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

PhD Dissertation

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS 4
INTRODUCTION 6
Regulation of the hypothalamo-pituitary-gonadal axis 6
Properties of the GnRH neuronal system 6
The gonadotropin-releasing hormone 7
The development, distribution and structural properties of GnRH neurons 8
The efferent projections of the GnRH neurons 10
Synaptic regulation of GnRH neurons 11
The main electrophysiological properties of GnRH neurons 12
GnRH neuron-related feedback mechanisms in reproduction 13
Classical and non-classical estrogen receptor signaling pathways 14
Influence of estrogen on functions of GnRH neurons 15
Metabolic signals 18
Glucagon-like peptide-1 signaling 19
The retrograde neurotransmission 21
The endocannabinoid system 21
The nitric oxide system 23
SPECIFIC AIMS 25
EXPERIMENTAL PROCEDURES 26
Experimental animals 26
Brain slice preparation and recording 26
Whole-cell patch clamp experiments 28
Loose-patch clamp experiments 30
Chemicals and reagents 31
Real-time PCR detection of Glp1r and Nos1 in GnRH neurons 32
Statistical analysis 33
RESULTS RELATED TO THE ESTRADIOL EFFECT DURING NEGATIVE FEEDBACK 35
Estradiol significantly decreases the firing rate and frequency of spontaneous and miniature postsynaptic currents in GnRH neurons of metestrous female mice 35

DOI:10.15774/PPKE.ITK.2018.010

The direct rapid effect of estradiol requires estrogen receptor beta 37
Retrograde endocannabinoid signaling is involved in estradiol-triggered decrease of miniature postsynaptic currents 40
RESULTS RELATED TO THE GLP-1 EFFECT IN GnRH NEURONS 42
The GLP-1 agonist Exendin-4 increases the firing rate and the frequency of miniature postsynaptic currents of GnRH neurons via GLP-1 receptor 42
Nitric oxide and 2-arachidonoylglycerol signaling mechanisms are involved in the action of Exendin-4 on GnRH neurons 46
The retrograde 2-AG pathway is regulated by anandamide-TRPV1 signaling 50
DISCUSSION 52
Estradiol suppresses the firing rate and frequency of postsynaptic currents in GnRH neurons in metestrous female mice 52
Estrogen receptor beta is required for the direct, rapid effect of estradiol on GnRH neurons 53
Retrograde endocannabinoid 2-AG signaling is involved in the estradiol-triggered reduction of miniature postsynaptic current frequency in GnRH neurons 54
GLP-1 is excitatory to GnRH neurons via GLP-1 receptor 57
Effect of GLP-1 is mediated partially by activation of the nitric oxide retrograde signaling on GnRH neurons 58
GLP-1 acts partially via retrograde endocannabinoid signaling pathway of GnRH neurons 59
The nitric oxide and endocannabinoid retrograde signaling pathways are simultaneously involved in the effect of GLP-1 60
NEW SCIENTIFIC RESULTS 63
ACKNOWLEDGEMENTS 65
REFERENCES 66
BIBLIOGRAPHY 80

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LIST OF ABBREVIATIONS

2-AG	- 2-arachidonoylglycerol
aCSF	- artificial cerebrospinal fluid
AEA	- anandamide (N-arachidonoylethanolamide)
AM251	- N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AMG9810	- (E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl) acrylamide
AMPA	- alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
cAMP	- cyclic adenosine monophosphate
CB1	- cannabinoid receptor type 1
CPTIO	- 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CREB	- cAMP response element binding protein
DAG	- diacylglycerol
DGL	- diacylglycerol lipase
DPN	- diarylpropionitrile
E2	- 17β-estradiol
EGTA	- ethylene-glycol-tetraacetic acid
ER	- estrogen receptor
ERα	- estrogen receptor alpha
ERβ	- estrogen receptor beta
Ex4	- Exendin-4
FAAH	- fatty acid amide hydrolase
FSH	- follicle-stimulating hormone
G1	- [(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanon
GABA	- gamma-aminobutyric acid
$GABA_A$-R	- gamma-aminobutyric acid receptor type A
GDP-β-S	- guanosine 5'-[β-thio] diphosphate
GFP	- green fluorescent protein
GLP-1	- glucagon-like peptide-1
GLP-1R	- glucagon-like peptide-1 receptor
GnRH	- gonadotropin-releasing hormone
GPCR	- G-protein-coupled receptor

GPR30	- G-protein-coupled receptor 30
GPR54	- G-protein-coupled receptor 54
GT1-7	- immortalized gonadotropin-releasing hormone neuron cell line
HEPES	-4-(2-hidroxietil)-1-piperazin-etánszulfonsav(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPG axis	- hypothalamo-pituitary-gonadal axis
KA	- kainate
KO	- knockout
KP	- kisspeptin
L-NAME	- Nω-Nitro-L-arginine methyl ester hydrochloride
LH	- luteinizing hormone
ME	- median eminence
NPLA	- Nω-Propyl-L-arginine hydrochloride
mGluR	- metabotropic glutamate receptors
mPSC	- miniature postsynaptic current
NMDA	- *N*-methyl-d-aspartate
NO	- nitric oxide
NOS	- nitric oxide synthase
NST	- nucleus of the solitary tract
OVLT	- organum vasculosum of the lamina terminalis
PCR	- polymerase chain reaction
PF3845	- N-3-Pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide
PHTPP	- 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl] phenol
PPT	- propylpyrazoletriol
PSC	- postsynaptic current
RP3V	- rostral periventricular area of the third ventricle
sGC	- soluble guanylyl cyclase
sPSC	- spontaneous postsynaptic current
THL	- tetrahydrolipstatin
TRPV1	- transient receptor potential vanilloid 1
TTX	- tetrodotoxin
vGlut	- vesicular glutamate transporter
V_{rest}	- resting membrane potential

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INTRODUCTION

The survival of species depends on the capability of adaptation and success of reproduction. Disorders affecting the units of the reproductive system can result in infertility. In mammals, reproduction is controlled by the hypothalamo-pituitary-gonadal axis (HPG axis) [1, 2]. Disorders affecting the reproductive axis result in infertility.

Regulation of the hypothalamo-pituitary-gonadal axis

There are complex and precisely regulated interactions between the different units of the HPG axis via hormone messengers. The HPG axis controls the ovarian cycle, sexual development, maturation and aging [1-5]. Gonadotropin-releasing hormone (GnRH; also called luteinizing hormone-releasing hormone) synthesizing neurons of the hypothalamus form the key central elements of the HPG axis [1, 2, 6]. The hypophysiotropic axons of GnRH neurons release GnRH into the fenestrated capillaries of the hypophyseal portal circulation in a pulsatile manner [7]. GnRH neurohormone reaches its target cells in the anterior pituitary gland via the long portal veins. Here, the episodically released GnRH stimulates its receptors on the surface of the gonadotroph cells, that results in a rhythmic discharge of gonadotropins, the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [2, 8]. FSH and LH are released into the systemic circulation and act on the gonads: FSH stimulates the maturation of ovarian follicles in females, the spermatogenesis in males and stimulates gonads to produce steroids. LH stimulates secretion of sex steroids from the gonads in both sexes. The gonadal steroids, in turn, exert negative and positive feedback actions on the hypothalamus and the pituitary, and regulate the synthesis of GnRH and the two gonadotropins [2, 9, 10] (Figure 1.). Gonadal steroid hormones also have a number of other important physiological effects upon almost all of the organs, including the brain.

Properties of the GnRH neuronal system

GnRH neurons play a decisive role in the regulation of the HPG axis and thus, in the control of reproduction. In this chapter, the properties and regulation of GnRH neurons will be discussed focusing on structural organization of the GnRH system, the operating signaling mechanisms of GnRH neurons and the molecular background of information processing.

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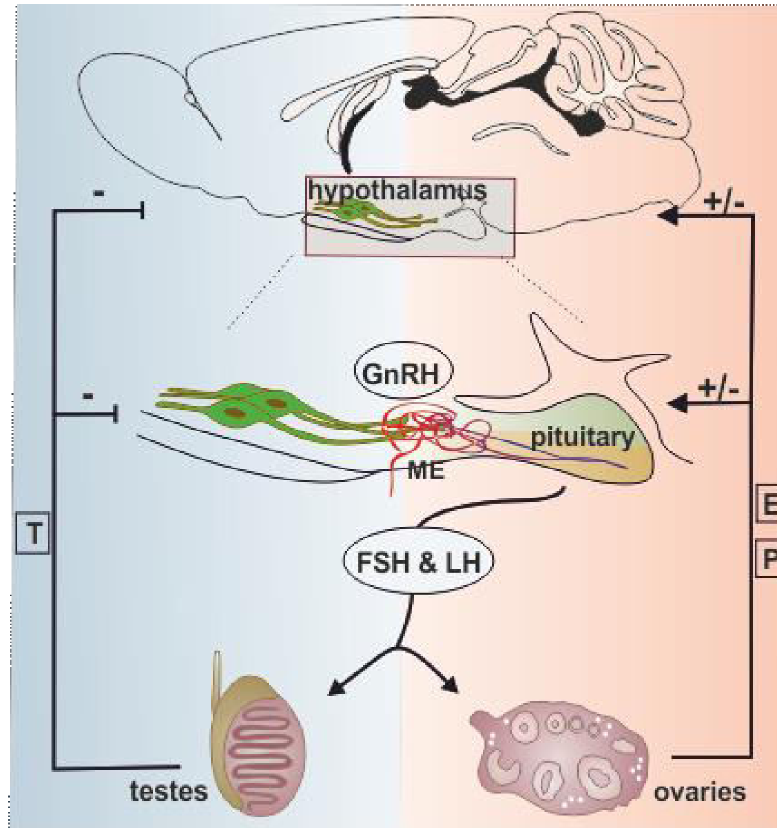

Figure 1. Relationship between the regulatory units of the reproductive axis. Hypothalamic GnRH neurons release GnRH neurohormone into the hypophyseal portal circulation. Via this portal system GnRH reaches its target cells in the anterior pituitary and regulates the synthesis and secretion of the gonadotropins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are released into the systemic circulation and act on the gonads to stimulate gonadal steroid secretion. Sexual steroid hormones, such as estrogen (E), progesterone (P), and testosterone (T) influence the hypothalamic and pituitary hormone secretions via feedback loops. Abbreviation: median eminence (ME).

The gonadotropin-releasing hormone

GnRH molecule was discovered in 1971 by Roger Guillemin and Andrew Schally [11]. Since the original discovery, different forms of GnRH have been found in vertebrates. GnRH-l is a decapeptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). The gene encoding *GnRH1* is on chromosome 8. GnRH-1 is a neurohormone, the hormone itself is synthetized in specific neurons of olfactory placode origin and is released at their terminals. The hypophysiotropic GnRH-1

population regulates the synthesis and release of pituitary gonadotropins [12]. The GnRH-1 neurosecretory system and the structure of GnRH-1 are evolutionarily conserved in vertebrates. In all mammals the amino acid sequence of GnRH-1 is the same [12]. The scope of this dissertation has been the hypophysiotropic GnRH-1 neurons (hereafter GnRH) and their involvement in the regulation of the HPG axis.

There are two additional GnRH molecules that form distinct populations in the brains of several species. GnRH-2 and GnRH-3 differ at several amino acids from GnRH-1. GnRH-2 is often referred to as chicken GnRH and GnRH-2 neurons are present in the midbrain tegmental area [12]. GnRH-3 can be found in the telencephalon of fish, amphibians and some mammals. This hormone is sometimes referred as salmon GnRH [12].

The development, distribution and structural properties of GnRH neurons

The GnRH neurons originate from the olfactory placode and migrate into the forebrain along the olfactory-vomeronasal nerves from the gestational day 11 in mice [13-16]. This phenomenon can be observed not only in rodents, but in humans [17], rhesus macaques [18], and chicken [19]. Disorders in the migration process can cause hypogonadotropic hypogonadism which is characterized by the lack of hypothalamic GnRH neurons in the forebrain leading to reproductive deficiencies. The human disease is called Kallmann syndrome, which often coincides with anosmia [20]. Migrating GnRH neurons settle down in the hypothalamic/median preoptic area by the time of birth [14]. According to the present literature there are approximately 800 GnRH neurons in the rodent brain, 1000-2000 in primates brain and their numbers are equal in both sexes [21-24]. Surprisingly, these small amount of GnRH neurons are sufficient to carry out the control of reproduction [25].

GnRH neurons have fusiform morphology. The perikarya of the cells are relatively small (10-15 μm). Being bipolar neurons GnRH cells have two processes emerging from the cell body: two dendrites or one dendrite and one axon. However, sometimes axon emanates from one of the main dendrites [26-28].

GnRH neurons form anatomical and functional networks. Communication among the cells is mainly achieved through axo-somatic and axo-dendritic connections [29], however other types of communication have also been observed. Some studies showed tight junctions between GT1-7 immortalized GnRH neurons [30]. The other connection type observed between GnRH nerve cells is formed by continuous intercellular bridges, where the cytoplasmic domains communicate with

each of them [31, 32]. These connections between GnRH neurons have a crucial role in the regulation of the synchronized operation of the system.

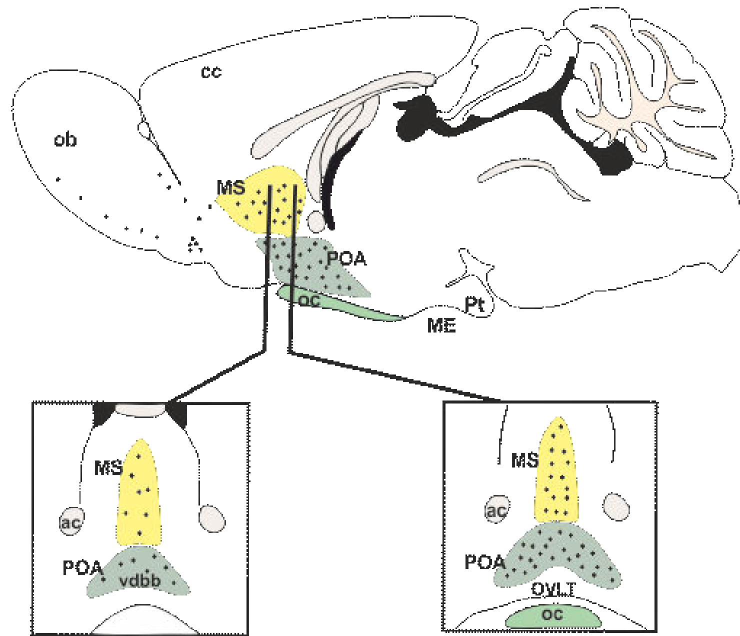

Figure 2. Distribution of the GnRH neurons in mice. Sagittal (top) and coronal (bottom) views of the distribution of GnRH neuron cell bodies (black dots). These neurons located mainly in the diagonal band of Broca (dbb), medial septum (MS) and the preoptic area (POA). Abbreviations: anterior commissure (ac); median eminence (ME); optic chiasm (oc); organum vasculosum of lamina terminalis (OVLT). The figure is based on the image of Dr. Michel Herde [33].

GnRH neurons are located in a relatively broad area in the brain. In rodents GnRH cells show a scattered distribution pattern, these neurons are located principally in the medial septum, the diagonal band of Broca, the medial and rostral preoptic area, in the vicinity of the organum vasculosum of the lamina terminalis (OVLT) [34, 35] (Figure 2.).

The efferent projections of the GnRH neurons

The major efferent targets of the GnRH neurons are two circumventricular organs: the median eminence (ME) and the organum vasculosum of lamina terminalis (OVLT) [15, 16]. The ME is a functional interface between the hypothalamus and the anterior pituitary gland. Hypothalamic neurons release peptides and small molecule neurotransmitters into portal vessels. The walls of the capillaries are fenestrated to ensure maximal hormone permeability, the axons of hypophysiotropic neurons terminate around this capillary network. The portal capillaries are reunited forming the long portal veins [36]. The ME has three parts: the ependymal layer, the internal zone and the external one. From 50% to 70% of all GnRH axons terminate in the external zone of the ME where GnRH is released into the portal capillaries in a pulsatile manner [7, 9, 37].

Besides the hypophysiotropic axon projections, GnRH fibers also innervate the OVLT. The OVLT is another sensory circumventricular organ of the brain, located along the ventral part of the anterior wall of the third ventricle [38]. It is made up of neurons, glial cells and ependymal cells. The ependymal cells form tight junction connections near their apical surfaces, which avert the free flow of the cerebrospinal fluid from the ventricle into the brain parenchyma. Being a circumventricular organ, OVLT contains a rich vascular network containing specialized fenestrated capillaries [38]. Multiple projections of GnRH neurons in the region of the OVLT and ME permit the sensing of peripheral signals due to the lack of the blood-brain barrier here [38].

There are less studied extrahypothalamic GnRH axon projections in addition to the above-mentioned ones in several areas of the brain. Although these cells make up 30 percent of all GnRH neurons, these non-hypophysiotropic GnRH cells and projections are rarely investigated [7]. A recent study showed that this kind of GnRH neurons communicate with approximately 50,000 neurons in 53 functionally diverse brain areas [39]. Moreover, a significant number of GnRH neurons are located in the medial septum and the olfactory tubercle, which innervate the olfactory bulb [35]. This suggests that the GnRH system may have a role in the transmission or modulation of olfactory stimuli, which is closely related to some reproductive functions and behavior [39].

Regarding the specific phenotype of GnRH efferent system, it has been shown that GnRH neurons express mRNA of vesicular glutamate transporter-2 (vGlut2) in rats [40]. This vesicular transporter is obligatory for accumulating glutamate into synaptic vesicles, thus it indicates that GnRH neurons are glutamatergic suggesting the possibility that GnRH neurons could release glutamate from their nerve terminals. Nevertheless, recently it has been also demonstrated that a subset of GnRH neurons is GABAergic in the mouse brain [41] indicating the chemical heterogeneity of GnRH neurons.

Synaptic regulation of GnRH neurons

One of the most significant regulatory neurotransmitters in the central nervous system, as well as in the hypothalamus, is *γ-aminobutyric acid (GABA).* GABA is the main neurotransmitter acting on GnRH neurons [42], as major proportion of synaptic contacts on GnRH neurons is GABAergic [43]. GnRH neurons express the ionotropic $GABA_A$ [44-46], and the metabotropic $GABA_B$ [47] receptors. The observed $GABA_A$ receptor-mediated postsynaptic currents [45, 46] also confirm the GABAergic input to these neurons. Although GABA is usually an inhibitory neurotransmitter in the adult central nervous system, early data seemed controversial whether GABA stimulates [45, 48] or inhibits [49] GnRH neurons. Now, it has been widely accepted that most mature GnRH neurons are excited by GABA [50-52]. The intracellular chloride ion concentration is responsible for determining the polarity of GABA response [53]. The hyperpolarizing (generally inhibitory) action of GABA exists when intracellular chloride ion concentration is low whereas the depolarizing (generally excitatory) action of GABA occurs when intracellular chloride ion concentration is high, such as in GnRH neurons. The excitation by the $GABA_A$ receptor activation is due to the high intracellular chloride level of GnRH neurons. On one hand this high intracellular chloride level is due to the absence or low expression levels of the K-Cl cotransporter 1 in GnRH neurons. This cotransporter excludes chloride ion from the cytoplasm in neurons. On the other hand high expression of the Na-K-Cl cotransporter 2 in GnRH neurons is responsible for maintaining high chloride level in GnRH neurons, since this cotransporter mediates inward transport of chloride ion [45]. $GABA_A$ receptor is a ligand-gated chloride channel. Activation of this receptor leads to the opening of the channel and results in chloride ion efflux and neuronal depolarization in GnRH neurons.

