The Sex-Specific Patterns of Changes in Hypothalamic-Pituitary-Gonadal Axis during Experimental Autoimmune Encephalomyelitis

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Running Head: EAE affects HPG axis

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Abstract

Multiple sclerosis develops during reproductive years in a sex-specific manner. Various neuroendocrine changes have been described in this inflammatory, demyelinating, and debilitating disease. We here aimed to determine the extent and sex specificity of alterations in the hypothalamic-pituitary-gonadal axis in the rat model of multiple sclerosis named experimental autoimmune encephalomyelitis (EAE). During the EAE course, the hypothalamic tissue showed transient upregulation of inflammatory marker genes *Gfap*, *Cd68*, *Ccl2*, and *Il1b* in both sexes, but accompanied by sex-specific downregulation of *Kiss1* (in females only) and *Gnrh1* (in males only) expression. In females, the expression of gonadotrope-specific genes *Lhb*, *Cga*, and *Gnrhr* was also inhibited, accompanied by decreased basal but not stimulated serum luteinizing hormone (LH) levels and a transient arrest of the estrous cycle. In contrast, *Fshb* expression and serum progesterone levels were transiently elevated, findings consistent with the maintenance of the corpora lutea, and elevated immunohistochemical labeling of ovarian StAR, a rate limiting protein in steroidogenic pathway. In males, downregulation of *Gnrhr* expression and basal and stimulated serum LH and testosterone levels were accompanied by inhibited testicular StAR protein expression. We propose that inflammation of hypothalamic tissue downregulates *Kiss1* and *Gnrh1* expression in females and males, respectively, leading to sex-specific changes downstream the axis.

1. Introduction

Over two million people around the world suffer from multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (CNS). Most probably, MS is triggered by the autoreactive T cells, which are activated in the periphery and reactivated in the CNS upon migration through the blood brain barrier. This causes inflammatory lesions in CNS, characterized by the presence of immune cells, including $CD4^+$ cells and macrophages, subsequent demyelination, axonal damage, and neuronal cell loss (Lassmann, 2014). Lesions are most often found in white matter of the brainstem, spinal cord, cerebellum, and in the optic nerve. However, pathological findings in MS are not restricted to the white matter; the gray matter pathology (which usually does not involve lymphocyte infiltration) was registered in the cerebral cortex, deep gray matter structures of the brain, and gray matter of the spinal cord (Hulst and Geurts, 2011; Lassmann, 2018).

Experimental autoimmune encephalomyelitis (EAE) is a widely exploited animal model of MS. Provoked by autoimmune responses to myelin proteins in certain strains of rats and mice and driven mainly by Th17 subset of $CD4^+$ T cells, EAE results in a disease that mimics many aspects of MS symptoms and pathology (Bjelobaba et al., 2018). Similar to MS, EAE is a complex disease that, besides motor abilities, affects multiple organs and systems, and imitates MS symptoms, including weight loss (Bernardes et al., 2016), perturbations in body temperature (Wrotek et al., 2014), bladder dysfunction (Altuntas et al., 2008), and dysregulation of the hypothalamic-pituitary-adrenal (Heesen et al., 2007) and HPG axes (Foster et al., 2003).

MS is more prevalent in women than in men, with an increasing ratio over the past decades. Nevertheless, disease progression, brain atrophy, and cognitive impairment appear to be worse in men (Harbo et al., 2013). Due to these facts, the roles of gonadal sex hormones in MS

have been extensively studied. For example, the decreased rate of relapses in late pregnancy is thought to be related to high estradiol and/or high progesterone levels, while the increased incidence of relapses postpartum was proposed to be a consequence of low estradiol and/or high prolactin levels (Confavreux et al., 1998; Runmarker and Andersen, 1995). Testosterone is generally considered as a protective factor in autoimmune diseases (Gubbels Bupp and Jorgensen, 2018; Spence and Voskuhl, 2012). Low testosterone levels were recently linked to an increased risk of disability in male MS patients (Bove et al., 2014) and male infertility appears to be associated to a higher risk for MS (Glazer et al., 2017).

Previous gonadal status is also crucial for EAE course and severity. Gonadectomy induces a more severe disease and greater CNS pathology in both sexes (Fillmore et al., 2004; Trooster et al., 1996), and an earlier onset of the disease in females (Jansson et al., 1994). However, females ovariectomized before puberty become less susceptible to the induction of EAE (Ahn et al., 2015). Also, MS is predominantly detected after puberty, leading to a hypothesis that neuroendocrine changes at this period might be important for the risk increase and disease course (Ahn et al., 2015; Chitnis, 2013).

During puberty, hypothalamic-pituitary-gonadal (HPG) axis is activated by increased secretion of gonadotropin-releasing hormone (GnRH), a decapeptide produced by a diffuse network of hypothalamic neurons. The function of GnRH neurons critically depends on *Kiss1* gene products, termed kisspeptins, secreted by hypothalamic neurons residing in the arcuate nucleus and in distinct areas of the preoptic region (Harter et al., 2018). Kisspeptins activate their cognate receptor, termed GPR54 (Muir et al., 2001), expressed in GnRH neurons (Messager et al., 2005) leading to phosphatidylinositol-4,5-bisphosphate hydrolysis, calcium mobilization, and GnRH release (Messager et al., 2005). GnRH reaches the pituitary through hypophyseal portal

circulation and binds to its calcium mobilizing receptor (GnRHR) expressed in gonadotropes. The signaling pathways triggered by the activation of this receptor control numerous cellular processes, including synthesis and/or release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that control follicular/sperm maturation and gonadal steroid hormones synthesis (Coss, 2018). The gonadal feedback to the hypothalamus relies on Kiss1 neurons (Harter et al., 2018).

Having in mind that research in animal models provides valuable guidance for translational research, we aimed to elucidate the status of HPG axis during EAE in postpubertal rats of both sexes. Previous observations in mice led to a hypothesis that EAE induces direct inhibitory actions on testicles, based on decreased serum testosterone levels coupled with an increase in serum LH levels. In contrast, serum estrogen and LH levels in female EAE mice were not affected (Foster et al., 2003).

We here show that EAE causes inflammation in the hypothalamic tissue and downregulates the hypothalamic expression of *Kiss1* mRNA in females and *Gnrh1* mRNA in males. Downstream the axis, EAE induces estrous cycle arrest in diestrus stage, lowers circulating levels of LH in both sexes, upregulates serum progesterone levels in females and downregulates serum testosterone in males. These novel data imply that the changes in HPG axis occur due to perturbations in centrally operated mechanisms, revising the current hypothesis.

2. Material and methods

2.1. Animals

This study was done on 9-12 week old female and male rats of Dark Agouti strain. The animals were housed 3-5 per cage, in standard laboratory environment (12 h dark/light cycle, at constant temperature/humidity), with unlimited access to chow and tap water. Experimental

procedures were approved by the Ethical Committee for the Protection of Welfare of Experimental Animals of the Institute for Biological Research “Sinisa Stankovic” (Approval No 02-07/15 and 06-10/19). Experiments were done in compliance with the Directive on the protection of animals used for experimental and other scientific purposes (2010/63/EU) and results reported in compliance with the ARRIVE guidelines.

