Adams et al., 2018). Therefore,
we have put the emphasis on the impact of proestrus, instead
of E2. Regarding the molecular events, we explored significant
differences in expression of genes encoding sodium, potassium,
and chloride and calcium ion channel-forming proteins in GnRH
neurons obtained from pro- and metestrous mice, respectively.
The differential expression of ion channel-coding genes in
proestrus elucidates the subtypes of ion channels that contribute
to the altered electrophysiology and function of GnRH neurons
prior to the GnRH surge.

AUTHOR CONTRIBUTIONS

CV designed and performed the experiments and analyzed
the data. NS carried out the bioinformatical analysis of
the microarray data. IF contributed to discussion of
electrophysiological significance of findings. ZL designed and
supervised the project and wrote the manuscript.

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REFERENCES

Adams, C., Stroberg, W., DeFazio, R. A., Schnell, S., and Moenter, S. M. (2018).
Gonadotropin-releasing hormone (GnRH) neuron excitability is regulated by
estradiol feedback and kisspeptin. J. Neurosci. 38, 1249–1263. doi: 10.1523/
JNEUROSCI.2988-17.2017
Arroyo, Á., Kim, B. S., Biehl, A., Yeh, J., and Bett, G. C. (2011). Expression
of Kv4.3 voltage-gated potassium channels in rat gonadotrophin-releasing
hormone (GnRH) neurons during the estrous cycle. Reprod. Sci. 18, 136–144.
doi: 10.1177/1933719110382306
Bosch, M. A., Kelly, M. J., and Ronnekleiv, O. K. (2002). Distribution, neuronal
colocalization, and 17beta-E2 modulation of small conductance calcium-
activated K+ channel (SK3) mRNA in the guinea pig brain. Endocrinology 143,
1097–1107. doi: 10.1210/endo.143.3.87808
Bosch, M. A., Tonsfeldt, K. J., and Ronnekleiv, O. K. (2013). mRNA expression of
ion channels in GnRH neurons: subtype-specific regulation by 17beta-estradiol.
Mol. Cell. Endocrinol. 367, 85–97 doi: 10.1016/j.mce.2012.12.021
Bosma, M. M. (1993). Ion channel properties and episodic activity in isolated
immortalized gonadotropin-releasing hormone (GnRH) neurons. J. Membr.
Biol. 136, 85–96.
Byers, S. L., Wiles, M. V., Dunn, S. L., and Taft, R. A. (2012). Mouse estrous cycle
cell identification tool and images. PLoS One 7:e35538. doi: 10.1371/journal.pone.
0035538
Carmel, P. W., Araki, S., and Ferin, M. (1976). Pituitary stalk portal blood
determination of gonadotropin-releasing hormone neurons during the estrous cycle.
Endocrinology 99, 243–248. doi: 10.1210/endo-99-1-243
Christian, C. A., Mobley, J. L., and Moenter, S. M. (2005). Diurnal and estradiol-
dependent changes in gonadotropin-releasing hormone neuron firing activity.
Proc. Natl. Acad. Sci. U.S.A. 102, 15682–15687. doi: 10.1073/pnas.0504270102
Christian, C. A., and Moenter, S. M. (2010). The neurobiology of preovulatory
