Effects of 17β-estradiol and glucagon-like peptide-1 on gonadotropin-releasing hormone neurons in mice

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Theses of the PhD Dissertation

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INTRODUCTION

Reproduction is controlled by the hypothalamo-pituitary-gonadal axis (HPG axis) in mammals. There are complex and precisely regulated interactions between the different units of the HPG axis via hormone messengers. Gonadotropin-releasing hormone (GnRH) synthesizing neurons of the hypothalamus form the key central elements of the HPG axis [1]. These neurons have a crucial role in the regulation of gamete production and synthesis of gonadal hormones in both sexes.

Disorders of the reproductive axis result in infertility which is due to diverse causes, including the malfunction of GnRH neurons. The function of GnRH neurons is influenced by a number of factors, including sex steroids, circadian rhythm, stress and metabolic states. Thus, it is important to examine these systems acting on the function of GnRH neurons. Therefore, in my doctoral thesis my goal is to give a more accurate picture about the role of the gonadal steroid, 17β estradiol (E2) and the metabolic hormone, glucagon-like peptide-1 (GLP-1) in the regulation of GnRH neurons using electrophysiological methods.

E2 is one of the primary regulators of GnRH cells and acts as a classic, homeostatic feedback molecule between gonads and brain. E2 is known to modulate GnRH output via positive and negative feedback mechanisms [2]. These feedback actions are crucial for the GnRH neurons to control the fluctuating levels of gonadal hormones. The actions of estrogen on GnRH neurons are mediated by estrogen receptors (ER). GnRH neurons express the beta form of ERs (ERβ) [3-5]. E2 exerts its effect through different signaling pathways: nuclear-initiated (classical) signaling [2, 6] and acute, membrane-initiated (non-classical) signaling [2, 7-10]. This rapid, non-classical action of E2 on GnRH neurons also effectively modulates their functions.

E2 at low physiological concentration (10 pM) has been shown to suppress the firing rate of GnRH neurons with involvement of fast neurotransmission [11]. GABA is the main regulatory neurotransmitter to GnRH neurons which is also an important player in the steroid feedback controlling GnRH neurons [12, 13]. Most mature GnRH neurons are excited by GABA due to the elevated intracellular chloride level in these cells [14, 15]. Thus, GABA has been suggested to be involved in the suppression of firing of GnRH neurons upon E2 administration [11]. Nevertheless, GABAergic input to GnRH neurons can be modulated by the retrograde endocannabinoid signaling mechanisms regulating GABA release from cannabinoid receptor type 1 containing axon terminals [16].

Therefore, it was hypothesized that the suppressing effect of E2 at low physiological concentration on GnRH neurons requires the activation of ERβ and retrograde endocannabinoid signaling mechanisms resulting in the repression of GABAergic neurotransmission onto these neurons.

Besides examining the role of E2 in negative feedback regulation of GnRH neurons, I also studied the effect of the glucagon-like peptide-1 (GLP-1), metabolic signal molecule, on these neurons. GLP-1 was shown to reduce food intake, inhibit gastric emptying and increase glucose-stimulated insulin secretion [17, 18].

In addition to regulating energy homeostasis, GLP-1 is a potent regulator of reproduction. Male GLP-1 receptor knockout mice showed reduced gonadal weights, and females possessed delay in the onset of puberty [19]. GLP-1 modified estradiol and progesterone levels [20]. Since GnRH neurons control the HPG axis, any GLP-1-induced modulation of the GnRH neuronal system itself has a major impact on reproductive physiology. The exact target and detailed molecular mechanism involved in the downstream actions of GLP-1 in GnRH neurons of the rodent preoptic area have not been elucidated, yet.

Although earlier studies described the modulatory effect of this gut hormone on reproduction, the direct effect of this peptide hormone on GnRH neurons has not been fully revealed so far. Therefore, my aim was to reveal the putative direct effects of GLP-1 upon electric activity of GnRH neurons.

SPECIFIC AIMS

The purpose of my doctoral thesis was to get a more accurate view about the operation of GnRH neurons using electrophysiological methods. In the first part of my work, I carried out detailed analyses to investigate the mechanisms of the negative estrogen feedback on GnRH neurons. To this end, the following essential questions have been raised and studied:

1. What is the effect of the estradiol on the function of GnRH neurons during the negative estradiol feedback period?
2. Which estrogen receptor is involved in the direct regulatory mechanism?
3. Does the retrograde endocannabinoid system play a role in the fast action of estradiol on GnRH neurons? If so, what are the molecular constituents and the presynaptic targets?