2.2. Induction and evaluation of EAE.

The animals were divided into two groups: an immunized group (EAE) and a peer group of naive rats (control). EAE was induced by a subcutaneous injection of encephalitogenic emulsion, prepared by mixing equal volumes of spinal cord homogenized in phosphate buffered saline (pH = 7.4) and complete Freund's adjuvant (CFA-F5881; Sigma, St. Louis, MO). Anesthetized animals received 150 µl of the emulsion into the hind hock. On the day of immunization, female rats were not synchronized i.e. they were at different stages of the estrous cycle. The rats were examined daily and scored based on neurological signs for 28-day post-immunization period. The scale used grades the disease as follows: 0 - unaffected, 1 - tail atony, 2 - hind limb weakness, 3 - total hind limb paralysis, 4 –moribund state. Moribund state was set as a humane endpoint. During paralysis, food was placed within rats’ reach, and water was given manually. The animals were euthanized by gradual asphyxia in CO_2 chamber at three time-points during the disease course, i.e. grade 1, grade 3, or total recovery, thus forming three EAE groups, marked as *onset*, *peak*, and *end*, respectively. Six animals per group were used in each experiment. In the first experiment, additional 12 female animals were injected with CFA only, to estimate its effect on estrous cycle and other parameters tested. To compare disease course between sexes, the area under curve of the daily clinical score was calculated for each recovered rat, using GraphPad Prism 5 program. To compare the severity of the disease between males and

females, the mean maximal clinical score was determined for both sexes, using individual maximal clinical scores. The first symptom on a given day for each animal was used to assess the mean day of onset.

2.3. Tissue sample collection, RNA isolation, cDNA synthesis, and qRT-PCR.

After blood collection, the animals were perfused with cold saline. The hypothalamic and anterior pituitary tissue were dissected on ice and stored in RNAlater® RNA Stabilization Solution (Ambion™, Applied Biosystems by Thermo Fisher Scientific, Waltham, MA). Boundaries of the hypothalamic blocks were set using coronal rat brain matrix; the first cut was made 2 mm anterior to the optic chiasm, and the second behind the mammillary bodies. Lateral borders were set to 2 mm lateral to the third ventricle. The top of the third ventricle served as a dorsal border for block sampling. RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany) or RNAqueous® Kit (Ambion™, Applied Biosystems by Thermo Fisher Scientific). RNA content was determined by measuring optical density (OD) on Nanophotometer® N60 (IMPLEN, Munich, Germany) at 260 nm, and the quality of the samples was evaluated by OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios. Reverse transcription was done with High Capacity cDNA Reverse Transcription Kit; quantitative real time-PCR (qRT-PCR) analysis was done using the QuantStudio™ 3 Real-Time PCR System with TaqMan® or SYBR™ Green reagents (all from Applied Biosystems by Thermo Fisher Scientific). The probes and primers are listed in Table 1 and Table 2, respectively. The expression levels of target genes were quantified by comparative $2^{-\Delta Ct}$ method, using glyceraldehyde 3-phosphate dehydrogenase gene (*Gapdh*) as a reference gene, which was recently shown to be valid reference gene for pituitary tissues and cultured pituitary cells (Janjic et al., 2019).

2.4. Assessment of the estrous cycle stage.

Non-invasive vaginal lavage, performed by flushing the vagina with saline, was done every day by 10 am. Smears were collected on microscopic slides and dyed with 0.5% methylene-blue (Sigma-Aldrich, St. Louis, MO). The stage of the estrous cycle was determined under a microscope, based on the cell type and morphology. A mean daily number of dietruses was determined for control and EAE group. 3 - 5% of rats in each experiment were acyclic before immunization, and excluded from the study. All cycling females were sacrificed on the morning of diestrus II.

2.5. Buserelin treatment.

In the third experiment, the animals were treated with a GnRH analogue, buserelin (Buserelin acetate salt (Sigma-Aldrich, St. Louis, MO)). Naïve and rats at the peak of EAE (both male and female) received a bolus injection containing 4 µg of buserelin in saline, intraperitoneally. Buserelin was left to circulate for one hour. The animals (6 per group) were subsequently sacrificed as described above. Additional 12 animals (6 females, 6 males) served as naïve controls.

2.6. Hormone measurements.

Blood was centrifuged at 3000 × g for 10 min and serum samples were stored at -80 °C. LH levels were measured using the Rodent ELISA kit (Endocrine Technologies Inc, Newark, CA). Intra-assay and inter-assay variations were 5.11% and 11.27%, respectively. Serum samples from buserelin-treated animals were diluted appropriately. Testosterone, progesterone, and estradiol levels were determined using Cayman Chemical Company ELISA kits (Ann Arbor, MI). Intra- and inter-assay coefficients of variability for estradiol, progesterone and testosterone were as follows: 9.53% and 15.58%; 6.11% and 14.96%; 5.15% and 6.47%, respectively.

2.7. Ovary and testis histochemistry and immunohistochemistry.

Ovaries and testicles were collected from control (n = 3) animals and animals at the peak of EAE (n = 3), fixed in Buin's solution and dehydrated in increasing concentrations of alcohol. Tissue was embedded in paraffin and 7 µm thick sections were cut on a microtome. After deparaffinization, tissue was routinely stained for hematoxylin/eosin.

For StAR immunolabeling, ovary and testicle sections were deparaffinized and rehydrated. Blocking was done in 0.3% H_2O_2 in methanol followed by 5% goat serum. StAR antibody (8449S, Cell Signaling Technology, Danvers, MA) was applied in 1:200 dilution, overnight. Sections were incubated in goat antirabbit antibody (sc-2004, Santa Cruz Biotechnology Inc, Dallas, TX) for two hours at room temperature. The color reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (SK-4100, Vector Laboratories, Burlingame, CA). Sections incubated without primary antibody served as a negative control. Slides were counterstained with hematoxylin, dehydrated, cleared in xylene and mounted with DPX (Sigma, St. Louis, MO).

Sections were observed under Leica DMRB microscope equipped with camera (Leica Microsystems, Wetzlar, Germany). Micrographs were sized, cropped and arranged in Photoshop CS (Adobe Inc. San Jose, CA).

2.8. Statistical analysis.

Data were analyzed using SPSS 20 (IBM, Armonk, NY, United States) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, United States) software. All datasets were tested for Gaussian distribution with the Kolmogorov-Smirnov normality test and homogeneity of variance using Levene's test. When all compared groups had passed the normality test and had the same variance, the significance of difference in means between: (i) two groups - was determined by Student's t test; (ii) multiple groups – was calculated by one-way ANOVA, followed by

Dunnett's post hoc test or Bonferroni's posttest analysis. Otherwise, the appropriate nonparametric test was carried out, i.e. Kruskal–Wallis test, followed by Dunn's post hoc test. The results are presented as mean ± SEM for each group of animals, and are considered statistically significant for $p < 0.05$. Linear correlations and Pearson r coefficient were calculated using the KaleidaGraph Program (Synergy Software).

