Reception and function of estrogen in the neuronal network regulating GnRH secretion

Ph.D. Thesis

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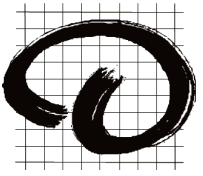

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Budapest, 2011

Introduction

Gonadotropin-releasing hormone (GnRH) producing neurons drive the hypothalamo-pituitary-gonadal axis. The regulation of the reproductive axis shows robust sex differences in rodents and there are species differences between rodents and humans, too. In general, the low level of estrogen is inhibitory whereas its high level is stimulatory within the axis. Function of GnRH neurons is influenced by estrogen through several direct and indirect manners. The estrogenic effects are mainly mediated by nuclear estrogen receptors (ERα and ERβ). Among these receptors, ERβ mRNA and protein has been detected in GnRH neurons *in vivo*, which can modulate gene expression and is also responsible at least in part for mediating the non-genomic rapid estrogenic effects. Neuroprotective action of estrogen may also take place in GnRH neurons similarly to the ones demonstrated for several other neurons involving ERβ in the central nervous system. This receptor subtype seems to be dispensable for sexual maturation and reproduction. In contrast, ERα is essential for reproduction, as this receptor subtype conveys both the negative and the positive feedback of estrogen. These data point to the fact that estrogenic feedback to the GnRH neurons happens through an indirect pathway involving neurons which innervate GnRH cells and express ERα. Neuronal population residing in the rostral periventricular region of the third ventricle (RP3V) is implicated in the generation of the preovulatory GnRH surge mechanism, whereas those in the arcuate nucleus (ARC) are responsible for the regulation of pulsatile secretion of this releasing hormone. Although the transmission of the estrogen signal to the GnRH neurons is an absolute prerequisite for generating the LH surge, in rodents, the surge event is also gated by a circadian oscillator in the suprachiasmatic nucleus (SCN). This gatekeeper ensures that the LH surge is precisely timed to occur on the day of proestrus within a 2- to 4-h window, near the onset of darkness. Candidates for mediating circadian signals to GnRH neurones include the vasopressin (VP)- and vasoactive intestinal polypeptide (VIP)- containing neurons of the SCN, but their precise role is not fully clarified. Some of the results emphasize the role of VP efferents from the SCN, but others support the primary mediator function of VIP. Moreover, differences between species are also observed. Studies indicate direct projection from the SCN to GnRH neurons in rats, in contrast such input to GnRH neurons was not detected in mice; instead a neuronal population residing in the RP3V has been implicated to relay circadian information to the GnRH neurons.

In the last ten years we have witnessed the emergence of kisspeptin (KP) as an indispensable element in the hierarchy of neuroendocrine factors governing reproductive functions. Results

to date made it clear that KP regulates both the surge and the pulsatile release of GnRH. In rodents the two main populations of KP neurons are located in the RP3V and in the ARC. The prior is responsible for the transmission of the positive feedback, whereas the latter conveys the negative feedback. In addition to KP, other neuropeptides known to participate in the regulation of GnRH secretion (i.e. neurokinin B, dynorphin, galanin, enkephalin) are variously expressed in neurons of the RP3V and the ARC. Recent investigations aim to determine the relative contributions of these peptides and the amine transmitters used in the neuronal network to regulate the pulsatile and/or surge release of GnRH.

Specific aims

1. To demonstrate a direct effect of estrogen in GnRH neurons by investigating the putative protection of GT1-7 cells against oxidative stress by selective and non-selective ER agonists *in vitro*.

2. To examine suprachiasmatic afferents of KP neurons in the RP3V in both male and female mice.

3. To characterise the chemical phenotype of KP neurons in the mouse RP3V and ARC, with special emphasis on the subpopulation innervating GnRH neurons.

4. To map the human KP system and to characterize the phenotype and connections of kisspeptin neurons with GnRH cells in the male and female hypothalamus.