In the second part of the dissertation, I present my results about the regulatory role of the metabolic hormone glucagon-like peptide-1 (GLP-1). Earlier studies described the modulatory effect of this gut hormone on reproduction, although targets and the involved molecular mechanisms have not been elucidated yet. Therefore, I sought the answers for the following questions:

1. Does GLP-1 directly affect the functions of GnRH neurons?
2. Which molecular pathways act downstream to the GLP-1 receptor in the GnRH neurons?
3. What sort of retrograde signaling mechanism relay the information to presynaptic regulators? What are the intermediate components of this regulation?

EXPERIMENTAL PROCEDURES

Animals

Adult (postnatal day 50-100), gonadally intact, female or male, GnRH-green fluorescent protein (GnRH-GFP) transgenic mice (n=228) bred on a C57Bl/6J genetic background were used.

Whole-cell patch clamp experiments

For electrophysiological experiments, 250 μm-thick coronal slices containing the medial septum/preoptic area were used. During whole-cell patch clamp experiments spontaneous and miniature postsynaptic currents were measured in acute brain slices. The postsynaptic current measurements were carried out with an initial control recording (5 min), then agonists (Table 1.) were added to the artificial cerebrospinal fluid (aCSF) in a single bolus onto the slice in the recording chamber and the recording continued for a subsequent 10 min. When the antagonists (Table 1.) were used, they were added to the aCSF 10 min before starting the recordings and then, they were continuously present in the aCSF during the electrophysiological recordings. Intracellularly applied drugs were added directly to the intracellular pipette solution.

Loose-patch clamp experiments

Measurements were carried out with an initial control recording (5 min) of action currents, then drugs were added to the aCSF in a single bolus onto the slice in the recording chamber and the recording continued for a subsequent 10 min. The antagonists were continuously present in the aCSF during the electrophysiological recording (Table 1.).

Table 1. The chemicals, agonists and antagonists used

Name	*Effect*	*Concentration*
17β-estradiol (E2)	non-selective estrogen receptor agonist	10 pM
AM251	Cannabinoid receptor type-1 inverse agonist	1 μM
AMG9810	transient receptor potential vanilloid 1 antagonist	10 μM
CPTIO	nitric oxide scavenger	1 mM
DPN	selective estrogen receptor β agonist	10 pM
Exendin-3(9-39)	GLP-1 receptor antagonist	1 μM
Exendin-4	GLP-1 receptor agonist	100 nM – 5 μM
Faslodex (ICI 182,780)	non-selective estrogen receptor antagonist	1 μM
G1	selective GPR30 receptor agonist	10 pM
GDP-β-S	G-protein inhibitor	2 mM
L-arginine	nitric oxide donor	1 mM
L-NAME	nitric oxide synthase inhibitor	100 μM
NPLA	neuronal nitric oxide synthase inhibitor	1 μM
PF3845	fatty acid amide hydrolase inhibitor	5 μM
PHTPP	selective estrogen receptor β antagonist	1 μM
PPT	selective estrogen receptor α agonist	10 pM
THL (tetrahydro-lipstatin/orlistat)	diacylglycerol lipase inhibitor	10 μM
TTX (tetrodotoxin)	selective blocker of voltage sensitive Na^+ channels	660 nM

Real-time PCR detection of Glp1r and Nos1 in GnRH neurons

mRNA content of individual GnRH neurons of male mice was harvested using patch clamp pipette. In order to successfully detect Glp1r in RT-PCR experiments, three pooled samples from three mice were used. Each pooled sample contained 10 GnRH neurons. Individual GnRH neurons were used (a total number of 30 separated neurons from five animals) to investigate Nos1 expression.

RESULTS

Thesis I. Estradiol directly suppresses the activity of GnRH neurons during the negative estrogen feedback period

Low physiological dose of estradiol significantly decreases the firing rate and the frequency of spontaneous and miniature postsynaptic currents of GnRH neurons in metestrous female mice. The decrease in frequency occurs within minutes indicating that the effect of estradiol was rapid on these cells.