and estradiol-induced gonadotropin-releasing hormone surges. Endocrinology
99, 243–248. doi: 10.1210/endo-99-1-243
Chu, Z., Andrade, I., Shupnik, M. A., and Moenter, S. M. (2009). Differential
regulation of gonadotropin-releasing hormone neuron activity and membrane
properties by acutely applied estradiol: dependence on dose and estrogen
receptor subtype. J. Neurosci. 29, 5616–5627. doi: 10.1523/JNEUROSCI.0352-
09.2009
Chu, Z., and Moenter, S. M. (2006). Physiologic regulation of a tetrodotoxin-
sensitive sodium influx that mediates a slow afterdepolarization potential in
gonadotropin-releasing hormone neurons: possible implications for the central
regulation of fertility. J. Neurosci. 26, 11961–11973. doi: 10.1523/JNEUROSCI.
3171-06.2006
Chu, Z., Takagi, H., and Moenter, S. M. (2010). Hyperpolarization-activated
currents in gonadotropin-releasing hormone (GnRH) neurons contribute to
intrinsic excitability and are regulated by gonadal steroid feedback. J. Neurosci.
30, 13373–13383. doi: 10.1523/JNEUROSCI.1687-10.2010
Chu, Z., Tomaiaulo, M., Bertram, R., and Moenter, S. M. (2012). Two types of burst
firing in gonadotropin-releasing hormone neurons. J. Neuroendocrinol. 24,
1065–1077. doi: 10.1111/j.1365-2826.2012.02313.x
Clarke, I. J., and Cummins, J. T. (1985). Increased gonadotropin-releasing
hormone pulse frequency associated with estrogen-induced luteinizing
hormone surges in ovariectomized ewes. Endocrinology 116, 2376–2383.
doi: 10.1210/endo-116-6-2376
Constantin, S., Jasoni, C., Romano, N., Lee, K., and Herbison, A. E. (2012).
Understanding calcium homeostasis in postnatal gonadotropin-releasing
hormone neurons using cell specific Pericam transgenics. Cell Calcium 51,
267–276. doi: 10.1016/j.cca.2011.11.005
Costantini, S., and Wray, S. (2016). Galanin activates G protein gated inwardly
rectifying potassium channels and suppresses kisspeptin-10 activation of gnrh
neurons. Endocrinology 157, 3197–3212. doi: 10.1210/en.2016-1064
Costantini, J. L., and Charles, A. C. (2001). Modulation of Ca(2+) signaling by K(+) channels in a hypothalamic neuronal cell line (GT1-1). J. Neurophysiol. 85,
295–304. doi: 10.1152/jn.2001.85.1.295
De Crescenzo, V., Fogarty, K. E., Leikowitz, J. J., Bellve, K. D., Zvaritch, E.,
Maclennan, D. H., et al. (2012). Type 1 ryamide receptor knock-in mutation
causing central core disease of skeletal muscle also displays a neuronal
phenotype. Proc. Natl. Acad. Sci. U.S.A. 109, 610–615. doi: 10.1073/pnas.
111511108
DeFazio, R. A., and Moenter, S. M. (2002). Estradiol feedback alters potassium
currents and firing properties of gonadotropin-releasing hormone neurons.
Mol. Endocrinol. 16, 2255–2265. doi: 10.1210/mce.2002-0155
Enyedi, P., and Czirják, G. (2010). Molecular background of leak K currents: two-pore domain potassium channels. Physiol. Rev. 90, 559–605. doi: 10.1152/physrev.00029.2009