As mentioned above GnRH neurons also express the metabotropic $GABA_B$ receptor [47, 54]. The functional $GABA_B$ receptor is a G-protein-coupled receptor linked to potassium channels or even calcium channels [55]. The receptor is a heterodimer formed by a GABA B1 and B2 subunit. Activation of $GABA_B$ receptors triggers inhibitory postsynaptic currents leading to reduced neuronal excitability of GnRH neurons [55]. GABA exerts its inhibitory effect via $GABA_B$ receptors on GnRH neurons [47, 56].

The major GABAergic innervation of GnRH neurons is supposed to arise from local GABAergic interneurons [10]. GABAergic inputs play major roles in the mediation of metabolic [46], circadian [57] and estrogen [58] signals to the GnRH system [57].

Another major neurotransmitter in the afferent control of GnRH cells is *glutamate* [59]. The markers of glutamate-secreting nerve terminals are the vesicular glutamate transporters (vGlut1, vGlut2 and

vGlut3). GnRH neurons receive synapses from vGlut2 containing glutamatergic neurons and these inputs were observed mostly on the dendritic compartment of GnRH cells [59]. Consistent with this, GnRH neurons express all ionotropic glutamate receptors, the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate (AMPA/KA) and *N*-methyl-d-aspartate (NMDA) receptors [44, 58, 60]. Whereas $GABA_A$ receptor mediated postsynaptic currents can be easily detected in all GnRH neurons, not all neurons exhibit postsynaptic currents mediated by these glutamate receptors [58, 60]. However, it should be noted that currents generated on the distal dendrites might be insufficient to be detected on the perikaryon [27], but may still have important role in the cell function. Whole-cell patch clamp studies reveal that glutamate transmission predominantly mediated by AMPA/KA receptors, but NMDA mediated postsynaptic currents were also observed in GnRH neurons [44, 58]. Activation of these ionotropic glutamate receptors contributes to the pulsatile [61] and also to the surge [62] release of GnRH.

In addition to the fast neurotransmission, slower glutamatergic neuromodulation is also present in GnRH neurons, for which metabotropic receptors are responsible. Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors (GPCRs). These can be grouped into three groups (Group I, II and III). Group I mGluRs are localized postsynaptically on neurons or glial cells. Activation of these receptors increases intracellular Ca^{2+} level. The presynaptically localized Group II/III mGluRs mediate feedback inhibition [63]. Bath application of Group II/III mGluR agonists decreased the frequency of GABAergic events on GnRH neurons. The mGluRs responsible for this synaptic inhibition are suggested to be located on the presynaptic GABA terminals and not on the GnRH neurons themselves [64].

The main electrophysiological properties of GnRH neurons

Our experiments were performed on GnRH-green fluorescent protein (GFP) transgenic mice, in which GnRH promoter is linked to a green fluorescent reporter molecule, allowing the microscopic detection of GnRH neurons in slices preparations [65], as the expression of the reporter is restricted to these neurons. Thus, GnRH neurons were visualized using fluorescence microscopy and the identified individual neurons were used for electrophysiological recordings and subsequent structural, molecular analysis. It is important to note that the main electrophysiological properties of GFP expressing GnRH neurons [44, 65], do not differ from the wild type cells using either brain slices [66], primary cell cultures [66, 67] or immortalized GnRH neuronal cell line (GT1) [68, 69].

Resting membrane potential (V_{rest}) ranges approximately -55 to -65 mV in GnRH-GFP cells [44, 65]. The input resistance shows the integrity of the cell, reflects the extent to which membrane

channels are open. Under physiological conditions the input resistance of GnRH neurons is high, approximately 1.60 GΩ [65]. This means that these cells have relatively few channels open at the resting membrane potential. The high input resistance also indicates that small currents can have a large impact on membrane potential.

GnRH neurons present periodic, spontaneous action potentials which could be blocked by tetrodotoxin (TTX) indicating that these are mediated by voltage-gated sodium channels [65] because TTX can inhibit the firing of action potentials by binding to voltage-gated sodium channels. The episodic, not continuous firing means that GnRH neurons showed quiescent periods with intermittent action potentials [65, 66]. The spontaneous action potentials were observed with an amplitude of >60 mV (76.9 ± 5.5 mV) [65].

Based on the firing patterns of adult GnRH neurons there are three different populations observed in acute brain slices. Cell-attached recordings revealed that majority of the GnRH neurons exhibit burst firing (~65%), another population remains silent, and the last one is a smaller group exhibiting continuous activity. Note that, *in vivo* experiments showed that only ~15% of GnRH neurons exhibit burst firing in mice [70]. These heterogenous firing patterns are observed in both gonadectomized and intact male and female mice. [66].

GnRH neuron-related feedback mechanisms in reproduction

The delicate balance of coordinated signals among the hypothalamus, pituitary gland and the gonads is strongly related to the precise function of GnRH neurons. The pattern of GnRH release forms the final output signal of the hypothalamus towards the pituitary gland. Thus, the secretion of gonadotropins (LH, FSH) from the anterior pituitary gland is influenced by hypothalamic GnRH pulse frequency and amplitude. GnRH pulses occur every 30-90 minutes and both the frequency and amplitude are crucial for normal gonadotropin release [9]. Low GnRH pulse frequency is required for FSH production and release, whereas high GnRH pulse frequency stimulates LH synthesis and release [9]. Furthermore, pulsatile GnRH secretion is indispensable to prevent the desensitization of GnRH receptors and thus to maintain the hormone sensitivity of neurons of the pituitary gland. Gonadotropins activate gonadal steroid hormone synthesis and these gonadal steroids exert negative (in both females and males) or positive (exclusively in females) feedback actions on the central components of the HPG axis (Figure 1.).

The negative feedback loop is the common regulatory mechanism in both sexes. In males sex steroids suppress GnRH neuron activity and GnRH release. After puberty, the testicular hormone genesis is continuous. FSH stimulates the spermatogenesis, while LH stimulates Leydig cells in

testes to produce testosterone. High levels of androgens exert a constant, direct/indirect inhibitory action on the hypothalamic GnRH neurons and pituitary via acting on the androgen receptors [71]. The GnRH and LH are secreted in pulsatile manner in males [72]. The orchidectomy of male animals and the testosterone treatment in females during the so-called critical postnatal period (postnatal day 5-10) has demonstrated that testosterone surge is responsible for the development of sexual dimorphism [73], including in brain structures. Consequently, testosterone and estrogen (which originates from testosterone by aromatase enzyme) have constant inhibitory effect on the reproductive axis through the androgen and estrogen receptors, respectively in adult rodents. The positive feedback does not occur in mature male animals.

Female animals have a cyclic pattern of reproductive function which is called estrus or ovarian cycle. A cycle lasts four days in mice and is characterized by four stages: proestrus, estrus, metestrus and diestrus. The pattern of GnRH and gonadotropin release and thus blood estrogen levels vary throughout the different stages. During the major part of the ovarian cycle (throughout the estrus-diestrus phase) the relatively low level of estrogen (approximately 10 pM) exerts negative feedback effect on the hypothalamus and pituitary. This means estrogen reduces GnRH pulse amplitude and frequency, leading to the suppression of LH release in the pituitary, thereby, repressing its own follicular synthesis [8, 74]. The negative feedback action of estrogen is modulated by peptides produced by the ovaries such as inhibin A, inhibin B, activin or follistatin [75, 76].

In proestrus, mature follicles show a dramatic increase in their estrogen secretion and a modest elevation in progesterone production. The response to this sustained elevating estradiol level is involved in the switch from negative to positive feedback in the hypothalamus. The positive feedback effect of estradiol initiates GnRH surge, a large increase in the volume of GnRH release [57, 77]. This can be explained by a fold elevation of amplitude and frequency of the GnRH secretory pulses [78]. Meanwhile, the responsiveness of the gonadotropin cells to GnRH increases [79] causing a surge in LH release from the anterior pituitary gland, initiating ovulation in the ovary [8, 77].

Classical and non-classical estrogen receptor signaling pathways

Estrogens in females are produced primarily by the ovaries, but the whole enzyme set is also expressed in neurons and astrocytes for estrogen synthesis in the central nervous system [80]. Sex steroids, including the three major natural estrogen – estrone (E1), estradiol (E2) and estriol (E3) – all derive from cholesterol through different steps of enzymatic reactions [80]. The major product from the whole biosynthesis process is the 17β-estradiol (E2) and it is the primary biologically

active and prevalent form of estrogen. E2 is produced by aromatase from testosterone or is converted from estrone at the end of the biosynthesis process [81]. E2 is one of the principal regulators of GnRH cells and acts as a classic, homeostatic feedback molecule between gonads and brain. Estradiol is critical in controlling GnRH neurons to exhibit fluctuating patterns of biosynthetic and secretory activity [10].

The actions of estrogen on neurons are mediated by estrogen receptors (ER). Estradiol mainly interacts with two types of classical estrogen receptors, ERα and the ERβ, each encoded by a separate estrogen receptor gene (ESR) 1 and 2, respectively. These receptors belong to the superfamily of nuclear receptors [82, 83]. E2 exerts its effect through two major signaling pathways: the genomic (classical, nuclear-initiated) signaling [84, 85] and the acute, non-genomic (non-classical, membrane-initiated) signaling [85-89]. In classical, genomic ER signaling pathway estrogen diffuses passively across the cell membrane then binds and activates ERs [82, 83]. Activation of ERs leads to a conformational change of the receptors and subsequent receptor dimerization. The two receptor types can form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. The dimeric receptors enter the nucleus and subsequently bind to the estrogen response element on the promoter regions of target genes leading to gene activation or repression [82, 83].

Additionally, estrogen is capable to bind and activate receptors associated with the plasma membrane, and thus exerts a rapid, direct effect (non-genomic, non-classical signaling), such as rapid increase in cAMP, or altered firing of neurons within seconds [85-89]. Receptors that are responsible for rapid action of estrogen are called extranuclear ERs. These ERs are associated with signaling complexes in the plasma membrane [90]. Most of the rapid effects of estrogen can be induced by selective ERα or ERβ agonists [91] or antagonized by the ER antagonists [92]. The rapid effects of estrogen are absent in ER mutant animals, since estrogen had no effect on an important target of the rapid estrogen action: the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) phosphorylation in double ERαβKO mice [93]. In addition to ERα and ERβ, other types of membrane associated receptors might also participate in the rapid estrogen effects, such as G-protein-coupled receptor 30 (GPR30) found in primate [94] and mouse [95] GnRH neurons.

Influence of estrogen on functions of GnRH neurons

Rapid action of E2 effectively modulates neuronal functions. Several studies have reported the non-genomic effect of estradiol in GnRH neurons. Estradiol increased the phosphorylation of CREB

[89]. The action of estrogen was rapid (<15 min) on CREB phosphorylation, indicating a non-genomic mechanism [89]. It has also been demonstrated that CREB within GnRH neurons is an important target for estrogen negative feedback actions [96]. In addition, estradiol increased calcium oscillations [86] and potassium currents [97-99] in GnRH neurons. ERβ has a role to play in mediating acute estrogen actions. Moreover, an *in vivo* study revealed that GnRH neurons respond to estrogen in a rapid manner through an ERβ-dependent mechanism in the mouse [89]. Nevertheless, the role of ERβ in GnRH neuron function is far from the fully elucidated.

Regarding the rapid effect of low physiological estradiol levels, it has been demonstrated, that estradiol (10 pM) inhibited GnRH neurons firing in a rapid manner when the fast synaptic GABA and glutamate receptors were left intact [100]. This indicates the involvement of fast neurotransmission in the rapid effect of estradiol and suggests an effect upstream of GnRH neurons.

As mentioned above, E2 can inhibit (negative feedback) or stimulate (positive feedback) GnRH release, which depends on estradiol concentration and the physiological state of the body. Estradiol feedback mechanisms alter the synaptic transmission to GnRH neurons and their intrinsic excitability [45, 100, 101]. The way that estradiol changes GnRH neuron functions is different in the two feedback mechanisms. During positive feedback action high physiological (preovulatory) concentrations of estradiol (approximately 100-200 pM in rodents) has a great influence on GnRH neurons and pituitary gonadotrophs to generate the preovulatory LH surge [8, 10, 57, 77]. According to a popular hypothesis, positive steroid feedback is achieved via presynaptic interneurons that are estradiol-sensitive. Estradiol may act via various ERs, mainly the ERα and ERβ. These receptors can act either on the DNA as transcription factors [84, 85] or estradiol is able to initiate membrane-associated signaling cascades via membrane-associated receptors [85-89].

One of the estradiol-sensitive presynaptic systems acting upstream the GnRH neurons during positive feedback is the kisspeptin (KP) neuron system. A population of KP neurons form a compact nucleus in the rostral periventricular area of the third ventricle (RP3V) in the mouse brain. The RP3V population is proposed to mediate the positive feedback of estrogen [85]. This is indicated by the fact that administration of KP shows a profound increase in serum gonadotropin levels via the stimulation of the secretory activity of GnRH neurons [102]. KP is the endogenous ligand of G-protein coupled receptor 54 (GPR54) [103]. GPR54 is highly expressed in GnRH neurons [102]. Mutations of KP [104] and GPR54 [105] cause hypogonadotropic hypogonadism with partial or moderate puberty in humans, while over activation of this system causes puberty praecox [106]. The direct effect of KP-producing neurons on GnRH neurons is supported by numerous observations. It was shown that KP axons innervate the perikaryon and dendrites of GnRH neurons

[107] and they respond to KP with increased neuronal activity [108]. The RP3V population has role in estrogen positive feedback [85], as KP neurons that contact GnRH neurons have been shown to express ERα and these neurons in the RP3V release KP in response to estrogen action [85].

During negative feedback mechanism low physiological level of estradiol (~10 pM) represses gonadotropin secretory activity in females by inhibiting hypothalamic GnRH secretion [10, 100]. Changes in the function of GnRH neurons are thought to be mediated by estradiol-sensitive afferents at this stage of the cycle, and the population of KP neurons located in arcuate nucleus are defined as the main regulator of estrogen negative feedback [85, 109]. ERα was detected exclusively in various synaptic afferent systems such as KP neurons of arcuate nucleus regulating GnRH neurons, but importantly GnRH neurons themselves do not express this receptor type [109-114]. Thus, the regulation of the negative feedback is more complex, several brain regions, cell types, and estrogen receptors could be involved in suppressing the activity of GnRH neurons. The important fact that GnRH neurons express the ERβ [112, 115-117] as a direct target of estradiol feedback further increases complexity of this system.

Data in the literature seems to be controversial regarding the role of the ERβ in the negative feedback. Experiments from female mice of different ERβKO mutant mouse lines [118-120] showed a range of reproductive phenotypes from mild subfertility [119] to complete infertility [120]. Examinations in global ERKO mice have shown the importance of ERα, and possibly ERβ, in reproductive regulation [113]. However, investigations in global knockout mice do not allow to make conclusions on a fine scale, and effects could be compensated by unknown mechanisms. In addition, neuron-specific deletion of ERα and ERβ in mice suggested that ERα seems to be essential for acute E2 negative feedback while ERβ particularly in GnRH neurons appears to be less critical [110]. These data suggest that ERβ may not be critical for central estradiol negative feedback of the HPG axis. In contrast, gonadotropin levels are less increased in ERα knockout versus the double (ERαβ) knockout mice, indicating that ERβ may still have a role in negative feedback of the HPG axis [121]. In addition, the exclusive role of the ERα in the negative feedback is questioned by a recent study in which KP-ERα knockout mice failed to show LH surges in response to estradiol but retained responsiveness to the negative feedback effects of estradiol [111]. Homozygous ERβKO female mice demonstrated subnormal fertility and had slightly elevated basal LH levels which suggests defective estrogen negative feedback [122]. These results indicate that estrogen negative feedback actions can be mediated by mechanisms that are independent of ERα and thus these other pathways may normally function as parts of the negative feedback mechanism. The fact that the ERβ is expressed in GnRH neurons suggests the physiological relevance and raises the possibility

of the direct role of the ERβ in feedback regulation. Thus, in this dissertation, I define one of the mechanisms present in negative feedback of E2.

Metabolic signals

Reproduction is an energy-demanding process which is related to the metabolic state of the body. The reproductive success of an individual is tightly linked to the nutritional state. Both obesity and malnutrition have been reported to disrupt reproduction. This means, if the correct utilization of metabolic resources is not ensured (as in the case of anorexia nervosa), or on the contrary, if there is a constant energy surplus available (such as obesity) the body must serve its priorities to obtain the physiological state ensuring survival. For instance, neuronal activity or blood circulation cannot be compromised, whereas thermoregulation, or growth can be reduced in a somewhat wider scale in metabolic stress.

This reproductive-metabolic connection requires a coordinated action of many central and peripheral regulators. Changes in energy homeostasis trigger fluctuations in hormonal (for example leptin, insulin and ghrelin) and nutritional (for instance glucose, lipids) signals that feedback mainly to the brain regions which regulates metabolism and fertility. These actions modulate function of different levels of the HPG axis, enabling the close cooperation between the energy level and gonadal function [123] (Figure 3.). However, the effects of metabolic signals on GnRH neurons are mostly unknown.

The adipocyte hormone *leptin* is a signal of energy sufficiency, suppresses feeding and increases energy expenditure [124]. The central effect of leptin was demonstrated by the experiment in which leptin receptor was ablated in a forebrain specific manner and as a result of it, mice became obese and infertile [125]. However, it was thought that GnRH neurons may not be targeted directly by leptin, because there was no conclusive evidence for leptin receptor expression in GnRH neurons [125]. Nonetheless, there is also a study in the literature claiming that leptin may act directly on GnRH neurons to alter postsynaptic responsiveness to GABA [46]. Nevertheless, there is no doubt about the physiological importance of leptin in the regulation of reproductive functions.

Insulin is another factor which conveys information between metabolic and reproductive system. Insulin is a pancreatic peptide hormone produced by beta cells of the pancreatic islets which modulates glucose homeostasis and body weight regulation. It has been shown, that neuron-specific insulin receptor-knockout mice exhibit hypogonadism [126]. Although insulin receptor is expressed in GnRH neurons, activation of it did not trigger any insulin-induced signal transduction pathway

such as phospho-Akt or phospho-extracellular-signal-regulated kinase 1/2 in GnRH neurons [127]. Another study has presented that specific deletion of insulin receptor in GnRH neurons did not modify puberty or fertility [128]. These data suggest that central insulin signaling on reproduction is not mediated directly via GnRH neurons.

In contrast to leptin and insulin, *ghrelin* is a signal of energy deficiency. Ghrelin is predominantly produced by the stomach but ghrelin-expressing neurons have also been detected in the central nervous system and specifically in the hypothalamus [129]. Several studies have demonstrated the negative effect of ghrelin at different regions of the HPG axis. It has been shown that ghrelin is able to suppress GnRH pulsatility and gonadotropin release [130]. First, ghrelin acts indirectly on GnRH neurons [131], on the other hand, our laboratory has provided evidence for direct inhibitory action of ghrelin on GnRH secreting neurons [132]. These studies clearly demonstrated that ghrelin plays a pivotal role in suppressing the reproductive axis during low energy conditions.