3. Results

3.1. EAE time-course in males and females is similar.

Figure 1 depicts a typical EAE experiment using adult DA rats of both sexes. The disease showed an acute monophasic course with complete recovery in both males and females (Fig. 1A). Both males and females exhibited a significant weight loss with a nadir at the peak of the disease (shown as % of weight change in Fig. 1B). Females show an earlier onset of the disease (10.46 ± 0.31), when compared to males (11.61 ± 0.23, $p<0.005$, by Student's *t*-test), in line with findings of others (Stojic-Vukanic et al., 2018). Using 9-12 week old animals (at the beginning of the experiment), we did not observe significant differences between males and females in the severity of the disease estimated by the evaluation of maximum severity score (3.07 ± 0.11 vs 3.07 ± 0.06, $p=0.73$, Fig. 1D, left) or the area under curve (AUC, 10.93 ± 1.57 vs. 10.83 ± 1.09, $p=0.96$, Fig. 1D, right). Administration of CFA alone did not induce any symptoms of EAE or weight loss (data not shown).

3.2. Estrous cycle in females is transiently disrupted during EAE.

Experiments were done using regularly cycling female rats prior to beginning of experiments (mean length 4.42 ± 0.64 days). We found that a transient loss of the estrous cycle is a consistent feature of the symptomatic phase of EAE. The mean length of the arrest in diestrus from a representative experiment is 8.0 ± 1.7 days (n = 6). In most cases, animals resumed to

cycle when EAE symptoms subsided to full or partial tail atony. Figure 1C represents daily number of diestruses (determined by vaginal smears) in one of the experiments. In each experiment, all cycling females were sacrificed at diestrus II stage; all EAE females at the peak of the disease were in a prolonged state of diestrus (4 – 6 days). Administration of the CFA alone did not disrupt the estrous cycle (data not shown).

3.3. EAE induces inflammation of hypothalamus and has sex-specific effects on Kiss1 and Gnrh1 expression.

The expression of several inflammatory marker genes was assessed by qRT-PCR (Fig. 2). In both female and male hypothalamic tissue, EAE induced an upregulation of *Gfap* and *Cd68* mRNA levels indicating activation of astrocytes and mononuclear phagocyte system, respectively. *Gfap* expression was significantly upregulated at the onset of the disease (~3x in both females and males), when compared to control levels, indicating that astrocytes increase their activity early during an inflammatory challenge. An induction of *Ccl2* and *Il1b* mRNA was also recorded. The trend of upregulation of *Il6* and *Tnf* at the onset did not reach statistical significance in either sex (data not shown). Two-way ANOVA analyses showed no significant interaction between sex and EAE stage and no effects of sex on the expression of inflammatory markers.

In females, *Gnrh1* mRNA levels were comparable to control levels at all stages of the disease (Fig 3A, left). However, *Kiss1* expression was significantly downregulated already at the onset of the disease (Fig. 3B, left). This decrease of *Kiss1* mRNA expression remained significant throughout the disease with a pronounced effect at the peak of the disease (~75% below the control levels). Despite the trend of recovery at the end of EAE, the decrease remains statistically significant in comparison to control animals. In contrast, a significant decrease in

Gnrh1 expression was observed in males at all stages of the disease (Fig. 3A, right), while the expression of *Kiss1* mRNA was not affected (Fig. 3B, right). The expression of *Pdyn* and *Tac3*, important for the regulation of kisspeptin-GnRH system, was not affected during EAE (data not shown). Together, these data indicate the sex-specific effects of hypothalamic tissue inflammation on the expression of two genes encoding peptides critical for reproductive function regulation.

3.4. EAE affects the expression of gonadotrope signature genes.

Because GnRH/kisspeptin neuroendocrine system controls the expression of *Gnrhr* and gonadotropin subunit genes, we next investigated the expression of gonadotrope marker genes in the anterior pituitary tissue (Fig. 4). At the onset of the disease in females, both *Fshb* and *Lhb* mRNA levels were significantly increased followed by a sharp decline (~50% compared to levels in controls) in *Lhb* mRNA levels at the peak of the disease (Fig 4A and 4B, left). *Cga* expression in females was also significantly downregulated at the peak of the disease (~40% decrease compared to control levels, Fig. 4C, left). In males, no significant changes in gonadotropin subunit genes expression could be observed (Fig. 4A, 4B and 4C, right).

We next examined the expression of *Gnrhr*, because GnRHR conveys GnRH actions on LH secretion; the expression of this gene is very tightly regulated by GnRH itself (Janjic et al., 2019). During EAE, both males and females showed a significant decrease in the expression of *Gnrhr*. While females showed a significant downregulation in *Gnrhr* mRNA levels at all investigated stages of disease, a significant *Gnrhr* downregulation was observed only at the peak of the disease in males (Fig. 4D).

Since *Fshb* mRNA in females was increased, we analyzed the expression of activin subunit genes in the pituitary tissue and found increased *Inhbb* expression at the onset of EAE. Conversely, in males, the expression of both *Inhba* and *Inhbb* was downregulated (Fig. 5).

3.5. EAE affects serum LH and gonadal steroid hormone levels.

Given that EAE interrupted the estrous cycle we estimated serum LH levels (Fig. 6A). In both females and males, serum LH levels drop significantly at the peak of the disease, when compared to levels in control animals. After complete cessation of EAE symptoms, serum LH levels recovered to control levels in females. At the end of the disease in males, serum LH levels also recovered when compared to the peak of EAE, but the recovery was incomplete in comparison to control.

To clarify whether the decrease in serum LH levels reflects decay in intracellular LH accumulation, in further experiments, we challenged EAE and control animals with a high dose of buserelin, a potent GnRHR agonist (Fig. 6B). A bolus injection of buserelin was able to induce a massive LH release in both diseased and control animals of both sexes. In females, there was no significant difference in serum LH levels between naïve and EAE buserelin treated animals (254 ± 9.9 vs. 212 ± 16.8 ng/ml). In comparison to naïve buserelin treated males, buserelin-treated EAE males showed almost 50-fold higher concentration of serum LH, implying that GnRHR remains operative in gonadotropes. However, in comparison to control buserelin treated males, buserelin treated EAE males showed significantly lower capacity to release LH from the pituitary (233 ± 28 vs. 143 ± 20.3 ng/ml; $p<0.05$), indicating that intracellular LH content may also be partially affected.

We also measured the serum levels of gonadal steroid hormones: progesterone and estradiol in females and testosterone in males (Fig 7). Estradiol levels were stable throughout the

time-course of the disease (Fig. 7B); however, progesterone levels were significantly elevated in females at the onset and peak of EAE (Fig. 7A). In males, testosterone levels were significantly lower at the onset and peak of the disease but recovered (incompletely) at the end of the disease (Fig. 7C); correlating with serum LH levels (Fig. 7D).

3.6. Gonadal tissue does not show signs of pathological changes but the pattern of expression of StAR protein is altered.

To investigate the status of gonadal tissue, the ovaries and testicles were histologically examined. When compared to the control sections (Fig. 8A), ovary sections from the animals at the peak of EAE (Fig. 8B) revealed no signs of degenerative changes. Follicles at different stages of development were present as well as numerous corpora lutea. Immunohistochemical labeling of StAR protein (Fig. 8C and D), a mediator of a rate limiting step in steroid biosynthesis revealed a more pronounced labeling of corpora lutea and lutein cells at the peak of the disease.