Farkas, I., Balint, F., Farkas, E., Vastagh, C., Fekete, C., and Liposits, Z. (2018). Estradiol increases glutamate and GABA neurotransmission into GnRH neurons via retrograde NO-signaling in proestrous mice during the positive estradiol feedback period. eNeuro 5:EUNEuro.0057-18.2018. doi: 10.1523/EUNEuro.0057-18.2018

Farkas, I., Varju, P., and Liposits, Z. (2007). Estrogen modulates potassium currents and expression of the Kv4.2 subunit in GT1-7 cells. Neurochem. Int. 50, 619–627. doi: 10.1016/j.neuint.2006.12.004.

Farkas, I., Vastagh, C., Sarvari, M., and Liposits, Z. (2013). Ghrelin decreases firing activity of gonadotropin-releasing hormone (GnRH) neurons in an estrous cycle and endocannabinoid signaling dependent manner. PLoS One 8:e78178. doi: 10.1371/journal.pone.0078178.

Finn, P. D., Steiner, R. A., and Cliffton, D. K. (1998). Temporal patterns of gonadotropin-releasing hormone (GnRH), c-fos, and galanin gene expression in GnRH neurons relative to the luteinizing hormone surge in the rat. J. Neurosci. 18, 713–719. doi: 10.1523/neurosci.18-02-00713.1998

Fukushima, A., Sano, A., Aiba, S., and Kimura, F. (2006). Male-specific role of GABA on GnRH neurons in the female mouse. Endocrinology 147, 1459–1466. doi: 10.1210/endo.147.8.145724

Gonzalez-Roca, E., Garcia-Albeniz, X., Rodriguez-Mulero, S., Gomis, R. R., Barra, J., and González, A. (2007). Characterization of Na(+) and Ca(2+) channels in gonadotropin-releasing hormone neurons. J. Neuroendocrinol. 21, 315–325. doi: 10.1111/j.1365-2826.2009.01849.x

Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Bradley, Y. D., Antonellis, K. J., Scherf, U., et al. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264. doi: 10.1093/biostatistics/4.2.249

Jentsch, T. J., Stein, V., Weinreich, F., and Zdebik, A. A. (2002). Molecular structure and physiological function of chloride channels. Physiol. Rev. 82, 503–568. doi: 10.1152/physrev.00029.2001

Kato, M., Tanaka, N., Ishii, H., Yin, C., and Sakuma, Y. (2009). Ca2(+) channels and Ca2(+)-activated K(+) channels in adult rat gonadotropin-releasing hormone neurones. J. Neuroendocrinol. 21, 312–315. doi: 10.1111/j.1365-2826.2009.01849.x

Kelly, M. J., Qiu, J., and Ronneklev, O. K. (2003). Estrogen modulation of G-protein-coupled receptor activation of potassium channels in the central nervous system. Ann. N. Y. Acad. Sci. 1007, 6–16.

Kobul, E. (1998). The neuroendocrine control of ovulation. Hum. Reprod. 3, 469–472. doi: 10.1093/oxfordjournals.humrep.a136730

Krsmanovic, L. Z., Hu, L., Leung, P. K., Feng, H., and Catt, K. J. (2010). Pulsatile GnRH secretion: roles of G protein-coupled receptors, second messengers and ion channels. Mol. Cell. Endocrinol. 314, 158–163. doi: 10.1016/j.mce.2009.05.015

Lee, K., Duan, W., Sneyd, J., and Herbsisson, A. E. (2010). Two slow calcium-activated afterhyperpolarizations control burst firing dynamics in gonadotropin-releasing hormone neurons. J. Neurosci. 30, 6214–6224. doi: 10.1523/JNEUROSCI.6156-09.2010

Levine, J. E. (1997). New concepts of the neuroendocrine regulation of gonadotropin surges in rats. Biol. Reprod. 56, 293–302. doi: 10.1095/biolreprod.56.2.293

Liu, X., and Herbsisson, A. E. (2008). Small-conductance calcium-activated potassium channels control excitability and firing dynamics in gonadotropin-releasing hormone (GnRH) neurons. Endocrinology 149, 3598–3604. doi: 10.1210/en.2007-1631

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

Mathie, A. (2007). Neuronal two-pore-domain potassium channels and their regulation by G protein-coupled receptors. J. Physiol. 578(PT 2), 377–385. doi: 10.1113/jphysiol.2006.121582

Miller, A. T., Sandberg, M., Huang, Y. H., Young, M., Sutton, S., Sauer, K., et al. (2007). Production of Ins(1,3,4,5)P4 mediated by the kinase Itpkb inhibits store-operated calcium channels and regulates B cell selection and activation. Nat. Immunol. 8, 514–521. doi: 10.1038/nait1458

Moenter, S. M., DeFazio, R. A., Pitts, G. R., and Nunemaker, C. S. (2003). Roles of the Kv4.2 subunit in GT1-7 cells. J. Neurosci. 23, 349–359. doi: 10.1523/JNEUROSCI.3739-11.2012