Glucagon-like peptide-1 signaling

Another candidate that responsible for transmitting metabolic information to GnRH neurons is the glucagon-like peptide-1 (GLP-1), which is one of the main target molecules of our study. Glucagon-like peptide-1 was originally described as a gut-derived peptide converted from the preproglucagon gene product and secreted from intestinal L-cells in response to food intake [133]. Being an incretin hormone GLP-1 is responsible for the control of insulin release following food intake from β cells of pancreatic islet in a glucose-dependent manner [134]. The mechanisms of GLP-1 controlling energy intake and nutrient assimilation are broad: enhances satiety, reduces food intake [135], inhibits gastric emptying [136], and increases insulin secretion in the presence of glucose [134]. GLP-1 exerts its biological effects by binding to GLP-1 receptor (GLP-1R), which is a member of the class B family of G-protein-coupled receptors [137]. Activation of this receptor is associated with increased intracellular calcium level, inhibition of voltage-dependent potassium currents and activation of gene expression through Erk1/2, protein kinase C, and phosphatidylinositol 3-kinase signaling pathways. GLP-1R signaling also triggers CREB phosphorylation [134]. GLP-1 and Exendin-4 (Ex4) a long lasting agonist of the GLP-1R can cross the blood-brain barrier [138], showing the ability to reach various control centers of homeostasis. The GLP-1 is produced not only in the periphery but also in neurons of the lower brain stem. These neurons are clustered in the nucleus of the solitary tract (NST) and the reticular nucleus of the medulla oblongata [139]. GLP-1 immunoreactive fibers and terminals were observed in various areas of the brain, for example hypothalamus, thalamus, septal regions, cortex and hindbrain (reviewed in: [139, 140]). GLP-1R is also widely expressed in numerous brain regions such as in neurons of the circumventricular organs,

amygdala, medulla oblongata, superior colliculus, NST, hippocampus, cortex [139], and in hypothalamic regulatory centers of glucose homeostasis [141] and feeding behavior [142].

In addition to modulating energy homeostasis, a large body of evidence indicates the regulatory influence of GLP-1 on reproduction. Intracerebroventricular administration of GLP-1 increased the plasma luteinizing hormone level of male rats, and concentration-dependent increase of GnRH was verified from cell clusters of immortalized GnRH-producing GT1–7 neurons [143]. GLP-1 doubled the amplitude of the preovulatory LH surge, changed the estradiol and progesterone levels leading to an increase in the number of mature Graafian follicles and corpora lutea in rats [144]. Experiments with male GLP-1R knockout mice showed reduced gonadal weights in males and delayed the onset of puberty in females [145].

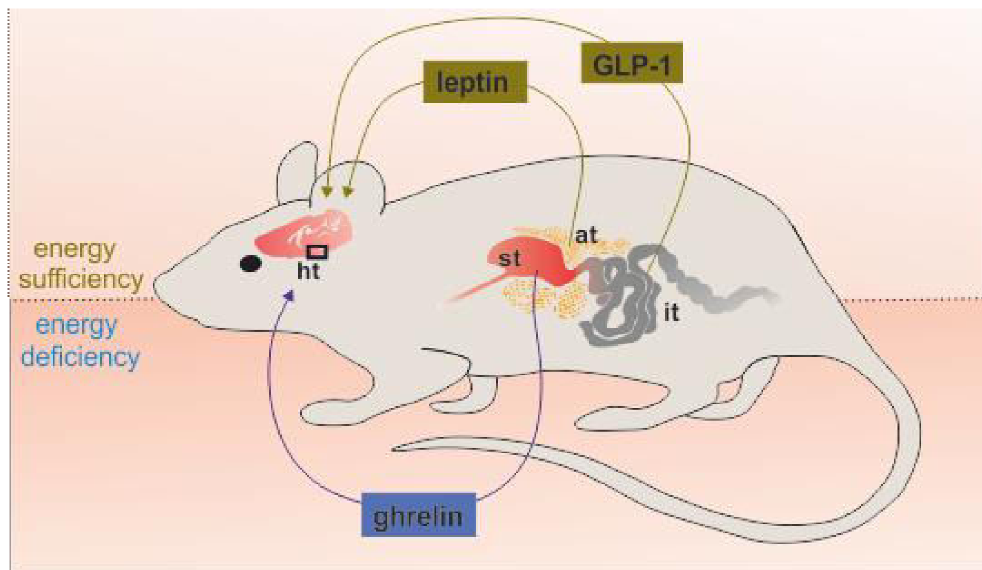

Figure 3. Metabolic factors affecting the hypothalamic regulation of reproduction. Under energy deficiency, the secretion of ghrelin by the stomach increases, the serum levels of leptin and GLP-1 decreases. Under energy sufficiency the GLP-1 production from the intestine rises and the serum level of leptin also increases, but ghrelin production by stomach decreases. These changes affect the different regions of the hypothalamus leading to modulation in the regulation of reproduction. Abbreviations: adipose tissue (at); glucagon-like peptide-1 (GLP-1); hypothalamus (ht); intestine (it); stomach (st).

Since GnRH neurons are key regulators of the HPG axis, any GLP-1-induced alteration of the GnRH neuronal system may have a major impact on reproductive physiology (Figure 3.). Some of the intracellular elements of the GLP-1 activated pathway have already been identified [134]. Elevated cytoplasmic cAMP level in the GT1–7 cells has been proved [143], but the exact target and detailed

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molecular mechanism involved in the downstream actions of GLP-1 in GnRH neurons have not been elucidated yet.

The retrograde neurotransmission

Although GABA is typical inhibitory neurotransmitter in the mature nervous system, GABA is excitatory on GnRH neurons via the ionotropic $GABA_A$ receptor [50-52]. The activity of GnRH neurons is also increased by the activation of ionotropic glutamate receptors [58, 60], therefore, these neurons need alternative mechanisms for their inhibitory regulation. Beside the inhibitory function of $GABA_B$ receptor, the retrograde endocannabinoid signaling can be a candidate to exert inhibitory tone on the excitatory afferents, since this machinery is one of the most widespread and efficient molecular pathway to control neurotransmitter release probability [146].

During a chemical synaptic transmission, a neurotransmitter is released from a presynaptic neuron and it diffuses to the postsynaptic neuron. Then the neurotransmitter binds to its receptor on the postsynaptic membrane and activates it. The postsynaptic neurons might synthetize and release diffusible messenger molecules from their postsynaptic dendrites or cell bodies back to the synaptic cleft. Next, the messenger travels "backwards" to the axon terminal of a presynaptic neuron, where it activates its receptors located in the membrane of the nerve terminals [146]. Activation of retrograde messenger receptors usually causes an alteration in synaptic transmitter release (Figure 4.) [146]. Retrograde signaling is known to play a role in long-term synaptic plasticity [146, 147]. In addition this mechanism has role on the short-term regulation of synaptic transmission [146, 147].

Mediators of the retrograde neurotransmission can be classified into different classes: molecules derived from lipids (endocannabinoids), gases (nitric oxide), conventional neurotransmitters (GABA), peptides (dynorphin), growth factors (brain-derived neurotrophic factor) [147]. Below, two of these retrograde molecules are described in more detail: the endocannabinoids and the nitric oxide.

The endocannabinoid system

The endocannabinoids are endogenous lipid-based messengers. 2-arachidonoylglycerol (2-AG) and anandamide (*N*-arachidonoyl ethanolamine, AEA) are the two most common endocannabinoids synthesized and released "on demand" by neurons in the brain. The endocannabinoid signaling system consists of two cannabinoid receptors, known as the cannabinoid type 1 and type 2 receptors (CB1 and CB2, respectively), their endogenous ligands (AEA, 2-AG) and the synthetizing and degrading enzymes that regulate the endocannabinoid synthesis and degradation [148]. Both

cannabinoid receptors are activated by all endocannabinoids and they are G-protein-coupled receptors. CB1 are abundant in the brain [149], while CB2 is mainly expressed in immune and blood cells, although it has been recently found in various brain areas.

The 2-AG and AEA both are arachidonic acid-containing lipid molecules generated from membrane glycerophospholipids, but their biosynthesis is different [148]. The depolarization of the postsynaptic cell - through different signaling pathways - leads to the activation of the phospholipase C and the generation of diacylglycerol (DAG) from the lipid phosphatidylinositol 4,5-bisphosphate. Next, DAG is converted into 2-arachidonoylglycerol by DAG lipase (DGL) [147]. Anandamide synthetized together with other N-acylethanolamines in a two-step process of Ca^{2+}-dependent N-acyltransferase and N-acylphosphatidylethanolamine-hydrolyzing phospholipase D activity [148]. Classical neurotransmitters and neuropeptides are stored in vesicles in the neurons. In contrast, endocannabinoids are not stored, but synthesized and released *in situ* from cells, followed by immediate action (including tonic one) as signaling molecules (Figure 4.).

Endogenous and exogenous cannabinoids (such as Δ-9-tetrahydrocannabinol, THC, the main psychoactive substance of the *Cannabis sativa* plant) known to modulate several endocrine functions under the control of the hypothalamus, and exert potent negative effects on reproduction in many species, like rodents, primates, and humans [150]. Endocannabinoid administration inhibited LH secretion from the adenohypophysis and reduced the concentration of sex steroids in the blood in both sexes [150]. Expression of CB1 receptors have been described in the hypothalamus, including the preoptic area [151], the main location of GnRH neurons [35]. Moreover, a previous study from our laboratory showed that the release of 2-AG from GnRH neurons caused a reduction in firing rate of GABAergic neurons and as a result a reduced GABAergic neurotransmission [152].

Various signals can modulate the endocannabinoid signaling in GnRH neurons. Contribution of endocannabinoids in GnRH neuron-GABAergic afferent local feedback circuits have been demonstrated and these local circuits can be altered by sex steroids [153]. This suggests the putative involvement of this retrograde signaling mechanism in the manifestation of feedback effects of estradiol on GnRH neurons. Endocannabinoids are also interplay with the modulation of other signals, such as metabolic factors. Farkas and colleagues for example showed that ghrelin decreased the activity of GnRH neurons in an endocannabinoid dependent manner [132, 154].

It has been shown that anandamide can also bind to and activate type-1 transient receptor potential vanilloid (TRPV1) channels in mammals [155]. The TRPV1 is a nonselective cation channel which is wildly expressed in the periphery and in the brain. There is also increasing evidence for the co-

localization of cannabinoid CB1 and TRPV1 [155]. Moreover, anandamide signaling modulates tonic 2-AG signaling via activation of TRPV1 receptors [156], thus, the TRPV1 plays a major role in controlling the endocannabinoid pathway.

The nitric oxide system

The free radical gas nitric oxide (NO) is another retrograde messenger in the central nervous system. The NO is membrane permeant and cannot be stored in neurons, thus it is also synthetized “on demand” from L-arginine by nitric-oxide synthase (NOS) [157]. Increased intracellular calcium levels trigger a cascade of events leading to NOS activation and NO synthesis. Most of the retrograde messenger molecules act via membrane-bound receptors, but since this is a low molecule weight gas, the main target of the NO is the soluble guanylyl cyclase (sGC) located in the cytoplasm [157]. Guanylyl cyclase catalyzes the synthesis of cGMP from GTP which leads to the activation of cGMP-dependent protein kinases (Figure 4.). There are several data about the role of NO in the modulation of reproductive axis at various levels, for example NO synthesis increases with the follicular development, NO regulates the GnRH synthesis and NO has a modulatory effect on sexual behavior [158]. In the central nervous system NOS shows high expression, *inter alia*, in numerous hypothalamic nuclei (for example, suprachiasmatic nuclei, supraoptic nuclei and paraventricular nuclei) and also in the diagonal band of Broca in rats [159], where many GnRH neurons are located [35]. In rats, the LH surge was inhibited by the blockade of NO synthesis [160]. Similar to these experiments using hypothalamic fragments containing the median eminence showed that stimulation of NO release increased the release of GnRH [160]. An *in vivo* study also demonstrated the role of NO in the regulation of the hypothalamic centers of reproduction when a NOS inhibitor was infused into the preoptic region of female mice. The abolishment of NO synthesis and thus NO signaling disrupted the estrous cycle and eventually caused infertility [161]. These data suggest that NO plays a fundamental role in the regulation of GnRH neuron.

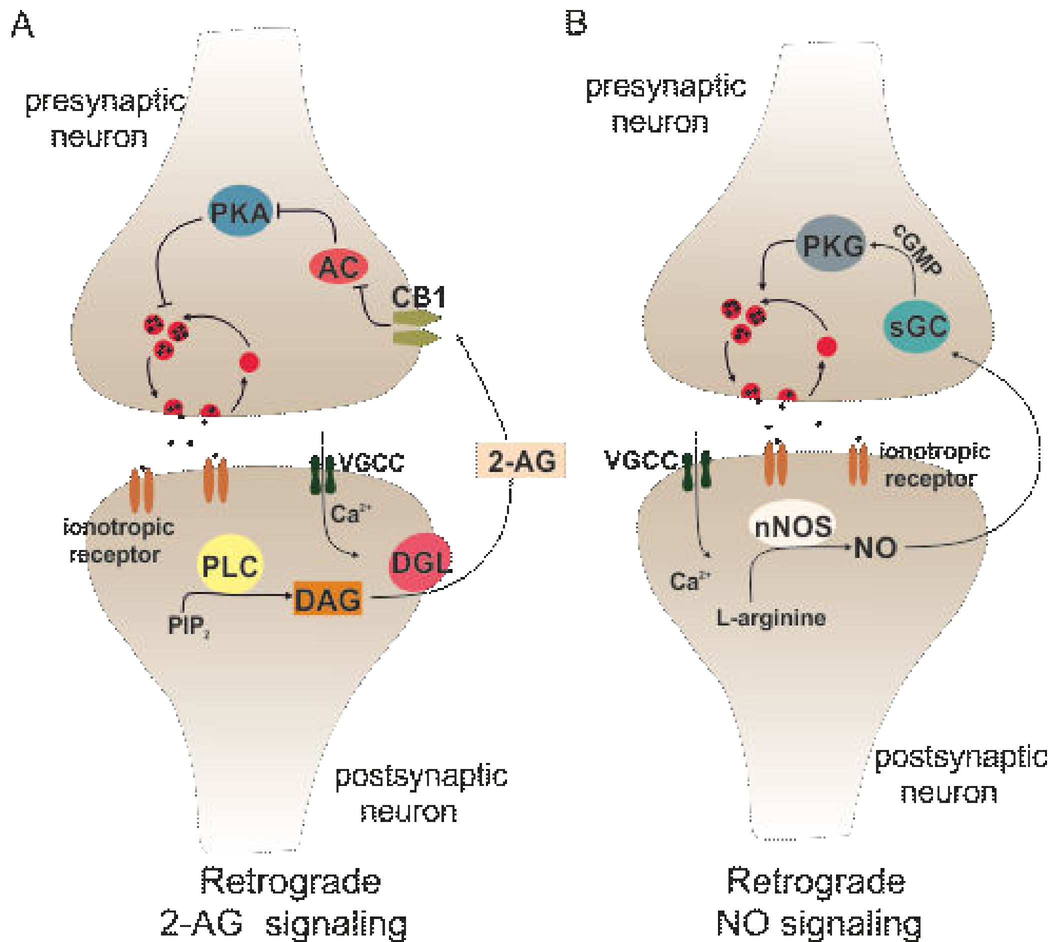

Figure 4. Schematic illustration of retrograde 2-AG and NO signaling pathways. **(A)** The retrograde 2-AG endocannabinoid signaling. The release of neurotransmitter from the presynaptic neuron leads to the depolarization of the postsynaptic neuron which results in elevations in intracellular calcium levels through activation of ionotropic receptors, and/or voltage-gated calcium channels (VGCC). This leads to the activation of PLC, which converts the phospholipid precursor PIP2 into DAG. The DAG is metabolized to 2-AG by DGL. 2-AG moves across the synaptic cleft and activates the CB1 receptors thereby inhibiting the adenylyl cyclase (AC), then PKA which ultimately suppresses the probability of neurotransmitter release. **(B)** The retrograde NO signaling. The neurotransmitter release from the presynaptic neuron activates the postsynaptically located ionotropic receptors. This leads to calcium entry into the postsynaptic neuron, which activates neuronal nitric oxide synthase (nNOS) to produce NO from the NO precursor, L-arginine. The NO moves across the synaptic cleft and activates the soluble guanylyl cyclase (sGC), which activates PKG ultimately increasing the probability of neurotransmitter release. Abbreviations: 2-arachidonoylglycerol (2-AG); adenylyl cyclase (AC); cannabinoid receptor type 1 (CB1); cyclic guanosine monophosphate (cGMP); diacylglycerol (DAG); diacylglycerol lipase (DGL); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); Phosphatidylinositol 4,5-bisphosphate (PIP2); protein kinase A (PKA); protein kinase G (PKG); phospholipase C (PLC); soluble guanylyl cyclase (sGC); voltage-gated calcium channels (VGCC).

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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. 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?

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EXPERIMENTAL PROCEDURES

All the following experiments were carried out with permissions from the Animal Welfare Committee of the Institute of Experimental Medicine Hungarian Academy of Sciences (Permission Number: A5769-01) and in accordance with legal requirements of the European Community (Decree86/609/EEC). All animal experimentation described here was conducted in accord with accepted standards of humane animal care and all efforts were made to minimize suffering. Attention was paid to use only the number of animals necessary to produce reliable results.

Experimental animals

Experiments were performed using adult (postnatal day 50-100), gonadally intact, female or male mice from local colonies bred at the Medical Gene Technology Unit of the Institute of Experimental Medicine. All mice were housed in the same room under same environmental conditions: animals were kept in 12/12h light-dark cycle (lights on at 06:00 h) and temperature controlled environment (22±2°C), with standard rodent chow and tap water available *ad libitum*.

GnRH-green fluorescent protein (GnRH-GFP) transgenic mice (n=228) bred on a C57Bl/6J genetic background were used. In this transgenic animal model, a GnRH promoter segment drives selective GFP expression in about 90% of GnRH neurons [65]. Visualization of GnRH neurons using fluorescence allows the identification of individual GnRH neurons for electrophysiological recordings and subsequent morphological analysis.

In one part of the experiment series, the gonadal phase of the female animals was important. In mice the estrous cycle lasts four days and is characterized as: proestrus, estrus, metestrus, and diestrus. These phases can be determined according to the cell types observed in the vaginal smear. Thus, the estrus cycle of mice was monitored by checking vaginal smears [162-164] and by visual observation of the vaginal opening using recently elaborated method [163, 164]. Metestrous mice were then chosen and used for testing how GnRH neurons react for the treatments during the negative estrogen feedback period.

Brain slice preparation and recording

Mice were decapitated in deep anesthesia by Isoflurane inhalation. All mice were sacrificed between 9 a.m. and 10 a.m. and all recordings performed between 11 a.m. and 4 p.m. period. After decapitation, brain was removed rapidly and immersed in ice cold sodium-free artificial cerebrospinal fluid (Na-free aCSF), which had been extensively saturated with carbogen gas, a mixture of 95% O_2 and 5% CO_2. Carbogen gas is indispensable to maintain oxygen saturation of

the solutions and the stability of pH value (pH 7). Sodium free solution is needed because the synaptic activity should be strongly reduced during slice preparation. In this solution, the low sodium concentration reduces presynaptic firing and glutamate release probability which otherwise would trigger sodium influx, water intake and subsequent swelling of cells leading to poor survival of neurons in the preparation. Thus, the composition of the solution helped to inhibit neuronal activity related to the extreme glutamate release and minimizing spontaneous activity and cell death. The temperature around the freezing point (2-4 °C) of the solution also contributed to the survival of the neurons during the sectioning. The Na-free solution contained the following components (in mM): saccharose 205, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 5, NaH_2PO_4 1.25, $CaCl_2$ 1, and glucose 10. The osmolarity of the solution was adjusted to 300 mOsm (Osmomat 3000, Gonotec GmbH, Germany).

Hypothalamic blocks were dissected and 250 µm-thick coronal slices containing the medial septum/preoptic area were prepared with a VT-1000S Vibratome (Leica GmBH, Germany) in ice-cold oxygenated Na-free aCSF. The slices were then transferred into normal aCSF (in mM): NaCl 130, KCl 3.5, NaH_2PO_4 1.25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 26, glucose 10, osmolarity adjusted to 300 mOsm saturated with carbogen gas and were incubated for 1 hour to be equilibrated. Electrophysiological recordings were carried out at 33°C, during which the brain slices were oxygenated in aCSF with carbogen gas. Axopatch 200B patch clamp amplifier, Digidata-1322A data acquisition system, and pCLAMP 10.4 software (Molecular Devices Co., CA, US) were used for electrophysiological recordings. Cells were visualized with a BX51WI infrared-differential interference contrast microscope (Olympus Co., Japan) installed on an anti-vibration table (Supertech Kft, Hungary).