Similarly, in the sections of rat testes, no pathological changes could be observed at the peak of EAE (Fig 9A and B). Normal structure of the seminiferous tubules could be observed in both control (Fig. 9A) and EAE animals (Fig. 9B). On the other hand, when compared to control sections (Fig. 9C), interstitial cells showed diminished staining for StAR protein (Fig. 9D), implying that EAE affects steroidogenesis in male and female gonadal tissue inversely.

4. Discussion

We here examined the effects of EAE on HPG axis in a systematic manner, using young adult rats of both sexes. We observed comparable time-courses of EAE in terms of neurological signs and body weight. Others also observed a notable weight loss of fat and muscle mass in EAE mice (Bernardes et al., 2016), while both cachexia (Kamalian et al., 1975; Van Waesberghe et al., 1999) and weight gain (Mokhtarzade et al., 2019) were reported in MS. More recent

studies also indicate that hypothalamic inflammation precedes both involuntary weight loss and obesity [(Clarke, 2010) and references within]. Our experiments also indicate that the phase in loss of body weight coincides with hypothalamic inflammation.

The HPG functions were also affected in both female and male rats, but in a sex-specific manner. We here for the first time show alterations in GnRH-kisspeptin system in the hypothalamus during EAE, a sustained downregulation of *Kiss1* expression in females and *Gnrh1* expression in males. At pituitary level, *Gnrhr* expression was downregulated for a prolonged period in females and only transiently in males. Furthermore, *Lhb, Fshb,* and *Cga* expression were transiently affected in females, but not in males. EAE effects on basal and stimulated serum LH levels were more pronounced in males than females. Finally, gonadal steroidogenesis was affected differently between sexes.

Both GnRH (Wu and Wolfe, 2012) and kisspeptin (Castellano et al., 2010; Iwasa et al., 2008) neurons are sensitive to inflammatory challenge, due to their specific positions in the brain. Namely, it is widely accepted that circumventricular organs are leaky, and that cells around these regions may “sense” molecules in the blood. This was specifically proposed for GnRH processes in median eminence (Prevot, 2011) and in vascular organ of lamina terminalis (Herde et al., 2011) and is supposed in general for the arcuate nucleus (Morita-Takemura and Wanaka, 2019), where a subpopulation of kisspeptin neurons resides. Various inflammatory challenges including Il-1β (Rivest and Rivier, 1993), LPS (Iwasa et al., 2014; Lee et al., 2019) and TNF (Watanobe and Hayakawa, 2003), were shown to affect GnRH release/expression. Animals of both sexes are rarely used, but similar downregulation of *Gnrh1* mRNA, in male mice only, was observed in the paradigm of high fat diet (Lainez and Coss, 2019; Lainez et al., 2018). It is also important to note that downregulation of *Kiss1* mRNA after high dose LPS

challenge occurs in both intact and ovariectomized female rats (Iwasa et al., 2014), implying that inflammation affects kisspeptin system regardless of the gonadal status.

In addition, it was shown that hypothalamic circumventricular organs in mice can serve as the sites of entry for autoreactive lymphocytes to the brain parenchyma during EAE (Schulz and Engelhardt, 2005). Both gray and white matter pathology in the hypothalamus of MS patients was also observed (Huitinga et al., 2001; Kamalian et al., 1975; Kantorová et al., 2017), correlating with disease activity and clinical outcomes (Huitinga et al., 2001; Kantorová et al., 2017). The upregulation of mRNA expression of several astrocyte and microglia/macrophage markers (*Gfap*, *Il1b*, *Ccl2* and *Cd68*) during EAE indicates that inflammation occurs in the hypothalamus. *Ccl2* mRNA, coding for Chemokine ligand 2, implicated as both astrocyte (Moreno et al., 2014) and microglia/mononuclear cells' (Pilipović et al., 2020) product in inflammatory events, was previously shown to be upregulated in the brain tissue even before the onset of EAE (Hasseldam et al., 2016). Early upregulation of expression of *Ccl2*, *Il1b* and *Gfap* implies that some inflammatory events in the hypothalamus may occur before visible manifestations of the disease. A more detailed study should be done to examine the extent of cellular and regional changes to provide rationale for sex-specific effects of EAE on GnRH and Kiss1 neurons.

It is reasonable to suggest that EAE-induced changes in gene expression and hormone release in pituitary gonadotropes reflect changes in hypothalamic *Kiss1* and *Gnrh1* expression. For example, it is well established that the expression of *Cga* and *Gnrhr*, but not *Lhb* (Janjic et al., 2019), as well as LH secretion (Stojilkovic et al., 1994) depend on GnRH concentrations and pattern of application. Therefore, the downregulation in *Cga* and *Gnrhr* expression in anterior pituitary and the decrease in basal serum LH levels also indicate an altered GnRH secretion.

We suggest that endogenous GnRH release pattern could be affected in EAE females due to the downregulation in *Kiss1* expression, whereas the amplitude of endogenous GnRH release in males could be affected due to the decay in *Gnrh1* expression. Because the effects on gene expression were more pronounced in females, we could further speculate that the pattern of endogenous GnRH release, rather than the amplitude of pulses, is more critical for the control of gene expression. In contrast, a nice parallelism in expression of *Gnrh1* and basal and stimulated blood LH levels in males is consistent with a hypothesis that the amplitude of GnRH response plays a more important role on synthesis and release of this gonadotropin. The responsiveness of gonadotropes to *in vivo* buserelin injection was robust in both females and males, but in males, lesser response suggests that LH protein synthesis or storage may also be affected, or the status of GnRHR may be more critical. Importantly, MS studies in men also reported lower (Safarinejad, 2008) or unchanged LH levels (Bove et al., 2014; Foster et al., 2003; Wei and Lightman, 1997), indicating hypothalamic-pituitary, rather than gonadal dysfunction, because primary hypogonadism is accompanied by high LH levels (Basaria, 2014). Further, Foster's hypothesis may be questioned regarding the status of the gonadal tissue i.e. our histological analyses found no gross pathological changes in testes. This also implies that the acute monophasic EAE does not last long enough to produce significant morphological changes in the male reproductive organs. However, consistent with lower LH (and testosterone) levels, interstitial cells at the peak of the disease show reduced staining for StAR protein.

Importantly, our results imply that EAE can faithfully represent alterations in hypothalamus and pituitary in a subpopulation of male MS patients that show lower LH and poorer response to GnRH analogues (Safarinejad, 2008).

The upregulation of *Fshb* mRNA in females only is peculiar; yet, GnRH is not the sole regulator of *Fshb* expression. *Fshb* expression is upregulated by activins even in the complete absence of GnRH [(Bernard and Tran, 2013) and our unpublished data)]. Activin-A is a member of the transforming growth factor-β superfamily of cytokines, released in response to TLR4 stimulation (de Kretser et al., 2012) and upregulated in autoimmune diseases like systemic lupus (Morianos et al., 2019). A small but significant upregulation in the pituitary *Inhbb* mRNA expression in female pituitaries was observed, while the expression of both *Inhba* and *Inhbb* mRNA in males is downregulated. Previously, the upregulation of Activin-A in macrophages was demonstrated in EAE (Ahn et al., 2012).