Moenter, S. M., Defazio, R. A., Straume, M., and Nunemaker, C. S. (2003b). Identified GnRH neuron electrophysiology: a decade of progress. Exp. Brain Res. 149, 3506–3509. doi: 10.1210/en.2003-0032

Noel, S. D., Keen, K. L., Baumann, D. I., Filardo, E. J., and Terasawa, E. (2009). Apamin inhibits slow afterhyperpolarization currents in rat gonadotropin-releasing hormone neurons. J. Neuroendocrinol. 21, 312–315. doi: 10.1111/j.1365-2826.2009.01849.x
Norberg, R., Campbell, R., and Suter, K. J. (2013). Ion channels and information processing in GnRH neuron dendrites. Channels 7, 135–145. doi: 10.4161/chan.24228

Numemaker, C. S., DeFazio, R. A., and Moenter, S. M. (2002). Estradiol-sensitive afferents modulate long-term episodic firing patterns of GnRH neurons. Endocrinology 143, 2284–2292. doi: 10.1210/endo.143.6.8669

Numemaker, C. S., DeFazio, R. A., and Moenter, S. M. (2003). Calcium current subtypes in GnRH neurons. Biol. Reprod. 69, 1914–1922. doi: 10.1095/biolreprod.103.019265

Pielecka-Fortuna, J., DeFazio, R. A., and Moenter, S. M. (2011). Voltage-gated potassium targets are targets of diurnal changes in estradiol feedback regulation and kisspeptin action on gonadotropin-releasing hormone neurons in mice. Biol. Reprod. 85, 987–995. doi: 10.1095/biolreprod.111.093492

R Core Team (2018) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. doi: 10.1095/biolreprod.111.093492

Radovick, S., Levine, J. E., and Wolfe, A. (2012). Estrogenic regulation of the GnRH neuron. Front. Endocrinol. 3:52. doi: 10.3389/fendo.2012.00052

Ronnekleiv, O. K., Bosch, M. A., and Zhang, C. (2010). Regulation of endogenous conductances in GnRH neurons by estrogens. Brain Res. 1364, 25–34. doi: 10.1016/j.brainres.2010.08.096

Ronnekleiv, O. K., Bosch, M. A., and Zhang, C. (2012). 17beta-estradiol regulation of gonadotrophin-releasing hormone neuronal excitability. J. Neuroendocrinol. 24, 122–130. doi: 10.1111/j.1365-2826.2011.02160.x

Ronnekleiv, O. K., Zhang, C., Bosch, M. A., and Kelly, M. J. (2015). Kisspeptin and gonadotropin-releasing hormone neuronal excitability: molecular mechanisms driven by 17beta-estradiol. Neuroendocrinology 102, 184–193. doi: 10.1159/000370311

Sarkar, D. K., and Fink, G. (1980). Luteinizing hormone releasing factor in neuroendocrinology. Int. J. Fertil. 25, 269–274. doi: 10.1007/s12576-010-0085-z

Spergel, D. J. (2007). Calcium and small-conductance calcium-activated potassium channels in GnRH neurons. Biol. Reprod. 76, 1914–1922. doi: 10.1095/biolreprod.111.093492

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 43, D447–D452. doi: 10.1093/nar/gku1003

Tanaka, N., Ishii, H., Yin, C., Koyama, M., Sakuma, Y., and Kato, M. (2010). Voltage-gated Ca2+channel mRNAs and T-type Ca2+currents in rat gonadotropin-releasing hormone neurons. J. Physiol. Sci. 60, 195–204. doi: 10.1007/s12576-010-0085-z

Taylor-Burds, C., Cheng, P., and Wray, S. (2015). Chloride accumulators NKCC1 and AE2 in mouse GnRH neurons: implications for GABAA mediated excitation. PLoS One 10:e0131076. doi: 10.1371/journal.pone.0131076

Thiel, G., Rubl, S., Lesch, A., Guethlein, L. A., and Rossler, O. G. (2017). Transient receptor potential TRPM3 channels: pharmacology, signaling, and biological functions. Pharmacol. Res. 124, 92–99. doi: 10.1016/j.phrs.2017.07.014