Patch electrodes (OD=1.5 mm, thin wall; Hilgenberg GmbH, Germany) were pulled with a Flaming-Brown P-97 puller (Sutter Instrument Co., CA). The resistance of the patch electrodes was 2–3 MΩ. GnRH-GFP neurons in the close proximity of organum vasculosum of the lamina terminalis (OVLT, Bregma 0.49-0.85 mm [165]) were identified by brief illumination at 470 nm using an epifluorescent filter set, based on their green fluorescence, typical fusiform shape, and characteristic topography (Figure 5.) [65]. After control recordings (5 min), the slices were treated with various drugs (see below) and the recordings continued for a subsequent 10 min.

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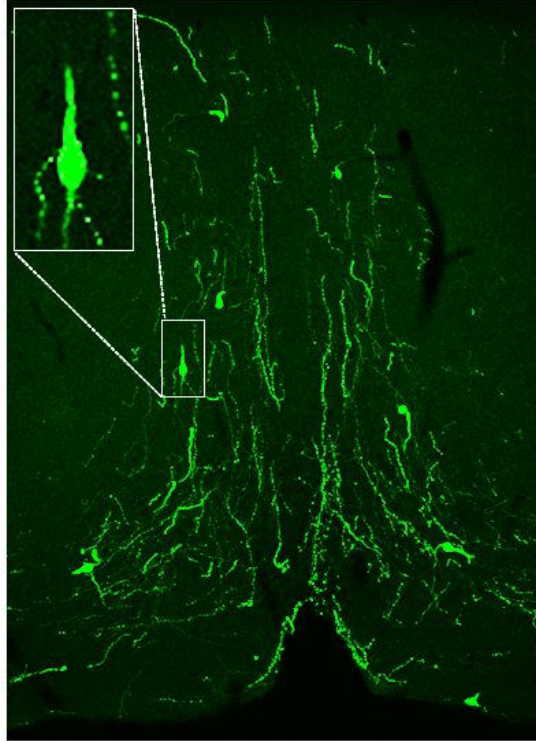

Figure 5. GnRH-GFP neurons and fibers in the organum vasculosum of the lamina terminalis. Visualization of GnRH neurons using fluorescence permits the identification of individual GnRH neurons for electrophysiological recordings and subsequent morphological analysis. Courtesy of Dr. Csaba Vastagh, IEM, HAS Laboratory of Endocrine Neurobiology

Whole-cell patch clamp experiments

Currently, the most widely used method for studying the electrophysiological properties of biological membranes and the currents that flow through their ion channels is the patch clamp technique [166]. In various configurations, this technique permits experimenters to record and manipulate the currents that flow either through ion channels or those that flow across the whole plasma membrane. Patch clamp technique can even allow low noise measurements of the currents passing through a couple of ion channels, by isolating a small patch of the membrane, which sometimes can contain solely a single channel. Here, a high-resistance (“giga ohm”) seal is formed between the pipette and the membrane of the cell. In the experiments whole-cell configuration of

the patch clamp methods was used. This means that the membrane within the pipette is ruptured while the gigaseal is still maintained. The main advantage of this method is the ability to manipulate of ionic or other composition of the intracellular milieu to aid isolation and detection of conductances via specific ion channels.

During whole-cell patch clamp experiments spontaneous and miniature postsynaptic currents were measured. Spontaneous postsynaptic currents (sPSC) are currents generated via mainly by action potential dependent presynaptic release of neurotransmitters in the absence of experimental stimulation. Miniature postsynaptic currents (mPSC) are currents observed in the absence of presynaptic action potentials; they are thought to be the response that is elicited by random release of neurotransmitter vesicles.

The parameters of the measurements were the following: during sPSC and mPSC measurements in GnRH neurons the cells were voltage clamped at -70 mV holding potential. The voltage clamp technique allows to "clamp" the cell potential at a chosen value, make it possible to measure how much ionic current crosses through the membrane of the cell at any given voltage values. Before the recording, pipette offset potential, series resistance and capacitance were compensated. Cells with low holding current (<50 pA) and stable baseline were used exclusively. Input resistance, series resistance, and membrane capacity were also measured before each recording by using 5 mV hyperpolarizing pulses. To ensure consistent recording qualities, only cells with series resistance <20 MΩ, input resistance >500 MΩ, and membrane capacity >10 pF were accepted. The intracellular pipette solution contained (in mM): HEPES 10, KCl 140, EGTA 5, $CaCl_2$ 0.1, Mg-ATP 4 and Na-GTP 0.4 (pH 7.3, osmolarity adjusted to 300 mOsm).

The postsynaptic current measurements were carried out with an initial control recording (5 min), then low physiological concentration of 17β-estradiol (E2, 10 pM), the GLP-1 analog Exendin-4 (1 μM), the NO-donor L-arginine (1 mM), the selective ERα agonist PPT (10 pM), the selective ERβ agonist DPN (10 pM) or the selective GPR30 receptor agonist G1 (10 pM) was added to the aCSF in a single bolus onto the slice in the recording chamber and the recording continued for a subsequent 10 min.

When the cannabinoid receptor type 1 inverse agonist AM251 (1 μM), the non-selective estrogen receptor antagonist Faslodex (1 μM), the ERβ antagonist PHTPP (1 μM), the NO-synthase (NOS) inhibitor L-NAME (100 μM), the GLP-1 receptor antagonist Exendin-3(9-39) (1 μM) or the nNOS inhibitor NPLA (1 μM) were used, they were added to the aCSF 10 min before starting the experiments and then they were continuously present in the aCSF during the electrophysiological recordings.

Intracellularly applied drugs, such as diacylglycerol lipase inhibitor tetrahydrolipstatin (THL, 10 μM), the membrane impermeable G-protein inhibitor GDP-β-S (2 mM), the membrane impermeable NO-scavenger CPTIO (1 mM), the transient receptor potential vanilloid 1 (TRPV1) antagonist AMG9810 (10 μM), NPLA (1 μM), or the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH) inhibitor PF3845 (5 μM) were added directly to the intracellular pipette solution. To minimize the spill of the intracellularly applied drugs, the GnRH cells were approached rapidly (< 1 min), and the flow rate of aCSF was increased from 5–6 to 8–9 ml/min. Just before releasing the positive pressure in the pipette, the flow rate was restored to 5–6 ml/min to avoid any mechanical movement of the slice. After achieving whole-cell patch clamp configuration, we waited 15 min to reach equilibrium in the intracellular milieu before starting recording.

In the experiments where any spike-mediated release of substances was to be inhibited, firing was blocked by adding the voltage-sensitive Na-channel inhibitor TTX (660 nM) to the aCSF 10 min before mPSCs or V_{rest} were recorded. The mPSCs recordings conditions used in our experiments were related to the conditions in which $GABA_A$-R activation occurs [46, 152], interestingly this GABAergic input via $GABA_A$-R is excitatory on GnRH neurons [50, 167, 168]. Nevertheless, it is important to note that GABA inhibits GnRH neurons via $GABA_B$-receptors [50, 169].

Resting potentials were recorded using current-clamp method. The current clamp technique records the membrane potential while injecting current into the cell through the recording electrode. This shows us the cell response when electric current enters the cell, therefore how neurons respond to substances that act by opening membrane ion channels. V_{rest} measurements were carried out at 0 pA holding current.

Loose-patch clamp experiments

In this type of recording, the pipette is pushed to the membrane not tightly but loosely without the formation of a tight gigaseal connection, and there is no direct exchange of cytoplasm and intracellular fluid. The action currents, which underlie action potential firing, can be recorded with this configuration. The advantage of the loose-patch technique is that the composition of the cytoplasm is not influenced, and the activity pattern of a cell can be observed for long time (even for hours) without changing the intracellular milieu. These experiments were carried out at 33 °C, pipette potential was set at 0 mV, pipette resistance 1–2 MΩ, and resistance of loose-patch seal varied between 7–40 MΩ. The composition of the pipette solution mimicking the extracellular milieu that contained the following (in mM): NaCl 150, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.3, HEPES 10, and glucose 10 (pH 7.3, osmolarity adjusted to 300 mOsm). Measurements were carried out with

an initial control recording (5 min), then E2 (10 pM) or Exendin-4 (100 nM – 5 μM) was added to the aCSF in a single bolus onto the slice in the recording chamber and the recording continued for a subsequent 10 min. In experiments to investigate the involvement of the GLP-1 receptor, its antagonist Exendin-3(9-39) (1 μM) was added to the aCSF 10 min before adding Exendin-4. The antagonists were continuously present in the aCSF during the electrophysiological recording.

Chemicals and reagents

Table 1. The chemicals, agonists and antagonists used

Name	*Effect*	*Concentration*	*Producer, Cat. No.*	*References*
17β-estradiol (E2)	non-selective estrogen receptor agonist	10 pM	Sigma, MO, US *# E2758*	[8, 100, 170]
AM251	cannabinoid receptor type-1 inverse agonist	1 μM	Tocris, UK *# 1117*	[132, 152, 156]
AMG9810	transient receptor potential vanilloid 1 antagonist	10 μM	Sigma, MO, US *# A2731*	[171-173]
CPTIO	nitric oxide scavenger	1 mM	Sigma, MO, US *# C221*	[174, 175]
DPN	selective estrogen receptor β agonist	10 pM	Tocris, UK *# 1494*	To keep comparability, all ER agonists were used at the same 10 pM concentration.
Exendin-3(9-39)	GLP-1 receptor antagonist	1 μM	Tocris, UK *# 2081*	[176-178]
Exendin-4	GLP-1 receptor agonist	100 nM – 5 μM	Tocris, UK *# 1933*	[176-178]
Faslodex (ICI 182,780)	non-selective estrogen receptor antagonist	1 μM	Tocris, UK *# 1047*	[100, 152]
G1	selective GPR30 receptor agonist	10 pM	Tocris, UK *#3577*	To keep comparability, all ER agonists were used at the same 10 pM concentration.
GDP-β-S	G-protein inhibitor	2 mM	Sigma, MO, US *# G7637*	[179-181]

L-arginine	nitric oxide donor	1 mM	Sigma, MO, US # *A8094*	[174, 182]
L-NAME	nitric oxide synthase inhibitor	100 µM	Sigma, MO, US # *N5751*	[174, 183]
NPLA	neuronal nitric oxide synthase inhibitor	1 µM	Tocris, UK # *1200*	[184-186]
PF3845	fatty acid amide hydrolase inhibitor	5 µM	Sigma, MO, US # *PZ0158*	[156]
PHTPP	selective estrogen receptor β antagonist	1 µM	Tocris, UK # *2662*	[187, 188]
PPT	selective estrogen receptor α agonist	10 pM	Tocris, UK # *1426*	To keep comparability, all ER agonists were used at the same 10 pM concentration.
THL (tetrahydro-lipstatin/orlistat)	diacylglycerol lipase inhibitor	10 µM	Tocris, UK # *3540*	[132, 152]
TTX (tetrodotoxin)	selective blocker of voltage sensitive Na^+ channels	660 nM	Sigma, MO, US # *T8024*	[132, 152]

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

Using patch-clamp technique, the electrical properties of neurons can be studied. Nevertheless, it also enables harvesting mRNA from a single neuron to study gene expression at the single-cell level.

Collecting mRNA for RT-PCR from neurons is a well-established method, including GnRH neurons [66, 189-193]. In our study, the mRNA content of individual GnRH neurons of male mice was harvested using sterile patch clamp pipette. Patch pipettes were pulled from capillaries sterilized at 180° C for 6 h and filled with sterile intracellular pipette solution. The solution consisted of the following chemicals (in mM) HEPES 10, K-gluconate 130, KCl 10, NaCl 10, EGTA 1 and $MgCl_2$ 0.1 (pH 7.3, osmolarity adjusted to 300 mOsm). The resistance of the patch electrodes was 2–3 MΩ. Harvesting of mRNA samples from GnRH-GFP neurons of acute brain slices was carried out in carbogen saturated aCSF at 33°C. After achieving the whole-cell patch clamp configuration, the cytoplasm was harvested by applying gentle negative pressure under visual control with extra care to avoid any glial RNA contamination with the protocol suggested by Fuzik et al. [194]. Cytoplasmic samples were collected by breaking the pipette tip into PCR tubes kept on dry ice [132, 195].

The subsequent PCR detection of Glpr1 and Nos1 RNAs (including cDNA synthesis, pre-amplification and real-time PCR) were done by my fellow colleague Csaba Vastagh. Briefly, ViLO SuperScript III cDNA reverse transcription (RT) kit (Thermo Fisher Scientific, MA, US) was used to reverse transcribe the cytoplasm directly in 20 μl reactions. The intracellular pipette solution was used as negative control. The resulting cDNA was used as template for the subsequent pre-amplification reaction using the Preamp Master Mix kit (Thermo Fisher Scientific, MA, US) according to the manufacturer's protocol. The pre-amplification products were then used in a 1:10 dilution (in 0.1x TE buffer) before use in qPCR. Real-time PCR was carried out using inventoried TaqMan gene expression assays (Thermo Fisher Scientific, MA, USA) using the following primers: Gnrh1 (assay ID: Mm01315604_m1), Glp1r (Mm00445292_m1), Nos1 (Mm01208059_m1), glial fibrillary acidic protein (GFAP) (Mm01253033_m1) and a housekeeping gene Gapdh (Mm99999915_g1). Each assay contained of a FAM dye-labeled TaqMan MGB probe and two primers. qPCR conditions were as follows: 2 min at 50 °C and 20 sec at 95 °C, followed by 40 cycles of 3 sec at 95 °C and 30 sec at 60 °C using the ViiA 7 real-time PCR platform (Thermo Fisher Scientific). All cDNA samples were checked for GFAP mRNA expression and only GFAP-negatives were used in the analysis of the expression of Nos1 in order to avoid glial contamination.

In order to successfully detect Glp1r in RT-PCR experiments, three pooled samples from three mice were used. Each pooled sample contained cytoplasm of 10 GnRH neurons. Individual GnRH neurons were used (a total number of 30 separated neurons from five animals) to investigate Nos1 expression.

Statistical analysis

Each experimental group contained 8–18 recorded cells from six to nine animals in the electrophysiological measurements. Responding cells in the E2-related experiments were defined according to definition of Chu et al. [100] with slight modification: cells were considered as responding ones if any negative change was detected in their frequency. Recordings were stored and analyzed off-line. Mean firing rate, sPSC and mPSC frequency were calculated as number of spikes divided by the length of the respective period (5 min "baseline value" and 10 min "agonist period", respectively). In GLP-1-related experiments bursts were defined according to Lee et al. [196]. In these experiments burst frequency was calculated by dividing the number of bursts with the length of the respective period. Intraburst frequency calculated by dividing the number of spikes with the length of the respective burst. Percentage changes resulted from drugs were calculated by dividing the value to be analyzed before (5 min) and after (the subsequent 10 min) the respective agonist administration. Each neuron served as its own control when drug effects were evaluated.

Event detection was performed using the Clampfit module of the PClamp 10.4 software (Molecular Devices Co.). Group data were expressed as mean ± SEM and percentage change in the frequency of the PSCs due to the application of various drugs was calculated. Statistical analyses were carried out using Prism 3.0 (GraphPad Software, Inc., GraphPad). In E2-related experiments statistical significance was analyzed using Kruskal-Wallis test followed by Dunns post-test for comparison of groups. In GLP-1-related experiments statistical significance was analyzed using one-way ANOVA followed by Newman-Keuls post-test. We considered as significant at $p < 0.05$ (i.e. 95% confidence interval).

RESULTS RELATED TO THE ESTRADIOL EFFECT DURING NEGATIVE FEEDBACK

Experiments concerning negative estrogen feedback period were carried out on adult, gonadally intact, metestrous female mice. During this gonadal stage, the concentration of the estradiol is the lowest in the blood and the negative feedback effect of estradiol takes place.

Estradiol significantly decreases the firing rate and frequency of spontaneous and miniature postsynaptic currents in GnRH neurons of metestrous female mice

I examined the effects of E2 on GnRH neurons of metestrous female mice using loose-patch experiments. In line with the original findings of Chu et al. [100], E2 at low physiological concentration (10 pM) diminished the action current firing activity of GnRH neurons (Figure 6.).

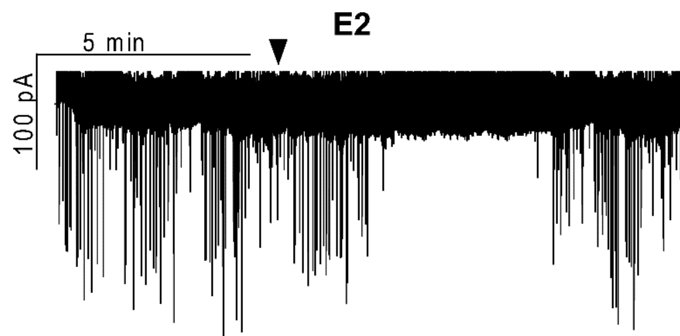

Figure 6. Effect of estradiol on the action current firing of GnRH neurons of metestrous female mice (a representative recording). Application of 10 pM E2 resulted in a significant decrease in the frequency of the action current firing on GnRH neurons. Arrowhead shows the onset of E2 administration.

Positive correlation between the firing rate and the frequency of GABAergic PSCs in GnRH neurons has been well established in the literature [64, 101, 132, 152], suggesting that a decrease similar to the one observed in firing might be found in PSCs. Whole-cell patch clamp method was used to investigate the action of E2 on sPSCs in GnRH neurons. In our experiments, the mean stochastic change in the frequency of the non-treated "responding" GnRH neurons was 83.7±3.8% which was used later as control value for the statistical analysis. Administration of E2 at 10 pM concentration resulted in a significant decrease in the sPSCs in 9 of 18 of examined GnRH neurons (49. 6±7.6%

of the baseline value 1.2±0.4 Hz; $p<0.05$) (Figure 7. A, C), whereas no change in amplitude of the sPSCs was observed suggesting role of a presynaptic process. E2 decreased the frequency of sPSCs within 1-2 minutes indicating that this phenomenon was due to the rapid, non-genomic effect of E2.