In EAE females, we observed a transient arrest of estrous cycle, followed by restoration of normal 4-5 days long cycles in recovered animals. In SJL/J mice, it was shown that after the symptomatic phase of EAE the estrous cycle shortens (Jaini et al., 2015). During EAE in C57BL/6 mice (Rahn et al., 2014), as well as in cuprizone-induced demyelination, arrest in diestrus was observed, but the hormonal status was not assessed (Taylor et al., 2010). Interestingly, some women report premenstrual worsening of MS symptoms (Zorgdrager and De Keyser, 1997; Zorgdrager and De Keyser, 2002); in a smaller study including MS patients with regular menstrual cycle and premenstrual worsening, ovulatory LH and estradiol levels were found to be lower than in control subjects (Guven Yorgun and Ozakbas, 2019).

We here report that during EAE estradiol levels are not affected, a finding consistent with literature (Foster et al., 2003), while for the first time we show increased progesterone levels. Several studies showed normal values for progesterone levels in women with MS (Guven Yorgun and Ozakbas, 2019; Kempe et al., 2018; Pozzilli et al., 1999; Tomassini et al., 2005). Progesterone levels were found to be decreased in both acute (Giatti et al., 2010) and chronic

EAE (Caruso et al., 2010). However, the comparison in these studies was done to females in proestrus, when progesterone levels in female rats are the highest (Smith et al., 1975), whereas we have done all comparisons to control groups in diestrus II stage. It is possible that upregulated *Fshb* mRNA expression in the pituitary in females leads to greater FSH synthesis and increase in serum FSH levels, because unlike LH, FSH is mostly released through constitutive secretory pathway (Anderson, 1996). This could explain higher progesterone levels in females, i.e. FSH alone can induce progesterone production in human granulosa cells (Oktem et al., 2017). Our histological and immunohistochemical analyses finally show the maintenance of corpora lutea and increased StAR expression, consistent with higher FSH levels on one end (Minegishi et al., 2002), and higher progesterone levels on the other. In addition, gross histological examination does not point to pathological changes in the ovary. Nevertheless, further studies are needed in order to elucidate the causes for unchanged estradiol levels.

5. Conclusions

In conclusion, EAE induces significant but acute and transient changes in HPG axis functions in a sex-specific manner.

Our data imply that these changes occur due to alterations in the hypothalamic functions, rather than on the level of pituitary and gonadal tissue. Our data are also consistent with a hypothesis that sex-specific changes in HPG axis reflect a sustained downregulation in *Kiss1* expression in females only and *Gnrhr* expression in males only, presumably affecting the pattern and amplitude of GnRH release. It is possible that some fine tuning of HPG axis suffers after the disease, given that some parameters do not recover in both males and females even after complete cessation of symptoms. Because we recorded additional differences down the axis,

further investigations will still be directed in two ways: detailed studies of hypothalamic inflammation and gonadal function during EAE.

Author Contribution: A.M., M.M.J., I.L., D.S. K.T., I.D.B., M.J., S.P., and I.B. performed experiments; I.B. conceptualization and the first draft of the manuscript; S.S.S, I.B., M.M.J. and A.M arranged the final version of the manuscript.

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Figures and Legends

Fig. 1. Scoring of EAE. **A,** Female (green circles) and male (blue circles) rats were scored for neurological signs and B, weighed (plotted as percent of weight change) every day until the animals recovered from EAE (after 29 days). **C,** Females were subjected to daily examination of vaginal smears. Daily number of diestruses per number of animals in the group was plotted on X-axis. Notice the temporary arrest in diestrus phase in EAE group of animals (n=6, green circles), compared to control animals that cycle normally (n=6, gray circles). **D,** No significant differences (Student's *t*-test) were observed between the sexes in disease severity as assessed by the maximum severity score (D, left) and area under curve (D, right). Plotted are the data from one of two similar experiments (presented as mean ± SEM in A, B and D).

Fig. 2. EAE upregulated the expression of inflammation marker genes *Gfap, Cd68, Ccl2* and *Il1b* in the hypothalamic tissue from female (green bars) and male (blue bars) rats. Abbreviations: C-control; O-onset; P-peak; E-end of the disease. Plotted data are from one of two similar experiments (n=6 animals per group) as mean ± SEM; *p<0.05, **p<0.01 and ***p<0.0001, by Kruskal–Wallis test followed by Dunn's post hoc test.

Fig. 3. The sex-specific effects of EAE on *Gnrh1* and *Kiss1* expression in the hypothalamic tissue of female (green bars) and male (blue bars) rats. **A,** Relative expression of *Gnrh1* did not change during the course of EAE in females, but was decreased in males at all stages of the disease. **B,** *Kiss1* mRNA levels dropped significantly at all stages of the disease in females, but were not significantly affected in males, although there was a trend of downregulation at the peak of the disease. Plotted data are from one of two similar experiments (n=6 animals per group) as mean ± SEM; **p<0.01 and ***p<0.0001, by ANOVA followed by Bonferroni's posttest.

Fig. 4. EAE altered the expression of gonadotrope-specific genes in the anterior pituitary gland of female (left panels) and male (right panels) rats. **A,** *Fshb* mRNA levels were upregulated in females at the onset of disease, but not significantly changed in males. **B,** In females, EAE induced a small but significant increase of *Lhb* mRNA expression at the onset and significant decrease at the peak of the disease. In males, no significant changes in *Lhb* mRNA were observed during the course of EAE. **C,** *Cga* mRNA expression was downregulated in females at the peak of the disease, but not in males. **D,** In females, EAE profoundly inhibited *Gnrhr* expression at all of the disease stages. *Gnrhr* mRNA levels were also downregulated in males, but only at the peak of the disease. Plotted data are from one of two similar experiments (n = 6 animals per group) as mean ± SEM; *p<0.05; **p<0.01; ***p<0.001, by ANOVA followed by Dunnett's post hoc test (A-D, left) or Kruskal–Wallis test followed by Dunn's post hoc test (A-D, right).

Fig. 5. Expression of activin subunits in the anterior pituitary tissue during EAE in females (green bars) and males (blue bars). **A,** *Inhba* expression was significantly upregulated in females at the end of the disease, while in males significant downregulation was recorded at the onset and peak of the disease. **B,** Females also showed a small but significant upregulation of *Inhbb* mRNA at the onset of the disease, while males exhibited downregulation at the same stage. Plotted data are from a representative experiment and presented as mean ± SEM, *p<0.05; **p<0.01, by ANOVA followed by Dunnett's post hoc test.