Thesis II. Execution of direct, rapid effect of estradiol requires ERβ in GnRH neurons during the negative feedback period

Electrophysiological experiments demonstrated that the beta type of estrogen receptor is mandatory for the observed rapid action of estradiol on GnRH neurons, since the subtype-selective ERβ agonist significantly decreased the mean frequency of the miniature postsynaptic currents. In addition, the effect of estradiol was significantly attenuated in the presence of the specific ERβ antagonist. In contrast, our data showed that other membrane-associated estrogen receptor agonists (ERα, GPR30) had no effect in mediating the observed rapid effect of the estradiol on GnRH neurons during the negative feedback period.

Thesis III. Retrograde endocannabinoid signaling is involved in the estradiol-evoked decrease of activity of GnRH neurons in the negative feedback period

We have proven the interaction between estradiol and endocannabinoid signaling mechanisms in GnRH neurons. The retrograde endocannabinoid signaling was blocked by cannabinoid receptor type 1 antagonist and by blockade of the synthesis of 2-arachidonoylglycerol which diminished the estradiol-triggered changes in GnRH neurons. The relationship between estradiol and endocannabinoid systems was confirmed when ERβ was not effective during the blockade of cannabinoid receptor type 1. These results support the view that 2-arachidonoylglycerol is synthetized in GnRH neurons and involved in the effect of estradiol suppressing GnRH activity when low physiological concentration of estradiol is used.