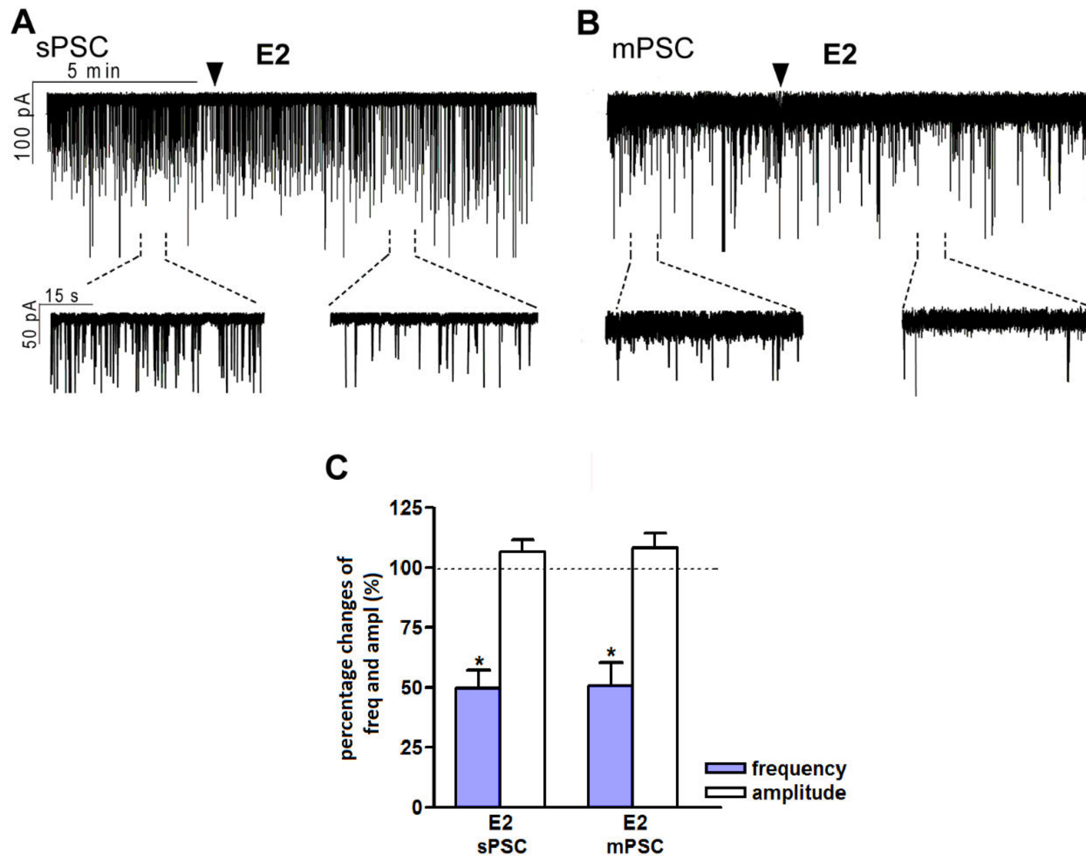

Figure 7. Effect of estradiol on the spontaneous and miniature postsynaptic currents of GnRH neurons of metestrous female mice. **(A)** E2 at low physiological concentration (10 pM) significantly decreased the frequency of the sPSCs with no change in the mean amplitude. One-minute-long periods of the recording before and after application of E2 are depicted under the recording. **(B)** E2 (10 pM) also decreased the frequency of the mPSCs, while the amplitude did not change. Representative zoomed intervals of the recording show the difference between the control vs. treated periods. **(C)** Bar graph summarizing the percentage changes in the frequency and the amplitude of the sPSCs and mPSCs resulted from E2 treatment. Arrowhead shows the onset of E administration. *$p<0.05$ as compared to the mean of stochastic control.

This result raised the question whether the effect of E2 on GnRH neurons is direct or indirect. To examine this, mPSCs were recorded in the presence of TTX (660 nM) to inhibit propagation of action potentials during whole-cell patch clamp recording. The main excitatory mediator of fast

synaptic transmission on GnRH neuron is GABA via $GABA_A$ receptor ($GABA_A$-R) and the recorded mPSCs observed under the circumstances used in our experiments were exclusively GABAergic [50, 152, 167, 168, 197]. Furthermore, our experiments showed that picrotoxin (100 µM) eliminated the mPSCs, demonstrating that the recorded mPSCS are GABAergic via $GABA_A$-R (not shown). Administration of E2 at low physiological concentration (10 pM) resulted in a significant decrease in the mean frequency of the mPSCs in 8 of 12 examined GnRH neurons. Frequency of the mPSCs declined to 50.7±9.6% (compared to the baseline value 2.2±0.4 Hz; $p<0.05$) (Figure7. B, C), while amplitude of the mPSCs showed no significant change (Figure 7. C, Table 2.). These results suggest that the effect of E2 is direct on GnRH neurons of metestrous female mice.

Table 2. Changes in spontaneous and miniature postsynaptic current amplitude on GnRH neurons. Table shows the mean amplitude before drug administration and the percentage change in this parameter resulted from the drug administrations.

		Amplitude (control; pA)	*Amplitude change (% of the control)*
sPSCs	E2	-37.0±5.0	104.7±3.6
mPSCs	E2	-31.6±3.0	102.4±5.3
	Faslodex *(non-selective ER antagonist)* + E2	-27.8±2.4	103±2.2
	AM251 *(CB1 inverse agonist)* + E2	-24.7±2.2	101.3±3.5
	THL *(DGL inhibitor)* + E2	-30.0±4.3	93.8±3.4
	DPN *(selective ERβ agonist)*	-36.6±6.7	102.9±5.1
	PHTPP *(selective ERβ antagonist)* + E2	-26.6±3.0	100.1±4.0
	AM251 *(CB1 inverse agonist)* + DPN *(selective ERβ agonist)*	-32.7±10.6	97.1±6.0
	PPT (*selective ERα agonist)*	-28.7±3.1	98.7±6.7
	G1 *(selective GPR30 receptor agonist)*	-33.0±2.6	98.8±2.2

The direct rapid effect of estradiol requires estrogen receptor beta

Since the administration of E2 at low physiological concentration (10 pM) resulted in a significant decrease in the sPSCs and the mPSCs of GnRH neurons, we were curious about the receptor involved in this signaling. Our primary candidates were the estrogen receptors. To demonstrate

involvement of the ERs in the action of E2 on GnRH neurons, the non-selective ER antagonist Faslodex (1 μM) was used in the presence of TTX. After E2 application, in the presence of Faslodex, the mean frequency of mPSCs (84.1±4.0% of baseline value 0.5±0.1 Hz; n=6) was significantly higher ($p<0.05$) compared to the value measured with E2 alone (Figure 8.). The amplitude did not change during the treatments (Table 2.). This result indicated that E2 acts via estrogen receptor(s) in this rapid effect.

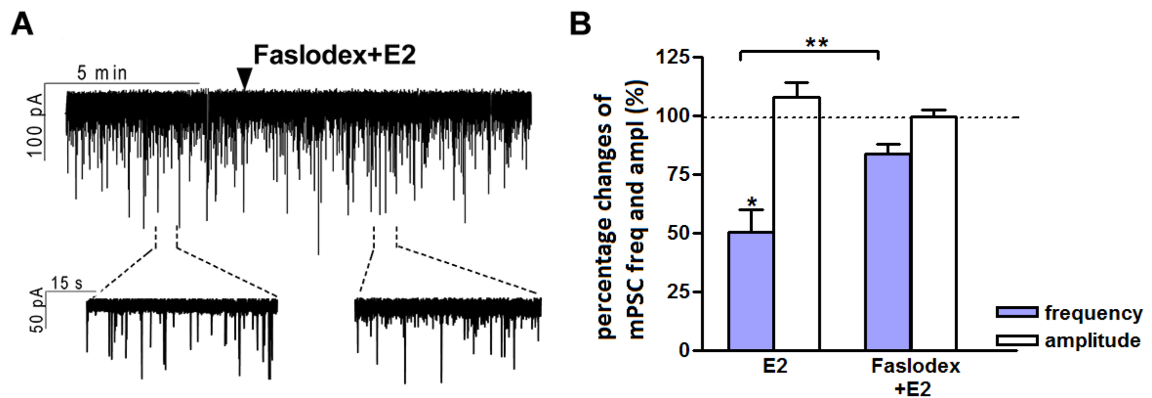

Figure 8. Effect of estradiol on the miniature postsynaptic currents of GnRH neurons in the presence of non-selective estrogen receptor antagonist. **(A)** Pretreatment of the brain slice with the non-selective ER antagonist Faslodex (1 μM) inhibited the effect of E2 (10 pM) on the mPSCs. One-minute long periods of the recording before and after application of the agonist are illustrated under the recordings. **(B)** Bar graph summarizing the percentage changes in the frequency and the amplitude of the mPSCs resulted from E2 treatment in the presence of non-selective ER antagonist Faslodex. Inhibition of the effect of E2 could be achieved with antagonizing the ERs. Arrowhead shows the onset of E administration. *$p<0.05$ as compared to the control; **$p<0.05$ as compared to the change evoked by the sole E2 treatment.

The action of E2 at low physiological concentration was rapid as the effect occurred within minutes, suggesting the activation of intracellular signaling pathways via membrane-associated estrogen receptors [85, 86, 88]. ERα and ERβ have already been shown to have plasma membrane coupled forms besides nuclear type ones. Alternatively, GPR30 could also be the source of the rapid signaling events [85, 87]. In order to identify which receptor(s) is/are involved in the mediation of E2 effect on GnRH neurons, we used subtype-selective ER agonists. We began the investigation by examining the putative involvement of ERβ as it is the most-known ER in GnRH neurons [112, 115-117]. The subtype-selective ERβ agonist DPN (and all other ER agonists in the subsequent experiments) was used at the same 10 pM concentration. DPN significantly decreased the mean frequency of the mPSCs in GnRH neurons (60.6±5.1% compared to the baseline value 2.1±0.6 Hz;

n=8; p<0.05) (Figure 9. A, E). The attenuating effect of E2 was significantly abolished (73.0±6.1% of baseline value 0.6±0.1 Hz; n=7; p<0.05) in the presence of the specific ERβ antagonist PHTPP (1 µM) (Figure 9. B, E). These results indicate that ERβ activation is required for the observed rapid effect of E2 in GnRH neurons.

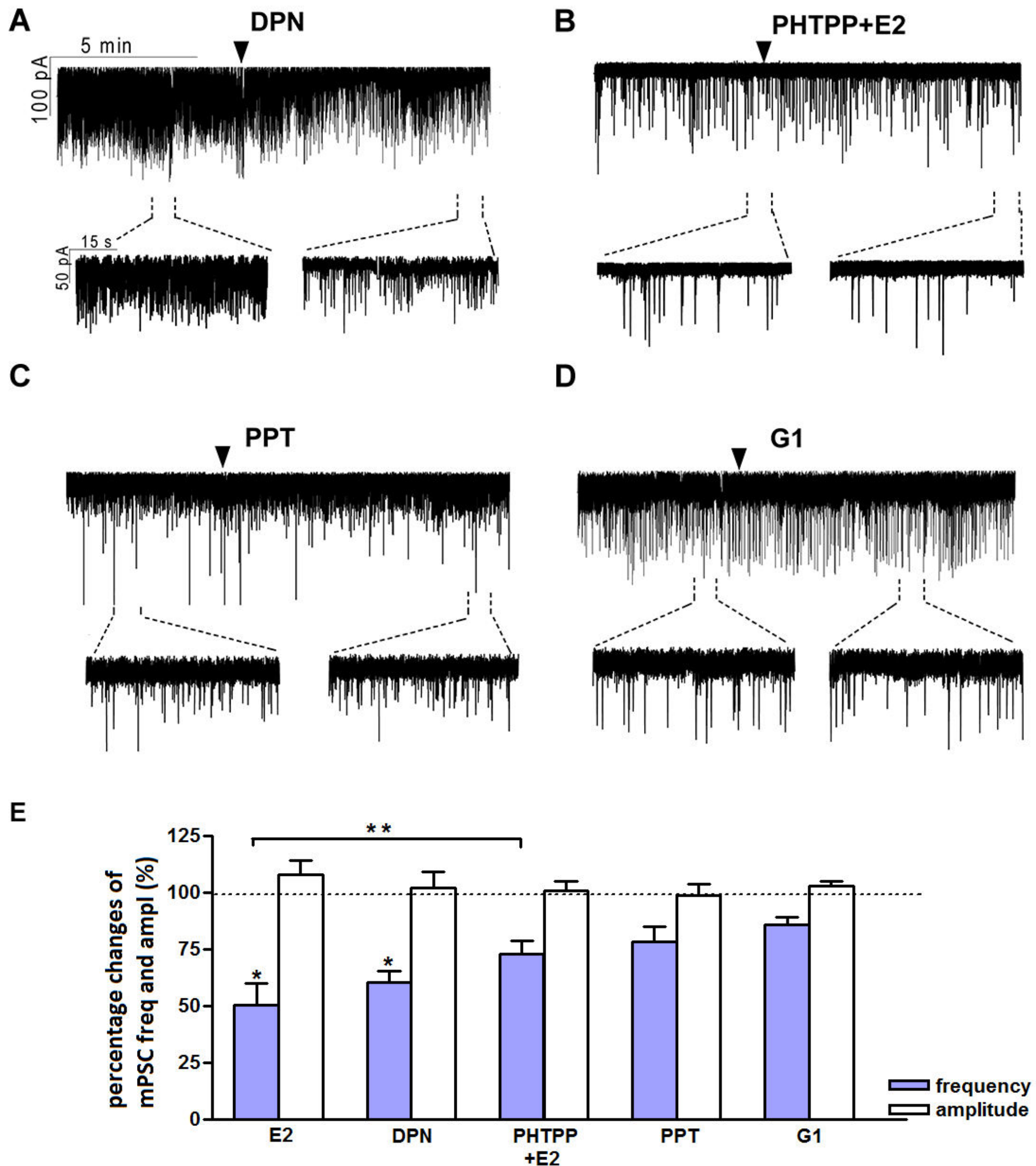

Figure 9. The effect of subtype-selective estrogen receptor agonists and antagonists on the miniature postsynaptic currents in GnRH neurons of metestrous female mice. (A) The subtype-selective ERβ agonist DPN (10 pM) significantly decreased the frequency of mPSCs. **(B)** Pretreatment of the brain slice with the selective ERβ receptor antagonist PHTPP (1 µM) inhibited the effect of E2 (10 pM) on mPSCs. **(C)** The selective ERα agonist PPT (10 pM) was unable to modify the frequency of mPSCs in the recorded

GnRH neurons. **(D)** Similarly, the GPR30 receptor agonist G1 (10 pM) did not modify the frequency of the mPSCs. **(E)** Bar graph summarizing the percentage changes in the frequency and the amplitude of the mPSCs resulted from selective ER agonists and various antagonists. The E2 and the selective ERβ agonist DPN significantly decreased the frequency of mPSCs. Effect of E2 could be inhibited by antagonizing selectively the ERβ by PHTPP. The selective ERα agonist PPT and the GPR30 receptor agonist G1 had no significant effect on the frequency of mPSCs. The amplitude of the mPSCs presented no change in any of the treatments. One-minute long periods of the recording before and after application of the agonist are illustrated under the recordings. Arrowhead shows the onset of E administration. *=$p<0.05$ as compared to the control; **=$p<0.05$ as compared to the change evoked by E2 treatment.

In contrast, the subtype-selective ERα agonist PPT, had no significant effect on the frequency of mPSCs in GnRH neurons (78.7±6.4% of baseline value 2.3±1.2 Hz; n=7; $p>0.05$) (Figure 9. C, E). Similarly, the application of the GPR30 selective agonist G1 (10 pM) could not exert any significant change on the frequency of the mPSCs (86.0±3.5% as compared to the baselined value 0.3±0.1 Hz; n=5; $p>0.05$) (Figure 9. D, E). These data show that ERα and GPR30 have no role in mediating the observed rapid effect of the E2 on GnRH neurons during the negative feedback period. There was no change in the amplitudes during any of the ER agonist treatments (Table 2.).

Retrograde endocannabinoid signaling is involved in estradiol-triggered decrease of miniature postsynaptic currents

Our laboratory has previously shown that endocannabinoid release from GnRH neurons could influence presynaptic neurotransmission to GnRH neurons [152]. Thus, the putative role of the retrograde endocannabinoid signaling mechanism was tested in the mediation of the effect of E2 on GnRH neurons by the CB1 inverse agonist AM251 (1 µM). Pretreatment of the slice (10 min) with this inverse agonist attenuated the decreasing effect of E2 on the frequency of mPSCs (86.9±3.5% of baseline value 0.8±0.2 Hz; n=5; $p<0.05$) on GnRH neurons (Figure 10. A, D), supporting the hypothesis that endocannabinoids were indeed involved in E2-evoked decrease of mPSC frequency.

Two main types of physiological ligands for the cannabinoid receptors are known in the central nervous system: anandamide and 2-AG. To identify which type of endocannabinoid is involved in the acute GnRH neurons, tetrahydrolipstatin (THL) was used. THL is the selective inhibitor of diacylglycerol lipase, the synthesizing enzyme of 2-AG. THL (10 µM) was applied intracellularly via the patch pipette and this pretreatment diminished the effect of E2 on the frequency of mPSCs (88.3±2.0% of baseline value 0.7±0.2 Hz; n=5; $p<0.05$) (Figure 10. B, D.), indicating that 2-AG synthesized by GnRH neurons was involved in the action of E2. The amplitude of the sPSCs and mPSCs presented no change in any of these treatments (Table 2.).

To strengthen our findings regarding the relationship between E2 and endocannabinoid systems, ERβ agonist DPN (10 pM) was used in the presence of AM251. The CB1 inverse agonist also attenuated the action of DPN on mPSCs (82.5±2.6% compared to the baseline value 1.0±0.3 Hz; n=5) (Figure 10. C, D). These results support the idea that retrograde endocannabinoid signaling mechanism is involved in the effect of E2 suppressing GnRH activity when low physiological concentration of estradiol is used.

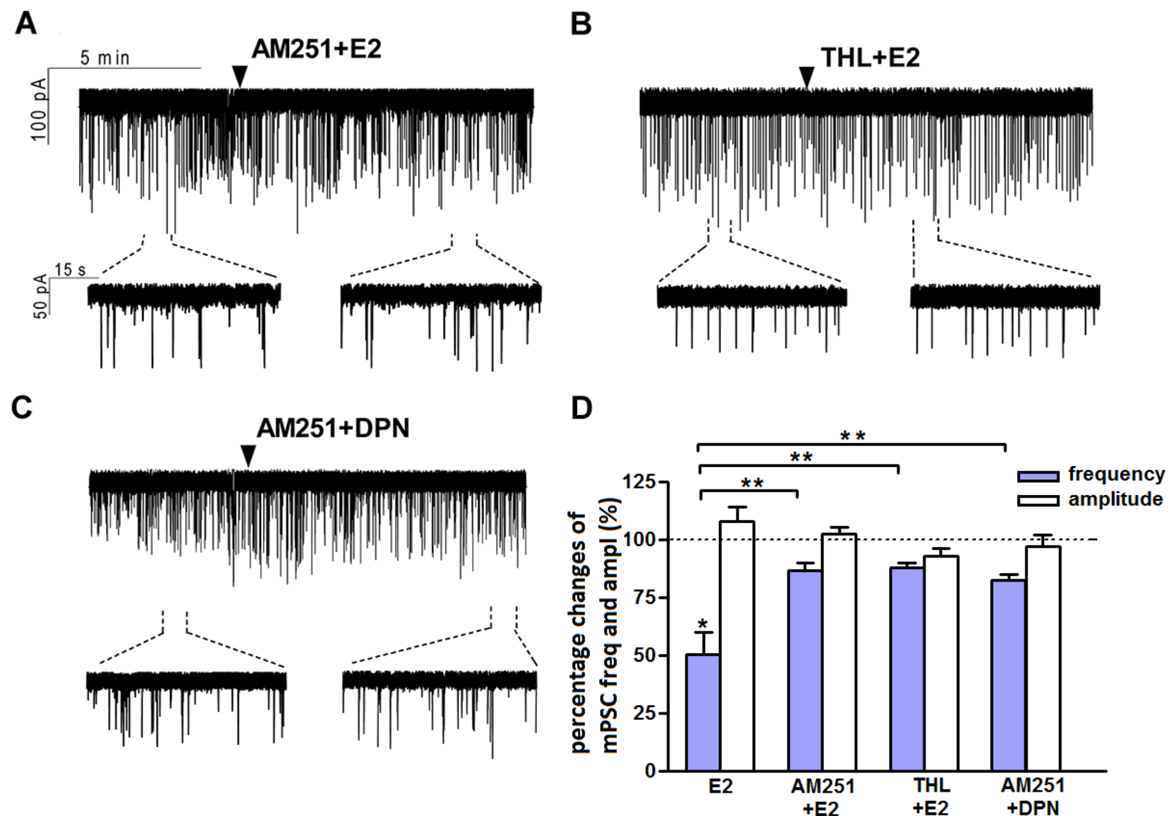

Figure 10. Effect of estradiol and DPN on the miniature postsynaptic currents of GnRH neurons in the presence of endocannabinoid receptor/synthesis blockers. (A) Effect of E2 on the mPSCs was abolished by the pretreatment with CB1 inverse agonist AM251 (1µM). One-minute long periods of the recording before and after application of the agonist are illustrated under the recordings. **(B)** Inhibition of the effect of E2 on the mPSCs could be achieved with intracellularly applied DAG lipase inhibitor THL (10µM). **(C)** ERβ agonist DPN (10 pM) had no significant effect on the frequency of mPSCs in the presence of AM251. **(D)** Bar graph summarizing the percentage changes in the frequency and the amplitude of the mPSCs resulted from E2 treatment in the presence of AM251 and THL. E2 significantly decreased the frequency of mPSCs. Inhibition of its effect could be achieved with antagonizing the CB1 receptors or blocking the intracellular 2-AG endocannabinoid synthesis. Effect of the ERβ agonist DPN was eliminated by the pretreatment with CB1 inverse agonist AM251.The amplitude of the mPSCs did not change in any of the treatments. Arrowhead shows the onset of E administration. *$p<0.05$ as compared to the control; **$p<0.05$ as compared to the change evoked by E2 treatment.