Fig. 6. Serum LH levels (in ng/ml) in female (green bars) and male (blue bars) rats during EAE. **A**, Females showed a significant drop in LH levels at the peak of the disease (0.26 ± 0.02 ng/ml) when compared to controls (2.08±0.23 ng/ml). LH levels recovered at the end of the disease. In males, LH levels were significantly lower at the onset and peak of the disease (0.74±0.14 and

0.47±0.08 ng/ml, respectively) than in controls (3.07±0.39 ng/ml). Notice that in females, but not in males, serum LH levels recovered completely by the end of experiment. Data plotted in A are combined data from two experiments. All data are presented as mean ± SEM, *p<0.05 ***p<0.001 by ANOVA followed by Bonferroni's post hoc test. **B,** Serum LH levels in animals treated with buserelin, a GnRH receptor agonist. Buserelin (B) was injected (4 μg/animal) intraperitoneal in controls (C) and animals at the peak of the disease (EAE). Female (green bars) and male (blue bars) rats were euthanized one hour after injection and LH serum levels were determined by ELISA. Note that buserelin treatment induced a massive release of LH in both control (C+B, n = 6) and EAE (EAE+B, n = 6) animals. The capacity of female pituitaries in terms of LH release was not altered by the disease. However, buserelin treatment in EAE males induced significantly lower LH secretion than in control animals. Data are plotted as mean ± SEM; *p<0.05 by Student's *t*-test.

Fig. 7. The profiles of gonadal steroid hormones during EAE in female (green bars) and male (blue bars and circles) rats. **A**, Serum progesterone levels were raised in onset and peak of the disease, whereas **B,** estradiol levels did not change during EAE when compared to levels in control animals. **C,** In contrast, testosterone serum levels were lower in onset and diminished at the peak of the disease when compared to levels in control animals. Plotted data are from one of two similar experiments (n=6 animals per group) as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001, by ANOVA followed by Dunnett's post hoc test. **D,** Data plotted for serum levels of LH on X axis and testosterone serum levels on Y axis; r^2= 0.98.

Fig. 8. Histological observation of the ovary from a control and rat at the peak of EAE by hematoxylin-eosin staining and StAR immunohistochemistry. **A,** Ovary of a control female at diestrus II stage. Follicles at early stages of development (*) and antral follicles (AF) present.

Large corpora lutea (CL) from the last cycle shows signs of fibrous tissue formation and fine vacuolation (higher magnification, right). **B,** Ovary of a female at the peak of EAE, 6 days into the diestrus, without signs of pathological changes. Follicles at different stages of development are present. Large protruding corpora lutea is still present. While some corpora lutea show clear signs of degeneration (arrows), some corpora lutea is still composed of cells with basophilic cytoplasm and plump cell bodies, indicating active steroidogenesis (arrowhead left and higher magnification at right).

C, StAR immunohistochemistry in the control rat ovary at diestrus II stage. The arrow points to a corpora lutea shown in higher magnification on the right, with lutein cells staining for StAR cells. **D,** StAR immunohistochemistry in the rat ovary at the peak of EAE (4 days into diestrus). The arrow points to a corpora lutea shown in higher magnification on the right. Note more intensive StAR labeling of corpora lutea and lutein cells at the peak of the disease.

Scale bar: 500 μm for the images on the left, 25 μm for the higher magnification images on the right.

Fig. 9. Histological observation of the testis by hematoxylin-eosin staining depicting tubular and intertubular space of a control and a rat at the peak of EAE. **A,** Cross section of a control rat testis showing seminiferous tubules (S) and interstitial cells (I). Stratified epithelium of seminiferous tubules in higher magnification (right) depicts spermatogenic (spermatogonia-SG, spermatocyte-SC, spermatide-ST, spermatozoa-SZ) and non-spermatogenic cells (Sertoli cell-S). Testosterone producing Leydig cells (L) are in the interstitium. **B,** Cross section of rat testis at the peak of EAE showing normal histological structure (left), with complete spermatogenic series and normal appearing interstitial space (right). **C,** StAR protein immunohistochemistry in control animals shows labeling of interstitial cells. **D,** At the peak of the disease, cell staining for

StAR is less pronounced. Scale bar in A, left (100 µm) applies to the images on the left. Scale bar in A, right (50 µm) applies also to B, right, Scale bar in C, right (25 µm) applies also to D, right.

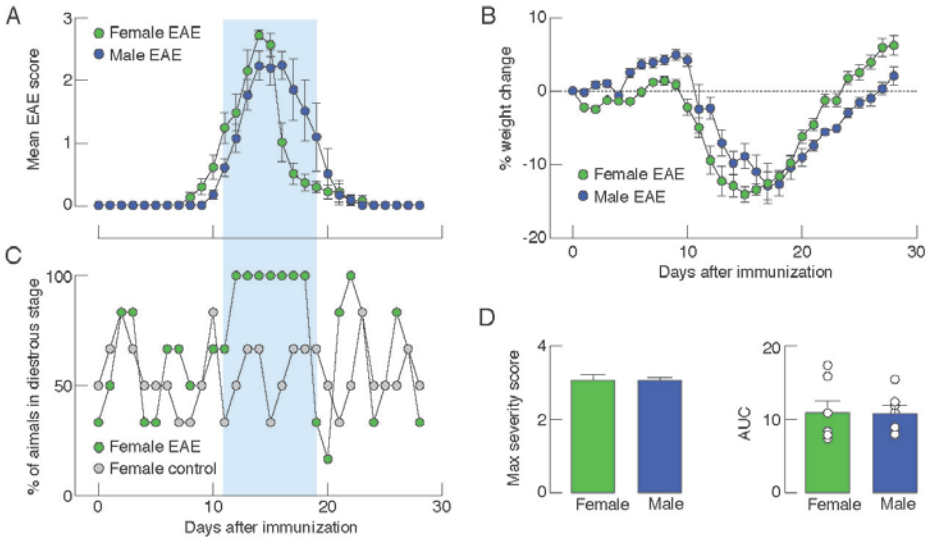
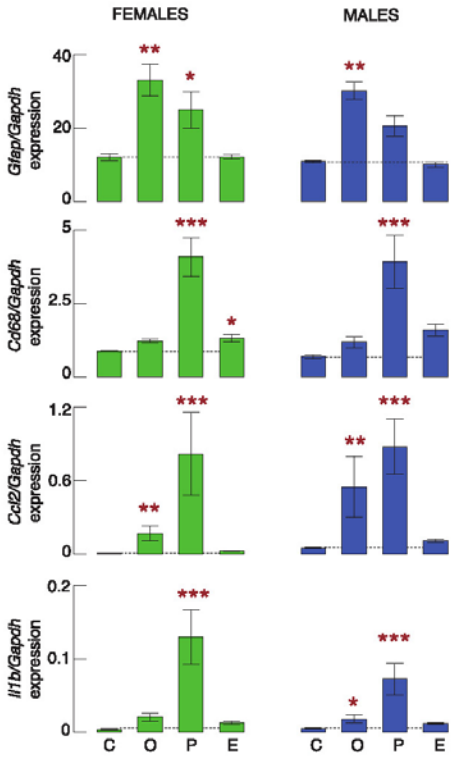

A
Mean EAE score
Female EAE
Male EAE
B
% weight change
Female EAE
Male EAE
Days after immunization
C
% of aimals in diestrous stage
Female EAE
Female control
Days after immunization
D
Max severity score
Female
Male
AUC
Female
Male

Fig. 1


FEMALES
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Cd68/Gapdh expression
Ccl2/Gapdh expression
Il1b/Gapdh expression
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E