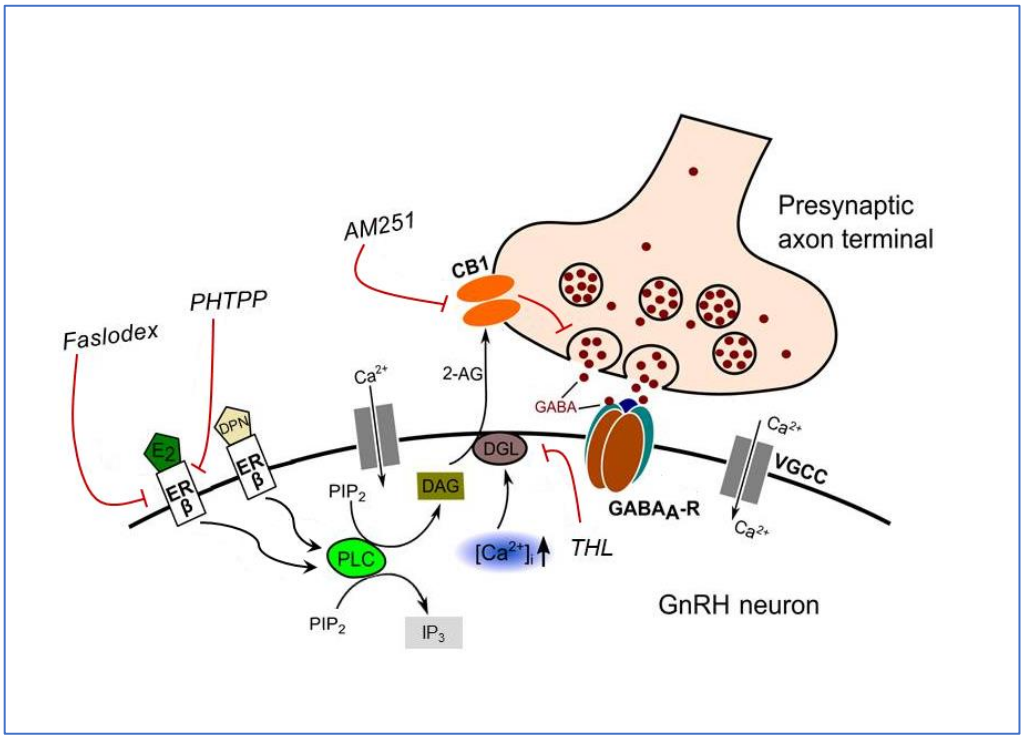

Figure 1. Schematic illustration of the relationship between estradiol and 2-AG endocannabinoid signaling in GnRH neurons of the metestrous female mice. E2 activation of ERβ causes the synthesis and release of 2-AG endocannabinoid from the GnRH neuron. Then, the released 2-AG binds to CB1 located in the presynaptic terminal of GABAergic afferents. This causes suppression of GABA release into the synaptic cleft and thus the attenuation of activity of GnRH neurons. The non-selective ER antagonist (Faslodex) or the selective ERβ receptor antagonist (PHTPP) is able to block the effect of E2. The CB1 inverse agonist (AM251) or the DAG lipase inhibitor (THL) also inhibit the signaling mechanism. Red lines represent inhibitory actions. Abbreviations: 17β-estradiol (E2); estrogen receptor beta (ERβ); subtype selective ERβ agonist (DPN); diacylglycerol (DAG); DAG-lipase (DGL); cannabinoid receptor type 1 (CB1); CB1 inverse agonist (AM251); non-selective estrogen receptor antagonist (Faslodex); subtype selective ERβ antagonist (PHTPP); 2-arachidonoylglycerol (2-AG); tetrahydrolipstatin (THL, a DAG-lipase inhibitor);, phosphatidylinositol 4,5-bisphosphate (PIP_2); inositol 1,4,5-trisphosphate (IP_3); phospholipase-C (PLC); $GABA_A$ receptor ($GABA_A$-R); intracellular free calcium ($[Ca^{2+}]_I$); voltage-gated calcium channel (VGCC).

Thesis IV. The GLP-1 is excitatory to GnRH neurons via GLP-1 receptor

The potent GLP-1 receptor agonist significantly increased the firing activity and the postsynaptic current frequency of GnRH neurons. The blockade of GLP-1 receptor by a specific antagonist could antagonizes these effects. Furthermore, the transcriptome of GLP-1 receptor mRNA was also detected in GnRH neurons. These results demonstrate the stimulatory effect of GLP-1 and the existence of functional GLP-1 receptors in GnRH neurons.

Thesis V. Nitric oxide and 2-arachidonoylglycerol signaling mechanisms are involved in the action of GLP-1 on GnRH neurons

Electrophysiological results revealed the involvement of NO retrograde signaling in the GLP-1-evoked action. NO synthase inhibitor or the intracellular scavenging of NO attenuated the excitatory effect of GLP-1 only partially. We demonstrated the expression of neuronal NO synthase mRNA in GnRH neurons. These results suggest that NO is synthetized by GnRH neurons. In addition, application of an NO-donor elevated the frequency of the postsynaptic currents, showing the mediating role of the NO system in function of GnRH neurons. Our results also demonstrate the involvement of 2-arachidonoylglycerol signaling mechanisms in GLP-1 action, since blockade of cannabinoid receptor type 1 partially eliminates the action of GLP-1. Nevertheless, the effect of GLP-1 was fully abolished by simultaneous blockade of both pathways. These results prove the simultaneous participation of NO and endocannabinoid retrograde signaling mechanisms in GLP-1 signaling.

Thesis VI. The retrograde 2-arachidonoylglycerol pathway is regulated by anandamide-TRPV1 signaling in GnRH neurons

We showed that stimulation of GLP-1R results in suppression of 2-arachidonoylglycerol endocannabinoid pathway. GLP-1 was unable to exert its 2-AG-related stimulatory effect when both the TRPV1 and the NO synthesis were inhibited. The role of anandamide in the activations of TRPV1 was also demonstrated in our experiments. These data indicate that suppression of 2-AG endocannabinoid signaling is mediated by the anandamide-TRPV1 pathway.