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RESULTS RELATED TO THE GLP-1 EFFECT IN GnRH NEURONS

In this chapter of the results, I show the effect of glucagon-like peptide-1 on GnRH neurons. To avoid the confounding effects of ovarian hormone changes during the estrous cycle, the experiments were performed on adult, gonadally intact, male mice. During our experiments, we used Exendin-4 which is a proven and widely used GLP-1 agonist [176-178].

The GLP-1 agonist Exendin-4 increases the firing rate and the frequency of miniature postsynaptic currents of GnRH neurons via GLP-1 receptor

The GLP-1 receptor agonist Exendin-4 on the function of GnRH neurons was studied by measuring the action current firing. Loose patch recordings revealed that spontaneously not firing - so-called "silent" - GnRH neurons (approximately 25 % of all GnRH neurons) could not be influenced by Exendin-4 administration, hence they were discarded from the subsequent analyses.

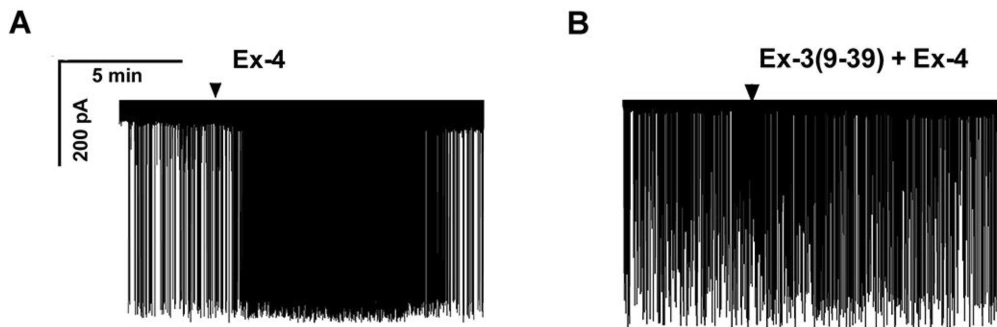

Figure 11. Effect of GLP-1 receptor agonist Exendin-4 on the firing of GnRH neurons of male mice. (A) Exendin-4 (1 μM) increases the firing rate in GnRH neurons. **(B)** Pretreatment of the brain slice with the GLP-1 receptor antagonist Exendin-3(9-39) (1 μM) inhibits the effect of Exendin-4 on firing. Arrowheads show application of Exendin-4.

All the firing GnRH neurons recorded were burst-type neurons. In these neurons Exendin-4 (1 μM) increased the mean firing rate to 434.2±69.9% of the control (n=10; $p<0.05$) (Figure 11. A, Table 3). Note that the effect of Exendin-4 was washed out within about 10 minutes (Figure 11. A). Compared to the control the average number of spikes within a burst increased to 162.3±32.9% (from 3.2±0.2 to 5.2±0.2; $p<0.05$), burst frequency increased to 381.1±65.2% (from 0.07±0.03 Hz to 0.2±0.02 Hz; $p<0.05$), and intraburst frequency increased to 172.4±54.7% (from 6.2±0.7 Hz to 10.7±0.5 Hz; $p<0.05$. Lower concentrations of Exendin-4 (100-500 nM) caused no significant change in the average firing rate (Table 3.). In contrast, a higher dose (5 μM) evoked a robust increase in the firing rate (Table 3.). Therefore, the 1 μM concentration was used in all subsequent

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experiments, in accordance with the doses used in other laboratories on other types of hypothalamic neurons [176].

Table 3. Changes in firing rate in GnRH neurons upon Exendin-4 administration at various concentrations of this agonist. The first column contains firing rate before any drug administration (basal firing rate), the second and third columns provide change in Hz and percentage in firing rate after the single bolus agonist administration. *p<0.05.

Dose	*Basal firing rate (Hz)*	*After agonist Exendin-4*	
		in Hz	*in %*
Ex-4 (100 nM)	0.57 ± 0.19	0.68 ± 0.22	121 ± 52.6
Ex-4 (500 nM)	0.50 ± 0.27	0.59 ± 0.23	118 ± 45.3
Ex-4 (1 µM)	0.52 ± 0.23	2.25 ± 0.18	434 ± 69.9*
Ex-4 (5 µM)	0.61 ± 0.31	3.91 ± 0.22	642 ± 57.1*

To test the involvement of GLP-1 receptor the brain slices were pretreated with the GLP-1R antagonist Exendin-3(9-39) (1 µM). No alteration in the basal firing rate was observed. Nevertheless, this pretreatment fully eliminated the effect of Exendin-4, the mean firing rate showed no change (98.3±8.1% of the control; n=10; p<0.05) (Figure 11. B). Burst parameters showed no change either.

A positive correlation between the firing rate and the frequency of mPSCs in GnRH neurons has already been proven [64, 101, 132, 152], and since Exendin-4 increased firing rate, loose-patch measurements were followed by whole-cell patch clamp recordings. The effect of Exendin-4 was further investigated by examining its action on the mPSCs, administration of Exendin-4 (1 µM) resulted in a significant increase in the mean mPSC frequency in all GnRH neurons studied, reaching 240.7±30.4% of control values (n=10; p<0.05) (Figure 12. A, D). Elevation of the frequency started to disappear after the 10 minutes washout period. Amplitude of the mPSCs however showed no significant alteration (Table 4.). Pretreatment of the brain slice with the antagonist Exendin-3(9-39) (10 min) caused no change in the basal mPSC frequency but abolished the Exendin-4 evoked frequency increase (Figure 12. B, D) providing evidence for involvement of the GLP-1R in the signaling (102.4±13.6 % of the control; n=11; p>0.05).

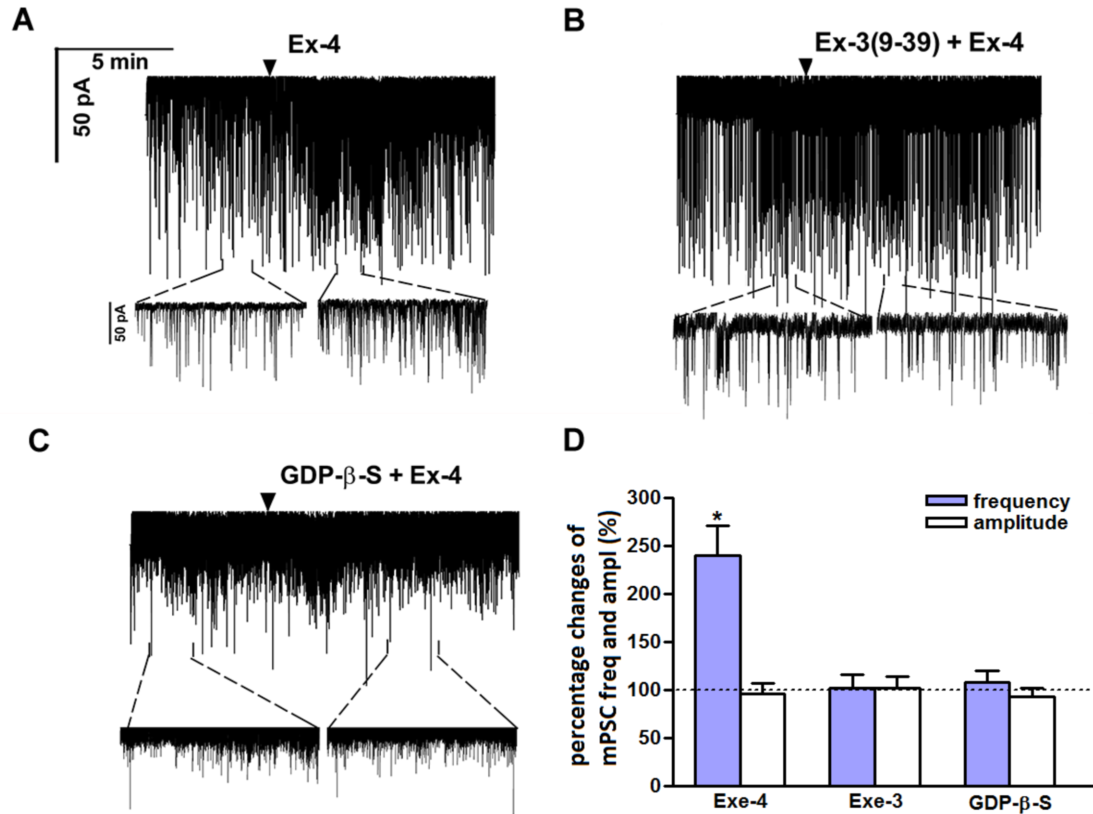

Figure 12. Effect of GLP-1R agonist and antagonist on the miniature postsynaptic currents in GnRH neurons of male mice. (A) Exendin-4 (1 μM) increased the frequency of the mPSCs with no change in the mean amplitude. **(B)** Effect of Exendin-4 on the mPSCs was abolished by the pretreatment with Exendin-3(9-39) (1 μM). **(C)** Exendin-4 was unable to modify frequency of mPSCs when G-proteins were intracellularly blocked by GDP-β-S (2 mM) in the recorded GnRH neuron. **(D)** Bar graph reveals that Exendin-4 significantly elevated the frequency of mPSCs. This effect could be inhibited by Exendin-3(9-39) pretreatment. Full inhibition could be achieved by antagonizing the GLP-1 receptor. The amplitude of the mPSCs did not change in any of the treatments. One-minute-long periods of the recording before and after application of Exendin-4 are drawn under the recordings. Arrowheads show application of Exendin-4. *$p<0.05$.

GLP-1R is a member of the G-protein-coupled receptor family, thus the G-protein blocker GDP-β-S is supposed to inhibit the function of the receptor. GDP-β-S was applied intracellularly to exert its effect exclusively in the recorded GnRH neuron, without affecting surrounding cells. To prove the direct action of Exendin-4 in GnRH neurons, its effect on the mPSCs was further examined in the intracellular presence of the GDP-β-S (2 mM). Intracellular application of GDP-β-S caused no change in the amplitude (Table 4.) but eliminated the effect of the GLP-1R agonist Exendin-4 on the frequency of the mPSCs (108.0±12.0% of the control; n=10; $p<0.05$) (Figure 12. C, D).

Table 4. Changes in miniature postsynaptic currents amplitude on GnRH neurons. The table shows the mean amplitude before Exendin-4 administration and the percentage change in these parameters resulted from the various antagonists or inhibitors.

	Amplitude (control; pA)	***Amplitude change (% of the control)***
Ex3(9-39) *(GLP-1R antagonist)*	-41.2 ± 6.2	96.8 ± 11.5
L-NAME *(nitric oxide synthase inhibitor)*	-35.4 ± 7.6	102.2 ± 12.2
AM251 *(CB1 inverse agonist)*	-32.2 ± 8.8	100.4 ± 11.6
L-NAME *(nitric oxide synthase inhibitor)* + AM251 *(CB1 inverse agonist)*	-39.6 ± 6.7	99.3 ± 8.7
GDP-β-S *(G-protein inhibitor)*	-35.0 ± 6.1	93.7 ± 9.3
CPTIO *(nitric oxide scavenger)*	-42.5 ± 5.5	110.5 ± 15.4
intraNPLA *(neuronal nitric oxide synthase inhibitor)* + AM251 *(CB1 inverse agonist)*	-43.1 ± 7.0	106.4 ± 12.3
L-NAME *(nitric oxide synthase inhibitor)* + AMG9810 *(TRPV1 antagonist)*	-43.3 ± 7.0	106.1 ± 12.3
NPLA *(neuronal nitric oxide synthase inhibitor)* + PF3845 *(anandamide degrading enzyme inhibitor)*	-40.4 ± 5.9	95.4 ± 8.9

RT-PCR experiment was used to examine the expression of the Glp1r in the GnRH neurons of adult male mice. In addition to Gnrh1 mRNA (cycle threshold value of 24.5±0.8), expression of Glp1r mRNA was detected in pooled, patch pipette-harvested GnRH-GFP neuron cytoplasm samples at Ct 32.7±0.4. The cycle threshold value of Gapdh was 22.3±0.1 (Figure 12.). None of the transcripts were detected in the negative control samples. Thus, the RT-PCR confirmed the expression of Glp1r genes in GnRH neurons of mice.

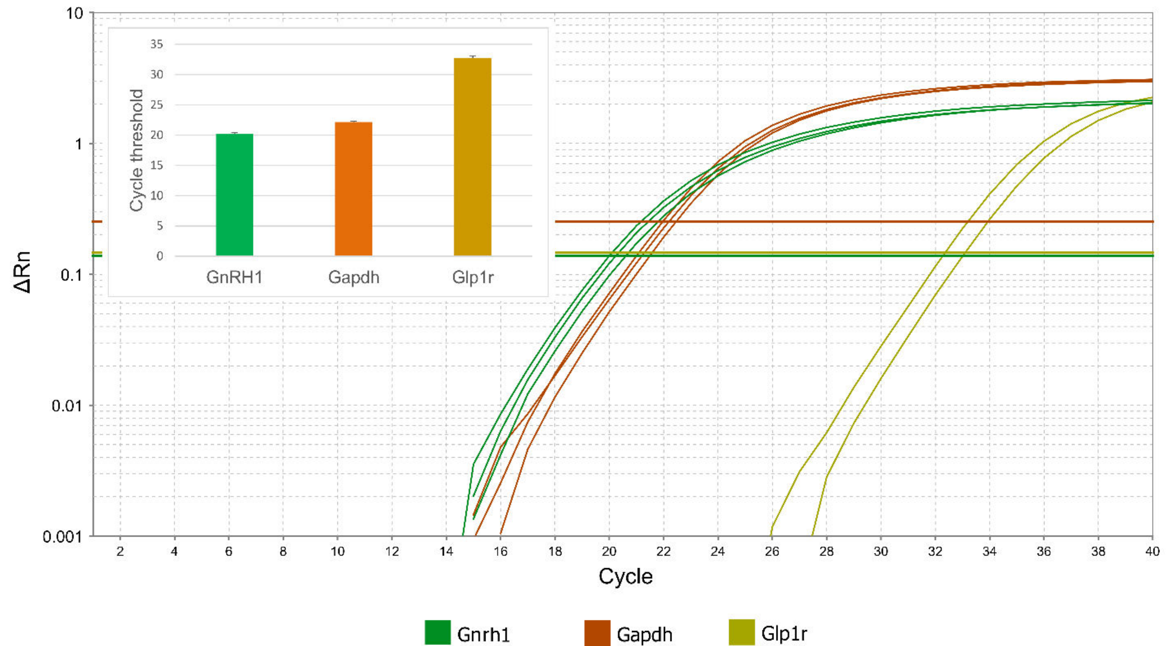

Figure 13. Expression of Glp1r mRNAs in GnRH neurons harvested by patch electrodes for single-cell qPCR. Real-time qPCR amplification of Gnrh, Gapdh, and Glp1r cDNA from GnRH neurons. Expression of Glp1r transcript was detected in 2 of 3 pools of GnRH neuronal cytoplasmic samples. The abundance of the Glp1r was low, indicated by its relatively high cycle threshold values (32.5 and 33.1) as compared to the housekeeping gene Gapdh (22.0-22.5). Column charts are in the insert to show quantitative results of the qPCR experiments.

Nitric oxide and 2-arachidonoylglycerol signaling mechanisms are involved in the action of Exendin-4 on GnRH neurons

Activation of the nitric-oxide system could increase the frequency of the GABAergic mPSCs in hypothalamic neurons suggesting that NO could be one of the candidates playing role in the effect of Exendin-4 [198]. Therefore, the involvement of this mechanism in the elevation of mPSC frequency after Exendin-4 application in GnRH neurons was examined. Slices were pretreated with nitric oxide synthase inhibitor L-NAME (100 μM, 10 min). This pretreatment alone caused no alteration in the amplitude (Table 4.). In the presence of L-NAME Exendin-4 was still able to increase the frequency of mPSCs to 144.5 ± 16.0% of the value measured prior to Exendin-4 application without affecting the amplitude (Figure 14. A, D). This percentage value was, however significantly lower ($n=12$; $p<0.05$) than the value obtained with Exendin-4 alone. Furthermore, full elimination of the Exendin-4 effect could not be achieved. The percentage elevation differed not only from the change observed in the absence of L-NAME, but also from the value when Exendin-4 was administered in the presence of GLP-1R antagonist ($p<0.05$).

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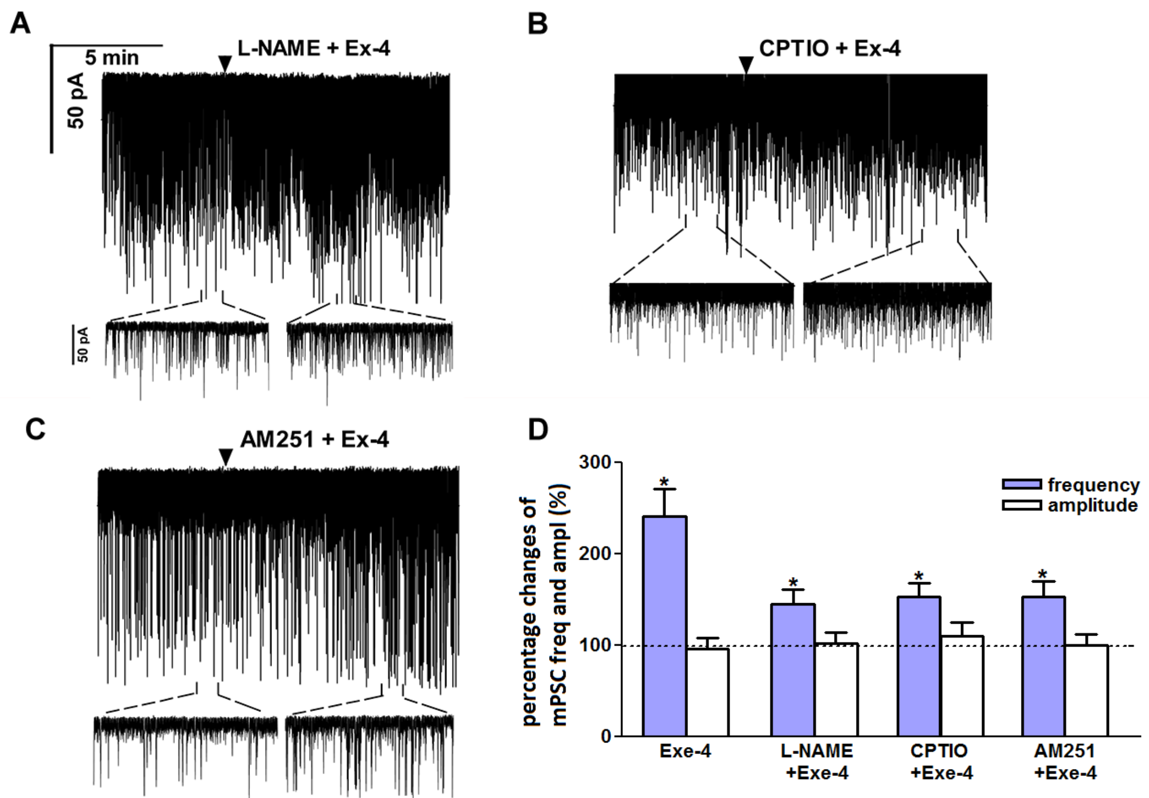

Figure 14. The effect of Exendin-4 could be partially inhibited by blocking retrograde signaling pathways. (A) Effect of Exendin-4 (1 µM) was eliminated only partially when slices were pretreated with the NO-synthase inhibitor L-NAME (100 µM). **(B)** Partial inhibition was observed when the NO-scavenger CPTIO (1 mM) was applied intracellularly in the GnRH neuron. **(C)** Similar partial inhibition was observed in the case of pretreatment with CB1 inverse agonist AM251 (1 µM). **(D)** Bar graph reveals that Exendin-4 significantly elevated the frequency of mPSCs. Blockade of either the NO or the endocannabinoid system resulted in partial inhibition only. The amplitude of the mPSCs did not change in any of these treatments. One-minute-long periods of the recording before and after application of Exendin-4 in the presence of L-NAME are drawn under the recording. Arrowheads show the onset of Exendin-4 administration. *p<0.05.