Fig. 2

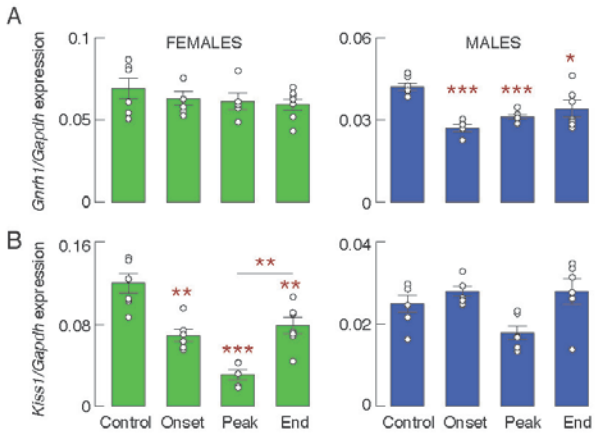
A
FEMALES
MALES
Gnrh1/Gapdh expression
0.1
0.05
0
0.06
0.03
0
***
***
*
B
Kiss1/Gapdh expression
0.16
0.08
0
0.04
0.02
0
**
**
**
***
Control
Onset
Peak
End
Control
Onset
Peak
End

Fig. 3

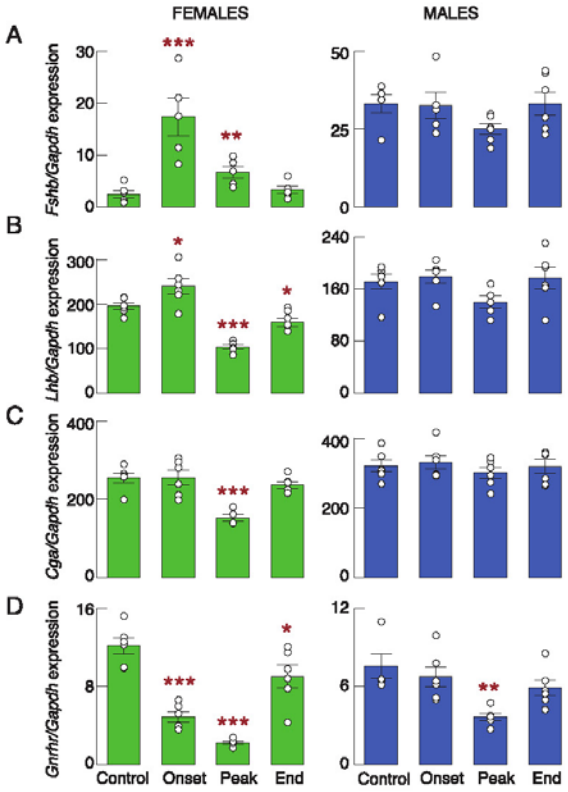
FEMALES
MALES
A
Fshb/Gapdh expression
B
Lhb/Gapdh expression
C
Cga/Gapdh expression
D
Gnrhr/Gapdh expression
Control
Onset
Peak
End

Fig. 4

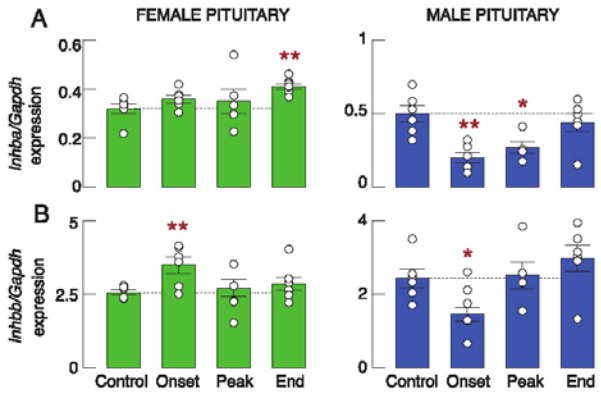
A
FEMALE PITUITARY
MALE PITUITARY
Inhba/Gapdh expression
0.6
0.4
0.2
0
1
0.5
0
**
**
*
B
Inhbb/Gapdh expression
5
2.5
0
4
2
0
**
*
Control
Onset
Peak
End
Control
Onset
Peak
End

Fig. 5

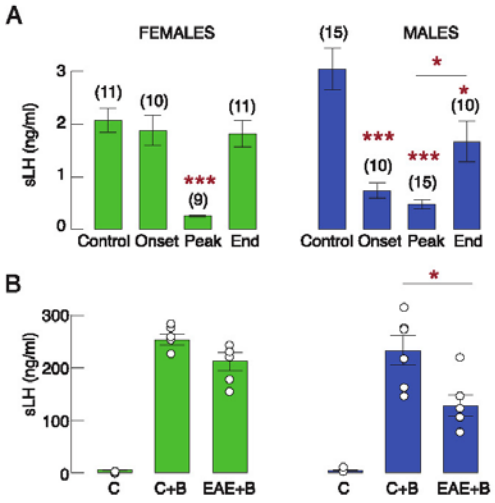
A
FEMALES
MALES
sLH (ng/ml)
3
2
1
0
(11)
(10)
(9)
(11)
(15)
(10)
(15)
(10)
***
***
***
*
*
Control Onset Peak End
Control Onset Peak End
B
sLH (ng/ml)
300
200
100
0
*
C
C+B
EAE+B
C
C+B
EAE+B