Toba, Y., Pakiam, J. G., and Wray, S. (2005). Voltage-gated calcium channels in developing GnRH-1 neuronal system in the mouse. Eur. J. Neurosci. 22, 79–92. doi: 10.1111/j.1460-9568.2005.04194.x

Van Goor, F., Krsmanovic, L. Z., Catt, K. J., and Stojilkovic, S. S. (1999). Control of action potential-driven calcium influx in GT1 neurons by the activation status of sodium and calcium channels. Mol. Endocrinol. 13, 587–603. doi: 10.1099/mend.13.4.0261

Vastagh, C., and Liposits, Z. (2017). Impact of proestrus on gene expression in the medial preoptic area of mice. Front. Cell Neurosci. 11:183. doi: 10.3389/fncel.2017.00183

Vastagh, C., Rodolosse, A., Solymosi, N., Farkas, L. Auer, H., Sarvari, M., et al. (2015). Differential gene expression in gonadotropin-releasing hormone neurons of male and metestrous female mice. Neuroendocrinology 102, 44–59. doi: 10.1159/000430818

Vastagh, C., Rodolosse, A., Solymosi, N., and Liposits, Z. (2016). Altered expression of genes encoding neurotransmitter receptors in gnrh neurons of proestrous mice. Front. Cell Neurosci. 10:230. doi: 10.3389/fncel.2016.00230

Wang, H. J., Hoffman, G. E., and Smith, M. S. (1995). Increased GnRH mRNA in the GnRH neurons expressing cFos during the proestrus LH surge. Endocrinology 136, 3677–3676. doi: 10.1210/endo.136.8.7628409

Wang, L., DeFazio, R. A., and Moenter, S. M. (2016). Excitability and burst generation of AVPV kisspeptin neurons are regulated by the estrous cycle via multiple conductances modulated by estradiol action. eNeuro 3:EN0094-16.2016 doi: 10.1523/EUNEURO.0094-16.2016

Wang, Y., Garro, M., and Kuehl-Kovarik, M. C. (2010). Estradiol attenuates multiple tetrodotoxin-sensitive sodium currents in isolated gonadotropin-releasing hormone neurons. Brain Res. 1345, 137–145. doi: 10.1016/j.brainres.2010.05.031

Xu, C., Roepke, T. A., Zhang, C., Ronnekleiv, O. K., and Kelly, M. J. (2008). Gonadotropin-releasing hormone (GnRH) activates the m-current in GnRH neurons: an autoregulatory negative feedback mechanism? Endocrinology 149, 2459–2466. doi: 10.1210/en.2007-1178

Zhang, C., Bosch, M. A., Levine, J. E., Ronnekleiv, O. K., and Kelly, M. J. (2007). Gonadotropin-releasing hormone neurons express K(ATP) channels that are regulated by estrogen and responsive to glucose and metabolic inhibition. J. Neurosci. 27, 10135–10164. doi: 10.1523/JNEUROSCI.1657-07.2007

Zhang, C., Bosch, M. A., Rick, E. A., Kelly, M. J., and Ronnekleiv, O. K. (2009). 17Beta-estradiol regulation of T-type calcium channels in gonadotropin-releasing hormone neurons. J. Neurosci. 29, 10552–10562. doi: 10.1523/JNEUROSCI.2962-09.2009

Zhang, C., Bosch, M. A., Ronnekleiv, O. K., and Kelly, M. J. (2013). Kisspeptin activation of TRPC4 channels in female GnRH neurons requires PI2 depletion and Csrc kinase activation. Endocrinology 154, 2772–2783. doi: 10.1210/en.2013-1180

Zhang, X. B., and Spergel, D. J. (2012). Kisspeptin inhibits high-voltage activated Ca2+channels in GnRH neurons via multiple Ca2+influx and release pathways. Neuroendocrinology 93, 68–80. doi: 10.1159/000335985

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