NO is widespread in the nervous system and it is synthesized on demand and cannot be stored as it is membrane permeable. To determine the cellular source of NO we dissected this regulatory mechanism further by applying the NO-scavenger CPTIO (1 mM) intracellularly in the GnRH neuron. Pretreatment of CPTIO alone exerted no effect on the amplitude and frequency values of GnRH neurons (Table 4.). However, after pretreatment of CPTIO, administration of Exendin-4 increased the frequency of the mPSCs to 153.3±20.0% of the value measured before agonist application (n=12; p<0.05), although it was still significantly lower than in the absence of CPTIO (p<0.05) (Figure 14. B, D). Although intracellular scavenging of NO by CPTIO only partially

attenuated the excitatory effect of Exendin-4, these results suggest that NO is synthetized by GnRH neurons. As the partial blockade by CPTIO was very similar to the effect of L-NAME we presumed that another pathway should act in parallel to the NO system. This result suggested the involvement of other signaling pathway(s) as well.

Previous work of our laboratory showed that tonic 2-AG release from a GnRH neuron could influence synaptic transmission to itself [152]. To examine whether modulation of this tonic endocannabinoid release was also involved in the effect of Exendin-4, the CB1 inverse agonist AM251 (1 μM) was applied on slice preparations. In accordance with our earlier results [152], blockade of the retrograde endocannabinoid signaling machinery elevated the basal mPSC frequency without affecting the amplitude (Table 4.), nevertheless, it attenuated but not eliminated the effect of Exendin-4 (Figure 14. C, D). The frequency of mPSCs was raised by Exendin-4 to 153.1±17.1% of the value recorded before Exendin-4 application. This percentage increase is significantly lower ($n=11$; $p<0.05$) that measured in the absence of AM251.

Similarly to the inhibition of the NO-release by L-NAME or intracellular scavenging of NO by CPTIO, the blockade of CB1 by the administration of AM251 did not fully eliminate the action of Exendin-4. Since the blockade of NO-production or the presynaptic CB1 could only partially inhibit the effects of Exendin-4 we tested the simultaneous blockade of both pathways.
Presence of both AM251 and L-NAME in the aCSF fully abolished the effect of Exendin-4 (102.2±12.8%; $n=10$, $p<0.05$) (Figure 15. A, D). This result suggests the simultaneous participation of both NO and endocannabinoid retrograde signaling mechanisms in GLP-1 signaling.

In order to confirm our results, the specific nNOS inhibitor NPLA was applied intracellularly (1 μM) in the extracellular presence of AM251 (1 μM) and then effect of Exendin-4 was examined on the mPSCs of GnRH neurons. The pretreatment alone elevated frequency of the mPSCs due to the inhibition of the tonic 2-AG release without affecting the amplitude (Table 4.). Simultaneous application of NPLA and AM251 fully abolished action of Exendin-4 on the frequency of mPSCs (104.8±6.1 % of the value before Exendin-4 was added, $n=9$, $p<0.05$; Figure 15. B, D), verifying that GnRH neuron was indeed the source of the released NO.

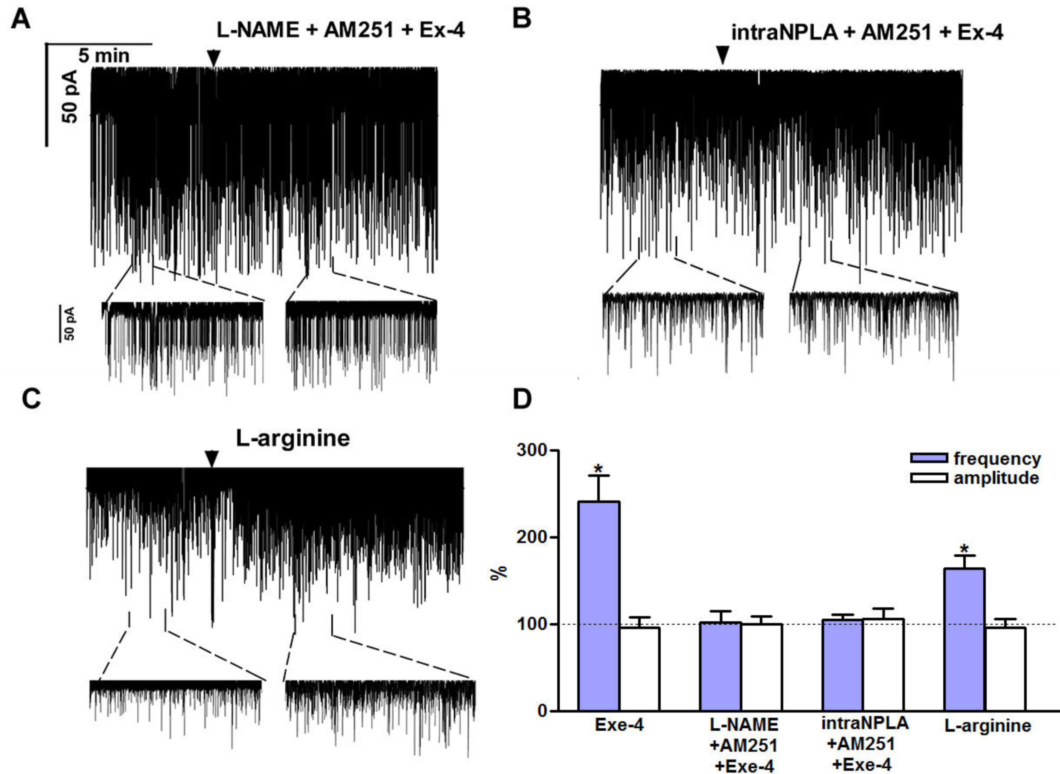

Figure 15. The effect of Exendin-4 can be completely inhibited by the simultaneous blockade of retrograde systems. (A) Full blockade of action of Exendin-4 could be accomplished by simultaneous blockade of the NO- (by L-NAME, 100 μM) and endocannabinoid (by AM251, 1 μM) signaling mechanisms. **(B)** Full inhibition was also achieved when nNOS was inhibited by the intracellularly applied NPLA (1 μM) and the endocannabinoid pathway was blocked by AM251 (1 μM). One-minute-long periods of the recording before and after application of Exendin-4 in the presence of the antagonists are drawn under the recordings. **(C)** The NO-donor L-arginine (1 mM) elevated frequency of the mPSCs. **(D)** Bar graph reveals that simultaneous blockade of the two retrograde systems abolished the effect of the GLP-1 agonist Exendin-4 on the mPSCs in GnRH neurons of male mice. The increased mPSC frequency resulted from L-arginine confirms the involvement of the NO system in the effect of GLP-1. The amplitude of the mPSCs did not change in any of the treatments. Arrowheads show the onset of Exendin-4 or L-arginine administration. *$p<0.05$.

The effect of nitric oxide in GnRH neurons has been examined by further experiments. The NO-donor L-arginine (1 mM) was applied in the aCSF and resulted in the elevation of frequency of mPSCs (164.3±15.1%; n=10; $p<0.05$) (Figure 15. C, D) with no change in the amplitude (Table 4.).

In addition, RT-PCR confirmed the expression of Nos1 genes in GnRH neurons of mice. Amplification curves showed that GnRH neurons expressed Nos1 mRNA (cycle threshold 29.5±1.5) (Figure 16.). Presence of Nos1 transcript was detected in 5 out of 11 GnRH cytoplasmic

samples harvested from GnRH neuros. The cycle threshold value of the housekeeping gene Gapdh was 23.3±0.3.

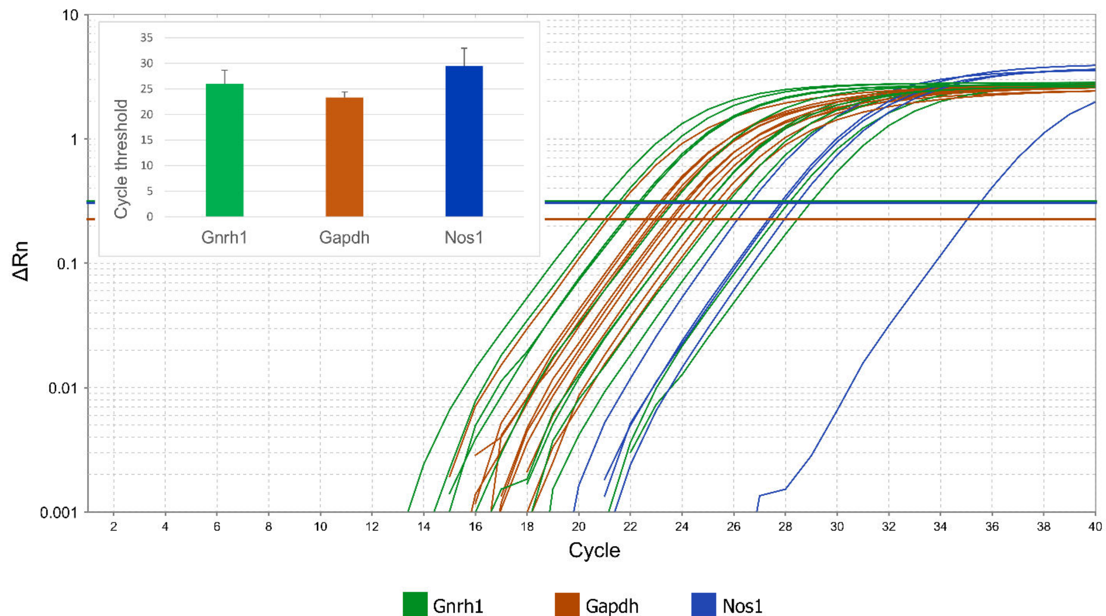

Figure 16. Expression of Glp1r and Nos1 mRNAs in GnRH neurons harvested by patch electrodes for single-cell qPCR. Real-time qPCR amplification of Gnrh, Gapdh, and Nos1 cDNA from GnRH neurons. Expression of the Nos1 gene in individually harvested glial fibrillary acidic protein (GFAP)-negative GnRH neuronal samples. The logarithmic scale of Rn and number of PCR cycles are indicated on the Y and X axes, respectively. The onset of the exponential phase (Ct) is indicated by horizontal lines for each target gene. Column charts in the inserts show quantitative results of the qPCR experiments.

The retrograde 2-AG pathway is regulated by anandamide-TRPV1 signaling

The involvement of the TRPV1 in the inhibition of 2-AG production and in the retrograde endocannabinoid signaling mechanism in hippocampal neurons was previously shown [156]. Hence, the hypothetic role of TRPV1 in the decreased tonic 2-AG production in GnRH neurons was examined. The TRPV1 antagonist AMG9810 (10 μM) was applied intracellularly in the presence of L-NAME. The amplitude did not change (Table 4.), but the effect of Exendin-4 on the frequency of mPSCs was completely abolished (106.0±9.8%; n=12, $p<0.05$) (Figure 17. A, C).

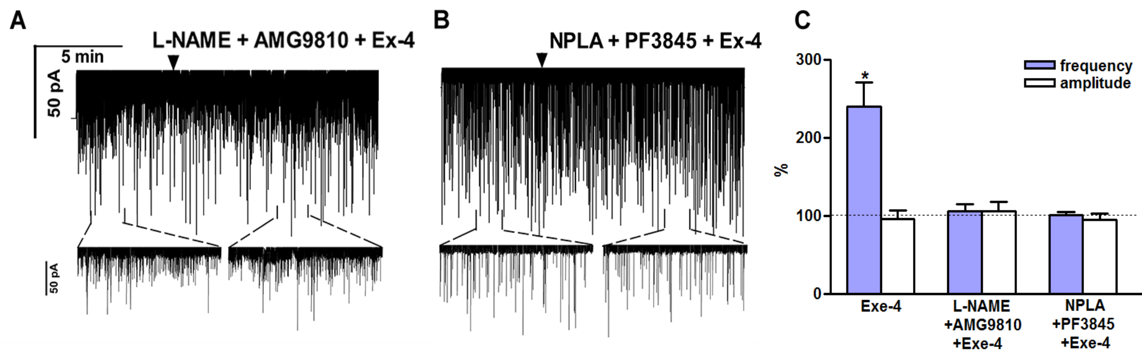

Figure 17. GLP-1R signaling involves the anandamide-TRPV1-coupled mechanism. **(A)** Simultaneous blockade of NOS by L-NAME (100 µM) and intracellular inhibition of the TRPV1 receptor by AMG9810 (10 µM) in GnRH neuron abolished the effect of Exendin-4. **(B)** Inhibition of both nNOS by NPLA (1 µM) and anandamide degradation by PF3845 (5 µM) resulted in full elimination of action of Exendin-4. **(C)** Bar graph summarizes these results. The amplitude of the mPSCs did not change in any of the treatments. Arrowheads show the onset of Exendin-4 administration. *p<0.05.

Anandamide is an endogenous ligand of TRPV1. Thus, we investigated its role in the activation of TRPV1 by inhibiting FAAH (degrading enzyme of anandamide). The FAAH inhibitor PF3845 (5 µM) was applied intracellularly whereas the NO signaling was blocked by the nNOS inhibitor NPLA (1 µM). Under these conditions the amplitude showed no change (Table 4.) but the stimulatory effect of Exendin-4 on the mPSC frequency was fully eliminated in GnRH neurons (101.0±4.3%; n=10, p<0.05) (Figure 17. B, C). These data indicate that suppression of 2-AG endocannabinoid signaling is mediated by the anandamide-TRPV1 pathway.

Finally, effect of Exendin-4 on the resting membrane potential was examined (Figure 18.). The measurements showed no significant change in this parameter demonstrating that ion channels contributing to the level of V_{rest} are not involved in the process.

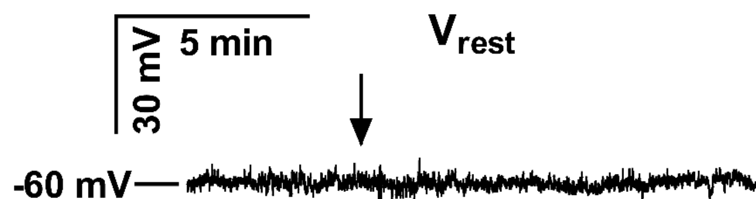

Figure 18. Effect of Exendin-4 on the resting membrane potential. Exendin-4 (1 µM) does not affect resting membrane potential (V_{rest}) in GnRH neurons. Arrow shows onset of the Exendin-4 application.

DISCUSSION

GnRH neurons are indispensable in the central regulation of reproduction. The function of these neurons is influenced by a number of factors, including sex steroids, circadian rhythm, stress and metabolic states. During my work, my goal is to give a more accurate picture about the role of the gonadal steroid, estradiol and the metabolic hormone, GLP-1 in the regulation of GnRH neurons using electrophysiological methods.

First part of the present dissertation demonstrates that the suppressive effect of estradiol on GABAergic neurotransmission on GnRH neurons requires the activation of ERβ and 2-AG signaling in metestrous female mice. The specific results include, (1) the firing rate and frequency of spontaneous and miniature postsynaptic currents significantly decrease upon estradiol treatment, (2) ERβ is required for the execution of this direct and rapid effect of estradiol, and (3) retrograde 2-AG endocannabinoid signaling has an integral role in the estradiol-evoked suppression of mPSC frequency of GnRH neurons.

Estradiol suppresses the firing rate and frequency of postsynaptic currents in GnRH neurons in metestrous female mice

Our experiments revealed the inhibitory effect of low physiological dose of E2 on the firing rate and the GABAergic neurotransmission on GnRH neurons. These results are consistent with earlier findings, which showed that estradiol at 10 pM concentration reduced the firing of GnRH neurons in the absence of ionotropic GABA and glutamate receptor inhibitors [100]. The suppressive effect of E2 has also been reported in other cell types of the hypothalamus and other brain regions. For instance, estradiol suppressed the neurokinin-B agonist induced firing rate in KNDY (kisspeptin/neurokinin-B/dynorphin-containing) neurons of the hypothalamic arcuate nucleus [114] and E2 also inhibited spontaneous firing activity in extrahypothalamic regions, such as the lateral habenula [199].

Additionally, our results showed that E2 administration decreased the frequency of the GABAergic postsynaptic currents of GnRH neurons in metestrous female mice. Similar effects of estradiol have been demonstrated in other brain regions: hippocampal CA1 neurons showed decreased postsynaptic current frequency upon estradiol treatment [200] and estradiol induced reduction of mPSC frequency was observed in kisspeptin neurons of the arcuate nucleus [201]. Our results are in line with previous findings, suggesting positive correlation between firing rate and frequency of postsynaptic currents in GnRH neurons [64, 101, 132].

According to a recent study, chronic E2 administration has no effect on ionotropic GABA and glutamate receptor synaptic transmission on GnRH neurons neither in negative nor in positive feedback [202]. This discrepancy between these and our results may arise from usage of two different experimental animal models. They used gonadectomized and estradiol replaced animals in order to mimic the negative and positive feedback periods, respectively [202]. On the contrary, we used intact metestrous mice model. The advantage of our model is the intact ovarian signaling mechanisms and the physiological concentration of estradiol in the blood circulation. Ovariectomy abolishes the natural estradiol signaling and ceases production of numerous other hormones which are indispensable for proper operation of HPG axis, such as progesterone, activin, inhibin, and anti-Müllerian hormone. In agreement with our present results it has been reported that the E2 diminished the firing of GnRH neurons which effect was arrested by the blockade of GABAergic and glutamatergic neurotransmission [100, 101]. These results show the essential role of the fast synaptic transmission in the action of estradiol on GnRH neurons.

Estrogen receptor beta is required for the direct, rapid effect of estradiol on GnRH neurons

Experiments using autoradiography combined with immunocytochemistry have not been able to detect the presence of estrogen receptors in GnRH neurons until the 1990s [203]. Certain neuron sets innervating GnRH neurons expressed nuclear ERα [10, 109, 114]. These observations were the basis of the general view that the function of GnRH neurons is regulated by estrogen via estrogen-sensing interneurons (expressing ERα) located in hypothalamic and several extrahypothalamic loci. The discovery of ERβ [204] led to the finding that ERβ was expressed in GnRH neurons both in rodents [112, 115, 116] and humans [117]. Since then numerous studies have demonstrated that estrogen has a direct effect on GnRH neurons [86, 88, 89, 96, 100]. My doctoral thesis extended these studies by examining the direct effect of E2 on GnRH neurons of metestrous female mice.