Fig. 7

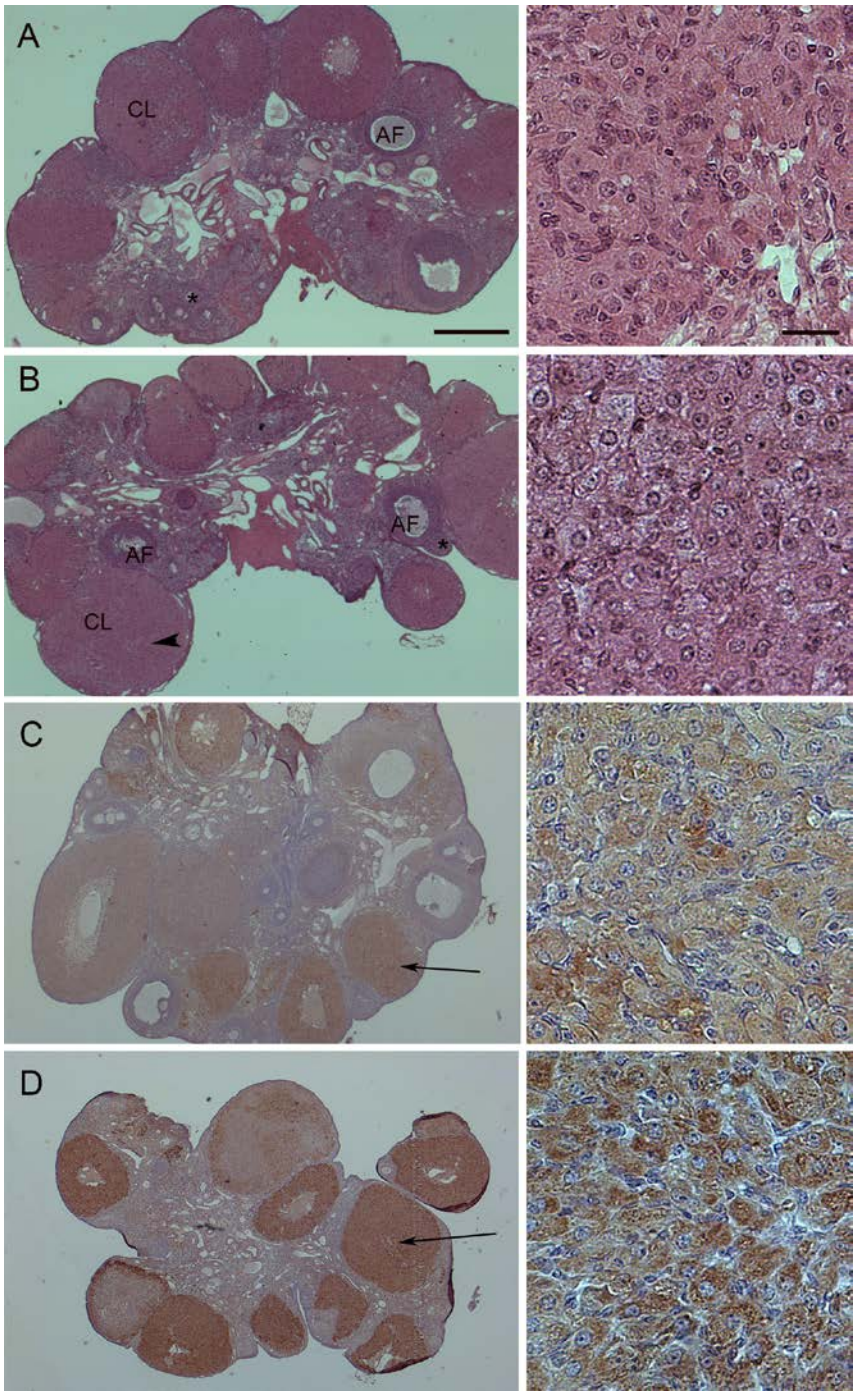

Fig. 8

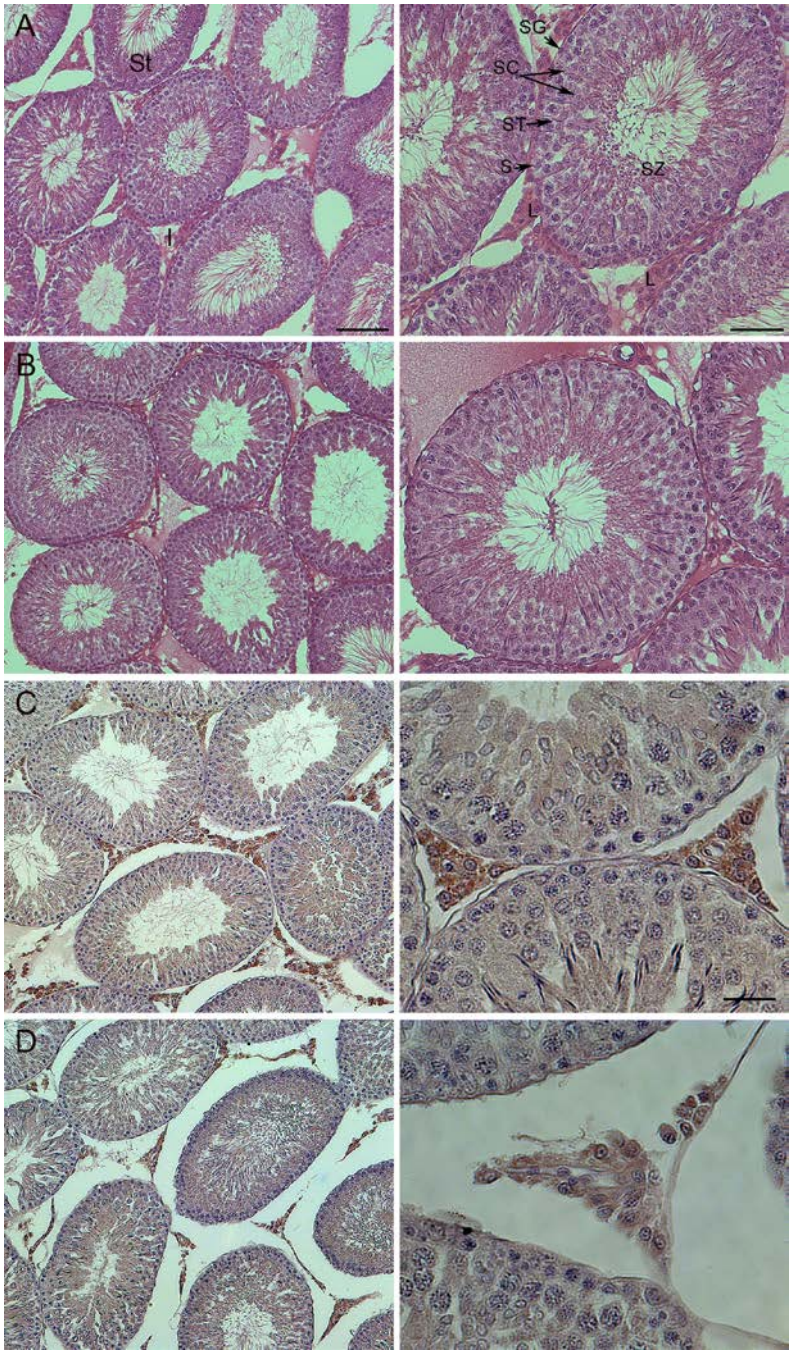

Fig. 9

Table 1. TaqMan probes

Target gene	Identification number
Cga	Rn01440184_m1
Fshb	Rn01484594_m1
Gapdh	Rn01462661_g1
Gfap	Rn01253033_m1
Gnrh1	Rn00562754_m1
Gnrhr	Rn00578981_m1
Kiss1	Rn00710914_m1
Kiss1r	Rn00576940_m1
Lhb	Rn00563443_g1
Pdyn	Rn00571351_m1
Tac3	Rn00569758_m1

Table 2. Primer sequences

Target gene	Sequence	Length	Accession number
Ccl2	F: TGATCCCAATGAGTCGGCTG R: TGGACCCATTCCTTATTGGG	127	NM_031530.1
Cd68	F: TGTGTGTCTGACCTTGCTGG R: AAGGATGGCAGAAGAGTGGC	90	NM_001031638.1
Gapdh	F: CAACTCCCTCAAGATTGTCAGCAA R: GGCATGGACTGTGGTCATGA	118	NM_017008.4
Il1b	F: CACCTCTCAAGCAGAGCACAG R: GGGTTCCATGGTGAAGTCAAC	79	NM_031512.2
Tnf	F: CCCCCATTACTCTGACCCCT R: CCCAGAGCCACAATTCCCTT	88	NM_012675.3
Il6	F: GCCCACCAGGAACGAAAGT R: GGCAACTGGCTGGAAGTCTC	86	NM_012589.2
Inhba	F: GCCTGAGACCACTCCTTTCC R: AAGGAGATGTTGAGGGCAGC	189	NM_012590.2
Inhbb	F: TCCTAGTGCCCTGCTGAGAT R: ACCCACAGGGACAACTTCTG	158	NM_080771.1