Materials and methods

Animals

All animal experiments were carried out on adult male and female mice (Charles River, Hungary, CD1, 25-30g body weight). They were housed under conditions of controlled lighting (12:12 light dark cycle, lights on at 07:00h) and temperature (22 ± 2°C), with food and water *ad libitum*. Human hypothalamic samples from male and female individuals were obtained at autopsy from the Forensic Medicine Department of Semmelweis University (Budapest, Hungary).

Cell culture

Immortalized GnRH-secreting GT1-7 cell line was used to investigate whether 24h pretreatment with 17β-estradiol or with selective ERα and ERβ agonist prevents the oxidative stress-induced decrease of the mitochondrial membrane potential ($\Delta\Psi_m$). Agonists were applied in 10pM concentration for 24h, which was followed by treatment with 100μM H_2O_2 for 50 minutes. The change of the $\Delta\Psi_m$ was detected by incubating cells in the solution of the lipophilic, cationic dye, DIOC6 and measurements of the fluorescence intensity, which accumulates in the mitochondria following the Nernst equation. Changes in the $\Delta\Psi_m$ alter the distribution of this dye between cytosol and mitochondria.

Anterograde tract-tracing

For morphological examination of neuronal contacts between fibers originating from the SCN and KP cells located in the RP3V, ovariectomised (OVX), estrogen-treated female mice were injected with the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHA-L). The injecting glass micropipettes were positioned stereotaxically into the SCN. Following 7 day of transport time, the animals were perfused. Brain sections were processed for single immunohistochemical labelling for the evaluation of the injection sites and PHA-L transport.

Multiple immunofluorescent labelling, confocal microscopic image analysis

1. In sections of PHA-L injected animals, PHA-L/VP and PHA-L/VIP double-labelled appositions were analysed on RP3V KP neurons in order to confirm the suprachiasmatic origin of the VP- and VIP-containing afferents.

2. Studies to quantify VPergic input on KP neurons relied on the observation that VP-synthesising neurones in the SCN unlike other VP systems projecting to the RP3V, do not express galanin. Sections from male and female mice immunolabelled for KP, VP, and galanin were used to count the VP-immunoreactive (IR) afferents of KP neurons with SCN origin. To determine the estrogenic regulation of this neuronal connection, analysis were carried out on three treatment-groups of female mice: intact diestrous (estrous cycles were monitored by cellular profile analysis and by measuring the electrical resistance of the vaginal mucosa), OVX and s.c. implanted with a capsule containing estradiol dissolved in sunflower oil (OVX+E2 group), OVX and s.c. implanted with a capsule containing oil (OVX+oil

group). Sections from these animals were also processed for double immunohistochemical labelling to reveal VIP-IR appositions on KP neurons.

3. To characterise neurochemical subpopulations of KP neurons located in the RP3V and the ARC, the galanin and neurokinin B (NKB) content of KP cells were examined. To optimize histological detection in these areas, animal models with the highest KP incidence were chosen for the analysis; OVX+E2 animals for the RP3V and the OVX+oil animals for the ARC. In addition, colchicine treatment was applied to enhance the perikaryal detection of neuropeptides.
To identify galanin and NKB content of KP-IR afferents to GnRH neurons sections from OVX+E2 and OVX+oil animals were processed for triple immunohistochemical labelling to detect GnRH, KP and galanin or NKB. The galanin and the NKB immunoreactivity of KP-IR appositions on GnRH neurons was counted.

4. NKB immunoreactivity of KP neurons located in anatomically analogous regions to the rodent RP3V and the ARC was investigated in humans. Following double immunofluorescent labelling on women hypothalamic sections, the incidence of KP-IR cell bodies and fibers immunopositive for NKB in the periventricular (Pe) and in the infundibular nucleus (Inf) was established.