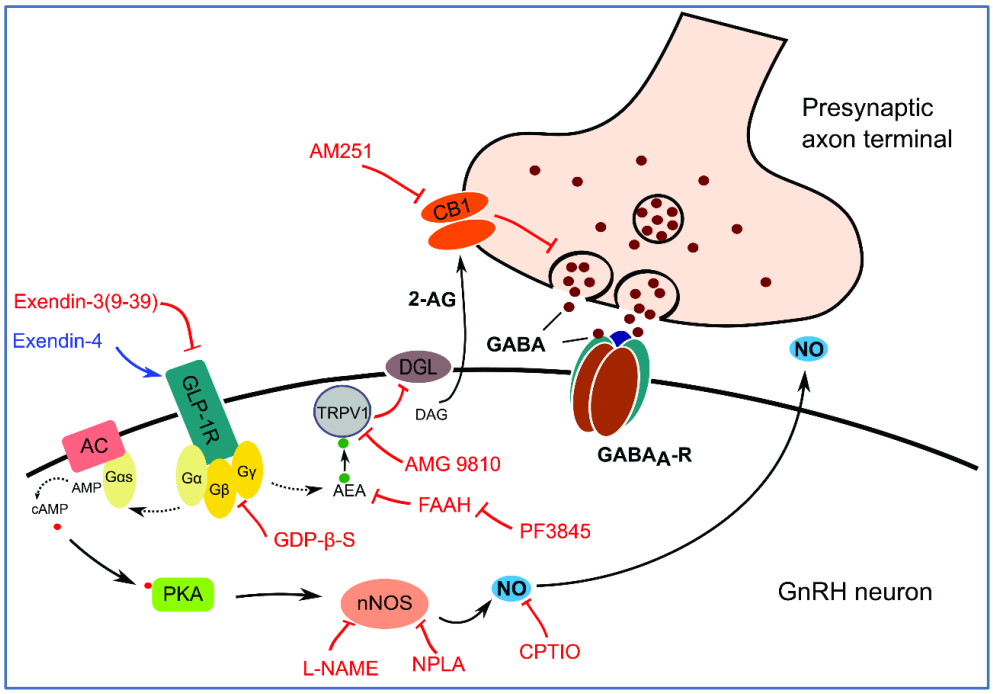

Figure 2. Schematic illustration of the proposed action of GLP-1 receptor signaling in GnRH neurons Effect of GLP-1R agonist (Exendin-4) is mediated by G-protein complexes which activate two retrograde signaling systems. The first one involves activation of nNOS, which leads to an increased NO production. Then NO diffuses from the postsynaptic GnRH neuron and subsequently increases the release probability and vesicular reuptake of GABA at the presynaptic terminal. The NO signaling was inhibited by the NO synthase blocker L-NAME, nNOS inhibitor NPLA or NO scavenger CPTIO. The second pathway is the activation of intracellular TRPV1 by anandamide. This suppresses the synthesis and release of 2-AG in the postsynaptic cell. It causes suppression of inhibition of the presynaptic GABA release. This signaling was blocked by FAAH inhibitor PF3845, TRPV1 antagonist AM9810, or the CB1 antagonist AM251. The effect of Exendin-4 was blocked by GLP-1R antagonist Exendin-3(9-39) or the G-protein inhibitor GDP-β-S. Red lines represent inhibitory actions, blue lines depict excitatory actions, and dotted lines denote putative actions. Abbreviations: glucagon-like peptide 1 receptor (GLP-1R); adenylate cyclase (AC); G-protein subunits (Gα, Gβ, Gγ); diacylglycerol (DAG); DAG lipase (DGL); cannabinoid receptor type 1 (CB1); CB1 antagonist (AM251); 2-arachidonoylglycerol (2-AG); $GABA_A$ receptor ($GABA_A$-R); protein kinase A (PKA); neuronal nitric oxide synthase (nNOS); L-NAME, a NOS inhibitor; NPLA, a nNOS inhibitor; GDP-Beta-S trilithium salt (GDP-β-S, a G-protein inhibitor); Carboxy-PTIO potassium salt (CPTIO, a NO scavenger); transient receptor potential vanilloid 1 (TRPV1); anandamide (AEA); TRPV1 antagonist (AMG9810); fatty acid amide hydrolase (FAAH); FAAH-inhibitor (PF3845).

POTENTIAL APPLICATIONS OF THE RESULTS

Infertility is one of the major health problems today, affecting approximately 20% of couples planning for pregnancy. Hence, it is critical to understand the central control of reproduction for new developments in infertility treatment. Since GnRH neurons orchestrate the HPG axis, studying and exploring neurons and hormones that affect GnRH neuron functions may provide therapeutic options and targets for treating menstrual disorders and infertility.

Both steroid and non-steroid substances influence the proper function of GnRH neurons. My results show the suppressive effect of estradiol on GABAergic neurotransmission on GnRH neurons. This action requires the activation of ERβ and 2-AG signaling during the estrogen negative feedback in mice. These results contribute to a better understanding of the estrous cycle, which is the basis of the proper female sex function. The discovery of the putative participation of the ERβ-2-AG signaling mechanism in GnRH neurons of humans raises a further possibility to detect disorders related to this signaling mechanism. The translation of these results to clinical science may initiate the development of new industrial strategies in the field of gynecology.

Further results showed the direct regulatory action of the GLP-1 metabolic signal molecule on GnRH neurons and the molecular mechanism in the downstream actions of GLP-1. This interaction between the metabolic and reproductive systems has a significant pathophysiological relevance. Reproductive maturation and fertility can be suppressed in conditions of energy deficit but can be modulated in energy excess like morbid obesity. By clarifying the effect of GLP-1 in the reproduction, we have contributed to a better understanding of the relation between nutritional status and gonadal function, which could later be part of the development of therapies for pubertal disorders or infertility due to improper nutritional status.

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List of publications not related to the subject of the thesis

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