Double immunohistochemical labelling, pre-embedding immuno-electronmicroscopy
In mice, the ultrastructural relationship between KP neurons immunostained with diaminobenzidine (DAB) and VP terminals labelled with silver-gold intensified Ni-DAB was investigated with pre-embedding immuno-electronmicroscopy. This method was applied for determining synapses between GnRH neurons and KP terminals, too.

Single and double immunohistochemical labelling, light microscopic image analysis
To determine the distribution KP-IR structures in the human hypothalamus KP immunoreactivity was detected on human male and female hypothalamic samples utilizing single-labelled immunostaining. The morphological relationship between GnRH and KP neurons was also investigated by using double immunohistochemical labelling.

Quantitative double *in situ* hybridisation

Galanin and KP mRNAs were detected in cells of the RP3V and the ARC. Percentage of KP neurons expressing galanin mRNA was determined in sections hybridized with digoxigenin-labelled KP and ^{35}S-labelled galanin probes in the RP3V of OVX+E2 mice and in the ARC of OVX+oil mice.

Statistics

Comparisons were made by means of one-way ANOVA, with a post-hoc Tukey–Kramer multiple comparison test, $p<0.05$ was considered statistically significant. Values are presented as the mean±SEM.

Results

1. GT1-7 cells exposed to oxidative stress accumulated remarkably less DIOC6 than control cells. This was indicated by a decrease in the fluorescence intensity. Pretreatment of neurons with estradiol or with selective ER agonists prevented the decrease.

2. VP-IR varicosities formed appositions onto KP-IR neurones of the RP3V in male and female mice. Galanin was absent from most of these VP-IR appositions, which support their suprachiasmatic origin. PHAL-VP double-labelled appositions were also detected on KP neurons, which confirm that VP neurones from the SCN innervate kisspeptin cells in the RP3V. Ultrastructural examination revealed symmetric synapses between VP-IR terminals and KP-IR neurones in the RP3V. Such synapses are considered to be inhibitory, which is in keeping with the GABAergic nature of almost all SCN neurones including VP cells. Estrogen increases the number of VP-IR appositions on KP neurons in female mice.

3. Almost all KP neurons in the RP3V produce galanin. In this region the lack of NKB cell bodies was also observed. In the ARC 12% of KP cells were immunolabelled for galanin, whereas nearly all of them were positive for NKB. Galanin mRNA was detected in nearly half of the KP neurones in both the RP3V and the ARC by using a dual-label *in situ* hybridisation technique. 6% of KP appositions on GnRH neurons exhibited galanin immunoreactivity, whereas 2% of them were positive for NKB. Estrogen administration increased the incidence of co-localisation in KP afferents with galanin, but not with NKB.

4. Mapping of KP-IR structures in male and female human samples revealed significantly higher number of fibers in the Pe and the Inf of females vs. males. The second important sex difference we identified was the KP-IR cell group in the rostral periventricular region in females, which was absent in males. Highest number of KP cell bodies was detected in the Ins and InfS in both sexes, about 7-fold more cell bodies could be visualized in the female Inf. Axoaxonic, axodendritic and axosomatic contacts were detected between KP fibers and GnRH cells. Higher percentage of GnRH neurons received KP-IR boutons in females than in males. In the female Inf, three quarters of KP neurons were immunopositive for NKB. One tenth of KP-IR fibers were immunolabelled for NKB in the periventricular region, whereas half of them were immunolabelled in the Inf.

Conclusions

1. Our *in vitro* experiments indicate that estrogen has a direct protective effect on GT1-7 cells. The low concentration of agonists and the high binding affinity of ERs suggest that both ER subtypes participate in the mediation of the protective effect against oxidative stress in GT1-7 cells.

2. Our morphological evidences support the hypothesis, that KP neurons in the RP3V are involved in a multisynaptic neuronal system, which integrates and then transmits the estrogenic and the circadian signal to the GnRH cells. Integration of this information is essential for normal reproductive cycle and for the generation of GnRH surge. According to our results, this pathway derives from the suprachiasmatic VP-IR neurons. Estrogen treatment increases the number of VP contacts on KP cells, indicating the enhancement of the SCN input at the critical time of proestrous. In males, innervation of KP cells by VP neurons may contribute to the regulation of diurnal GnRH expression.

3. In neurons of the ARC, which mediates the negative feedback of estrogen, and those of the RP3V, which transmit the positive feedback, KP is co-produced with other neuropeptides also having influence on GnRH function. Their co-release at the very proximity of GnRH neurons may influence the function of GnRH cells at different phases of the gonadal cycle or at various pathophysiological conditions including fasting, stress or inflammation.

Our morphological results contribute to the well-accepted model, which conceptualize the hypothamic interplay of KP and GnRH neurons. According to this model, when estrogen levels decline, KP neurons in the ARC become spontaneously active. This activity would be amplified by an autosynaptic feedback action of these neurons ending in pulsatile release of KP and NKB. Both neuropeptides target GnRH fibers in the median eminence resulting in pulsatile GnRH secretion. At the time of high estrogen levels KP neurons in the RP3V are active and KP/galanin input from the RP3V is enhanced on GnRH cell bodies. KP and galanin acting through GPR54 and galanin receptors evoke prolonged activation of GnRH neurons leading to GnRH surge. The detection of KP-NKB double-labelled appositions on GnRH perikarya indicates that some of the KP inputs to GnRH cells are of ARC origin. The significance of this innervation requires further investigation.

4. KP-IR structures were detected extensively in the human hypothalamus, which indicates that besides the regulation of GnRH neurons, KP has diverse effects in the brain. Numerous contacts between GnRH neurons and KP fibers indicate significant and multiple communications between the two systems. Sex differences regarding the distribution of KP cell bodies and the density of KP fibers can underlie the sex differences in the function of the reproductive axis of humans. The co-localisation of KP and NKB at the level of cell bodies and fibers points to the fact that these neuropeptides may release and act on so far unidentified neurons together.

List of publications underlying the thesis

1. B Vida, L Deli, T Kalamatianos, E Hrabovszky, A Caraty, C W Coen, Z Liposits, I Kalló
Evidence for suprachiasmatic vasopressin neurons innervating kisspeptin neurons in the rostral periventricular area of the mouse brain: regulation by oestrogen
J Neuroendocrinol. 2010 Sep;22(9):1032-9.
IF 3.7

2. I Kalló, B Vida, L Deli, CS. Molnár, E Hrabovszky, A Caraty, P Ciofi, CW C, Z Liposits
Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurons
J Neuroendocrinol 2011
IF 4.65

3. E Hrabovszky, P Ciofi, B Vida, MC Horvath, E Keller, A Caraty, SR Bloom, MA Ghatei, WS Dhillo, Z Liposits, I Kalló
The kisspeptin system of the human hypothalamus. Sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons
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Front Genom Endocr 2011
IF 0 (journal was first published in 2011)

List of publications related to the subject of the thesis

5. B Vida, E Hrabovszky, T Kalamatianos, C W Coen, Z Liposits, I Kalló
Oestrogen receptor alpha and beta immunoreactive cells in the suprachiasmatic nucleus of mice: distribution, sex differences and regulation by gonadal hormones.
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IF 3.25

6. I Farkas, I Kalló, L Deli, B Vida, E Hrabovszky, C Fekete, SM Moenter, MWatanabe, Z Liposits
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IF 4.752

List of publications unrelated to the subject of the thesis

7. I Kalló, C Jekkel, Hrabovszky, Z Jurányi, B Vida, A Járási, T Wilheim, LG Harsing Jr, Z Liposits
Immunohistochemical and *in situ* hybridization studies on glycine transporter 1 after transient ischemia in the rat forebrain.
Neurochem Int. 2008 Mar-Apr; 52(4-5):799-808.
IF 3.228

Cumulative IF: 23