Administration of low physiological concentration of E2 onto the brain slices resulted in a significant decrease in the mean frequency of the mPSCs on GnRH neurons within few minutes, indicating that the observed effect of E2 was direct on these neurons. This effect was inhibited by the administration of the non-selective estrogen receptor antagonist Faslodex, suggesting the involvement of estrogen receptor(s) in this rapid effect. The intracellularly applied 2-AG endocannabinoid synthesis blocker THL eliminated the effect of E2 on mPSCs, also confirming that indeed the effect of E2 on GnRH neurons was direct. The action of E2 was observed within minutes indicating that this effect occurs via rapid non-genomic estrogen signaling, since it was detected much earlier and lasted significantly shorter than expected for a transcriptional mechanism of a

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nuclear receptor. Both ERα and ERβ have membrane-associated forms, and along with other membrane-associated receptors such as GPR30, these mediate intracellular signaling pathways during the rapid action of E2 [85-88, 90, 96].

Next, we examined the effect of various subtype-selective ER agonists to identify the ERs involved in the mediation of estradiol effect on GnRH neurons. The ERβ agonist DPN significantly decreased the mean frequency of the mPSCs in GnRH neurons. Moreover, the ERβ specific antagonist PHTPP also significantly blocked the suppressive effect of E2. In contrast, agonists of other membrane-associated receptors did not show suppressive effect. Neither the ERα agonist PPT nor the GPR30 selective agonist G1 had any significant effect on the frequency of the mPSCs on GnRH neurons. Taken together these results revealed the exclusive role of ERβ in the observed rapid effects of E2 in GnRH neurons. This finding is in a good agreement with an *in vivo* study in which they found that GnRH neurons responded to estrogen in a rapid and direct manner via an ERβ-dependent mechanism [89]. Our experiments provided further evidence about the pivotal role of ERβ in the mediation of the rapid effect of E2 in GnRH neurons.

Retrograde endocannabinoid 2-AG signaling is involved in the estradiol-triggered reduction of miniature postsynaptic current frequency in GnRH neurons

Low dose of estradiol inhibited GnRH neurons in a rapid manner when the fast synaptic transmission was left intact [100]. This indicated the involvement of fast neurotransmission in the rapid action of estradiol and suggested an effect upstream to GnRH neurons.

Fast synaptic transmission on GnRH neurons is mediated via the ionotropic GABA and glutamate receptors. GABA neurotransmission has been implicated as one of the major signaling in the regulation of GnRH neurons since the early 2000s. Whereas GABA is usually an inhibitory neurotransmitter in the adult central nervous system, mature GnRH neurons show the unaccustomed character of being excited by GABA via postsynaptic $GABA_A$-R channels in adult rodents [45, 50-52, 167, 168]. An earlier study from our group showed that 2-AG endocannabinoid release from GnRH neurons was able to influence synaptic transmission to the GnRH cells, reducing the firing rate and the GABAergic neurotransmission via $GABA_A$-R. Both the CB1 antagonist AM251 and the 2-AG endocannabinoid synthesis inhibitor THL could influence the GABAergic input of these cells, providing evidence that 2-AG endocannabinoid tonically inhibits $GABA_A$-R drive onto GnRH neurons [152]. Therefore, the estradiol-evoked reduction in the firing rate and the frequency of the mPSCs in metestrous female mice may suggest the essential role of the retrograde endocannabinoid system in the manifestation of the suppressing effect of estradiol. This is in good agreement with a previous study demonstrating that release of endocannabinoids indeed regulates the function of

GnRH neurons [153]. Thus, our finding that both the blockade of endocannabinoid receptor and inhibitor of 2-AG synthesis attenuated the effect of the low physiological dose of estradiol in the period of negative feedback suggest the role of retrograde 2-AG signaling mechanism in the output of estradiol-triggered changes in the postsynaptic currents of the GnRH neuron. A study from the rat hippocampus strengthens our findings, in which the key role of retrograde endocannabinoid system was shown to be involved in the estradiol-dependent suppression of inhibitory GABAergic neurotransmission to CA1 pyramidal neurons [200]. Our results showed that inhibition of 2-AG synthesis by the intracellularly applied THL attenuated the effect of estradiol on GnRH neurons. This suggests that estradiol induces 2-AG synthesis in GnRH neurons. In line with this observation, 2-AG synthesis in immortalized GnRH neurons has already been shown [205]. The mechanism we have now revealed is very similar to other endocannabinoid-regulated systems in the brain, including hypothalamus [149, 206, 207]. Our model of estradiol action on GnRH neuron is shown in Figure 19. Briefly, GnRH neurons synthetize and release 2-AG endocannabinoid upon estradiol action via membrane associated ERβ. Next, the released 2-AG diffuses to the axon terminal of a presynaptic GABA afferent where it activates CB1 endocannabinoid receptor. This leads to attenuation of GABA release into the synaptic cleft repressing the excitatory action of GABA and thus decreasing the electric activity of GnRH neurons. Such interaction between estradiol and endocannabinoid signaling mechanisms represents a novel regulatory machinery in the execution of the negative estrogen feedback to GnRH neurons.

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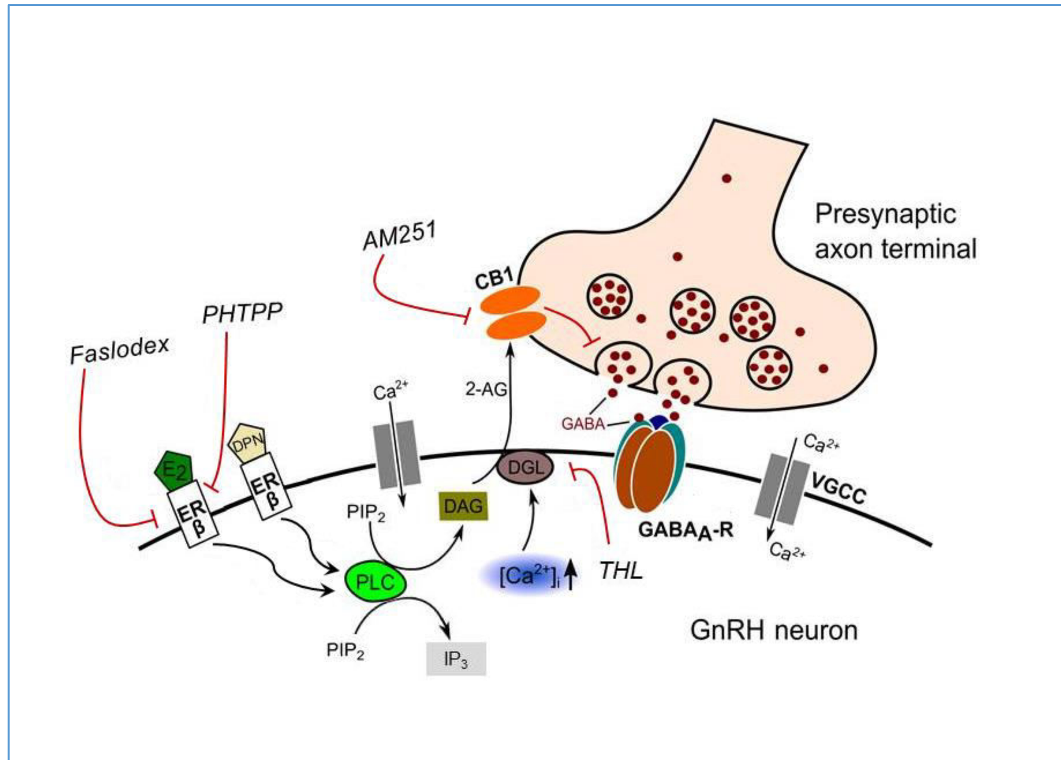

Figure 19. Schematic illustration of the relationship between estradiol and 2-AG endocannabinoid signaling in GnRH neurons of the metestrous female mice. E2 activation of ERβ causes the synthesis and release of 2-AG endocannabinoid from the GnRH neuron. Then, the released 2-AG binds to CB1 located in 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).

Besides the role of E2 in negative feedback regulation of GnRH neurons, I also studied the effect of the GLP-1 metabolic signal molecule on these neurons. While 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. The second part of my dissertation shows the direct regulatory action of GLP-1 on GnRH neurons. Accordingly, (1) GLP-1 increased the firing rate and frequency of GABAergic mPSCs via GLP-1R; (2) the molecular mechanism in the downstream actions of GLP-1 contains two retrograde pathways: activation of NO- and suppression of 2-AG signaling mechanisms; (3) inhibition of 2-AG pathway is mediated via anandamide-TRPV1 signaling.

GLP-1 is excitatory to GnRH neurons via GLP-1 receptor

GLP-1 receptor agonist Exendin-4 increased the firing rate of GnRH neurons and frequency of GABAergic mPSCs, demonstrating that GLP-1 has stimulatory effect on GnRH neurons. Elevated firing activity and mPSC frequency correlates well due to the excitatory nature of the GABA via $GABA_A$-R in GnRH neurons [46, 152]. Similar stimulatory effect of GLP-1 was observed in the paraventricular nucleus, the bed nucleus of the stria terminalis and the hippocampus [208]. GLP-1 was also shown to activate kisspeptin neurons in the arcuate nucleus directly [209].

Regulatory effect of GLP-1 on the reproductive axis has already been proposed [143-145]. Our finding is strengthened by other studies showing that GLP-1 increases spike and mPSC frequency in hypothalamic hypocretin/orexin neurons [176]. Alteration of $GABA_A$-R mediated synaptic currents by GLP-1 has also been detected in hippocampus [210], suggesting similar mechanism in different area. The GLP-1 receptor antagonist Exendin-3(9-39) inhibited the excitatory action of GLP-1 both on firing and mPSCs frequency of GnRH neurons. This suggests an essential, direct role of this receptor in this action. Furthermore, our RT-PCR experiments confirmed the expression of Glp1r genes in GnRH neurons of mice. The fact, that the intracellularly applied G-protein inhibitor GDP-β-S was able to attenuate the Exendin-4 triggered elevation of the mPSC frequency provides further evidence for the existence of functional GLP-1R in GnRH neurons. Experiments with the intracellularly applied NO-scavenger CPTIO, the TRPV1 inhibitor AMG9810 and the FAAH inhibitor PF3845 all confirm the direct action of Exendin-4 in GnRH neurons, since we could demonstrate that all these chemicals efficiently blocked this intracellular pathway. Thus, we revealed that GLP-1 agonist has direct effect on GnRH neurons besides the indirect action via kisspeptin neurons suggested by others [144].

One of the putative sources of GLP-1 to GnRH neurons was also identified. GLP-1-immunoreactive (IR) fibers reached a subset of GnRH neurons in mouse samples showing the assumed role of brain-born GLP-1. Several hypothalamic regions are innervated by GLP-1-containing fibers originating from the NST [211, 212]. GLP-1-IR axons also innervate brain regions where the GnRH neurons are located. Thus, these results support our hypothesis, that GnRH neurons receive direct inputs from the periphery by the gut-born GLP-1, and also receive direct/indirect inputs from the NST by brain-born GLP-1.

Effect of GLP-1 is mediated partially by activation of the nitric oxide retrograde signaling on GnRH neurons

Our electrophysiological results demonstrated the involvement of the NO signaling pathway in the Exendin-4 evoked action. The Exendin-4 increased the frequency on mPSCs in GnRH neurons, but this effect was partially eliminated when the NO synthesis inhibitor L-NAME was administered. Similar partial inhibition was observed when the membrane-impermeable NO-scavenger CPTIO was intracellularly applied in the GnRH neuron. These results suggest that the increased mPSC frequency is due to an elevated NO level at least partially. Our experiment using the NO scavenger CPTIO also demonstrated that the measured GnRH neuron itself was the source of NO in this effect. The interaction between the NO signaling mechanism and the GLP-1 action has already been reported in other brain areas [213, 214]. Our data indicate presynaptic stimulatory role of NO, since there was no change in the amplitude or rise/decay tau along with the elevated mPSC frequency. NO acts as retrograde messenger, and it is released from the postsynaptic cell and travels to the presynaptic axon terminals [215]. Similar NO-related retrograde mechanism in the hypothalamus has already been described [216].

Although NO has an integral role in reproduction, its effects seem to be rather complex. Stimulated GnRH release was observed after NO-donor sodium nitroprusside treatment in hypothalamic explants [217]. Similar increased GnRH secretion was detected following L-arginine (a precursor of NO) administration in immortalized GnRH-producing neuronal cell line [218]. In addition, that immortalized cell line expressed the neuronal nitric oxide synthase [218].

Counter to these findings, repressive effect of NO was shown on LH secretion [219]. Inhibition of spontaneous firing activity of GnRH neurons was observed by NO [220]. Earlier studies could not detect nNOS in GnRH neurons [221]. Nevertheless, our RT-PCR experiments provide evidence for the expression of nNOS mRNA in GnRH neurons. Furthermore, our work revealed for the first time the presence of nNOS protein in mouse GnRH neurons using immunoelectron microscopy. This result was described in our paper [222]. The different experimental design used by us may explain

why earlier studies – based on light microscopy – reported the absence of nNOS protein in GnRH neurons [159, 221]. Our electrophysiological results provided evidences for NO synthesis in GnRH neurons and showed its facilitatory action on presynaptic GABAergic axon terminals targeting these neurons. Experiments using NO-donor L-arginine provided further evidence for functional NO-pathway in GnRH neurons since L-arginine treatment resulted in elevated mPSC frequency. Expression of nNOS in immortalized GnRH-producing GT1–7 neuronal cell line was also detected [223]. A very recent paper also proved the presence of nNOS in GnRH neurons of ewes [224] confirming the possibility that GnRH neurons contain nNOS.

Previous findings showed that activation of NO inhibited the spontaneous firing of GnRH neurons [220]. The reason behind the discrepancy might be that different experimental conditions such as recording temperature (room temperature vs. 33°C) were used. Since enzymatic processes, ion pumps and exchanger fluxes are extremely sensitive to temperature, this might explain the differences. The intracellular (pipette) solution was also different between studies. Their measurements were carried out using low-chloride (10 mM) pipette solution [220], whereas we used high-chloride concentration (130 mM) intracellular solution in our experiments. This is an important difference, since mature GnRH neurons maintain high intracellular chloride level [45], thus our experimental model may mirror the physiological conditions of GnRH neurons.

GLP-1 acts partially via retrograde endocannabinoid signaling pathway of GnRH neurons

Our experiments also provide evidences for the important role of retrograde endocannabinoid signaling in the GLP-1 action. The endocannabinoid receptor blocker AM251 partially inhibited the Exendin-4 evoked elevation of the mPSC frequency on GnRH neurons. Since this treatment did not cause full inhibition, similarly to the effect of nNOS inhibitor L-NAME, we could come to the conclusion that both retrograde systems are partially involved in the GLP-1 modulated function of GnRH neurons. Indeed Exendin-4 action was completely eliminated by simultaneous blockade of NO and 2-AG signaling. Furthermore, this effect is completely mimicked by using GLP-1R antagonist. These results support our model and the conclusion that GnRH neuron was the source of not only the NO but also the endocannabinoids. As mentioned above, it has already been demonstrated that tonic 2-AG retrograde endocannabinoid signaling is present on GnRH neurons and its activation decreased the activity of the excitatory GABAergic input [152]. The results shown in the first part of this thesis revealed the involvement of 2-AG endocannabinoid signaling in the suppressive effect of estradiol on GnRH neurons. In addition, the interaction between metabolic

signals and 2-AG mechanism was also reported in several neurons, including GnRH cells [132, 225].

The 2-AG retrograde signaling inhibits GABAergic input to GnRH neurons [132, 152], however, Exendin-4 could stimulate these cells, even when NO signaling was blocked by L-NAME or by the intracellularly applied CPTIO. Thus, we can deduce that stimulation of GLP-1R results in the suppression of the 2-AG pathway. A recent study showed the involvement of TRPV1 in the inhibition of 2-AG production and in the retrograde endocannabinoid signaling mechanism [156]. Stimulation of GPCRs such as muscarinic acetylcholine receptors or metabotropic glutamate 5 receptors can activate TRPV1 by elevating anandamide level [226, 227]. TRPV1 decreases the activity of DGL and thus 2-AG production and eventually suppressing GABAergic transmission in the striatum [226, 227]. These data are in line with our finding that the GLP-1R can diminish the tonic 2-AG suppression of the GABAergic neurotransmission to GnRH neurons via an anandamide-TRPV1 pathway.

The anandamide is also able to activate the CB1. Then how is it possible that the mPSC frequency was not reduced by anandamide, as theoretically, increased level of anandamide may indeed mimic 2-AG effects and act on presynaptic CB1? It may be explained by that the degrading enzyme of anandamide (FAAH) is postsynaptic, while the degrading enzyme of 2-AG (MGL) is presynaptic [228] and the 2-AG concentration is 170 times higher in the brain than anandamide concentration [229]. In addition, the synthesizing enzyme of 2-AG (DGL) is located postsynaptically where the FAAH can also be found [228]. These data suggest that 2-AG is more likely to reach presynaptic CB1 receptors. Therefore, the anandamide could not exert a significant direct effect on the presynaptic CB1. Rather, postsynaptically elevated anandamide level could possibly interfere with the mobilization of 2-AG by decreasing DGL activity instead of acting on the presynaptic CB1.

The nitric oxide and endocannabinoid retrograde signaling pathways are simultaneously involved in the effect of GLP-1

Similar partial inhibition of Exendin-4 action was observed when the nNOS or the CB1 was blocked. Complete inhibition could only be achieved by blocking the two retrograde signalings simultaneously. These results indicate that the downstream actions of GLP-1R on GnRH neurons involve both pathways. Activation of GLP-1R triggers production of NO, on the other hand it triggers suppression of tonic 2-AG, as illustrated in Figure 20. The proposed mechanism of GLP-1 action on GnRH neurons involves the following steps: binding of GLP-1, or its receptor agonist Exendin-4 to GLP-1R activates nNOS via a G-protein mediated pathway. As a result, the level of NO increases and it diffuses from GnRH neuron stimulating the presynaptic GABAergic input.

Binding of Exendin-4 to GLP-1R simultaneously induces another pathway, it suppresses the 2-AG synthesis via an anandamide-TRPV1-controlled pathway. The tonic endocannabinoid 2-AG release reduces $GABA_A$-R drive onto GnRH neurons [152], conversely inhibition of the 2-AG synthesis eventually causes facilitation of GABA release from the presynaptic terminal into the synaptic cleft.

Similar simultaneous involvement of both retrograde signaling mechanisms has already been observed in the hypothalamus [198]. In that study, the NO release and endocannabinoid synthesis simultaneously mediated the glucocorticoid-induced suppression of glutamatergic drive and the facilitation of $GABA_A$-R drive onto magnocellular neurons of the rat supraoptic nucleus [198]. These glucocorticoid-induced events are similar but not entirely the same as those ones we have observed in GnRH neurons, since the glucocorticoids activated both NO and endocannabinoid retrograde mechanisms whereas activation of GLP-1R evoked NO release but suppressed 2-AG production. This difference can be explained by the special excitatory role of $GABA_A$-R in GnRH neurons.

Elevated GABAergic neurotransmission could by itself indicate the presence of a direct presynaptic effect of GLP-1. Nevertheless, this can be excluded, since inhibition of cannabinoid and NO pathways together or G-proteins in the postsynaptic GnRH neurons both blocked the action of GLP-1 in our experiments. Our results suggest for the first time a direct postsynaptic effect of GLP-1 by triggering two parallel retrograde signaling mechanisms in GnRH neurons.

The integral role of GLP-1 in the regulation of human and rodent fertility has been described [230]. These observations suggest that GLP-1 peptide is capable to modulate the reproductive axis. GLP-1R KO mice are fertile [231], male mice exhibit reduced gonadal weight and female mice show delayed puberty [145]. Our study provided evidences for the direct effect of GLP-1 on GnRH neurons. This effect is mediated by the stimulation of retrograde NO and anandamide-TRPV1-mediated 2-AG endocannabinoid signaling mechanisms providing options to fine-tune the reproduction-specific effects of GLP-1.

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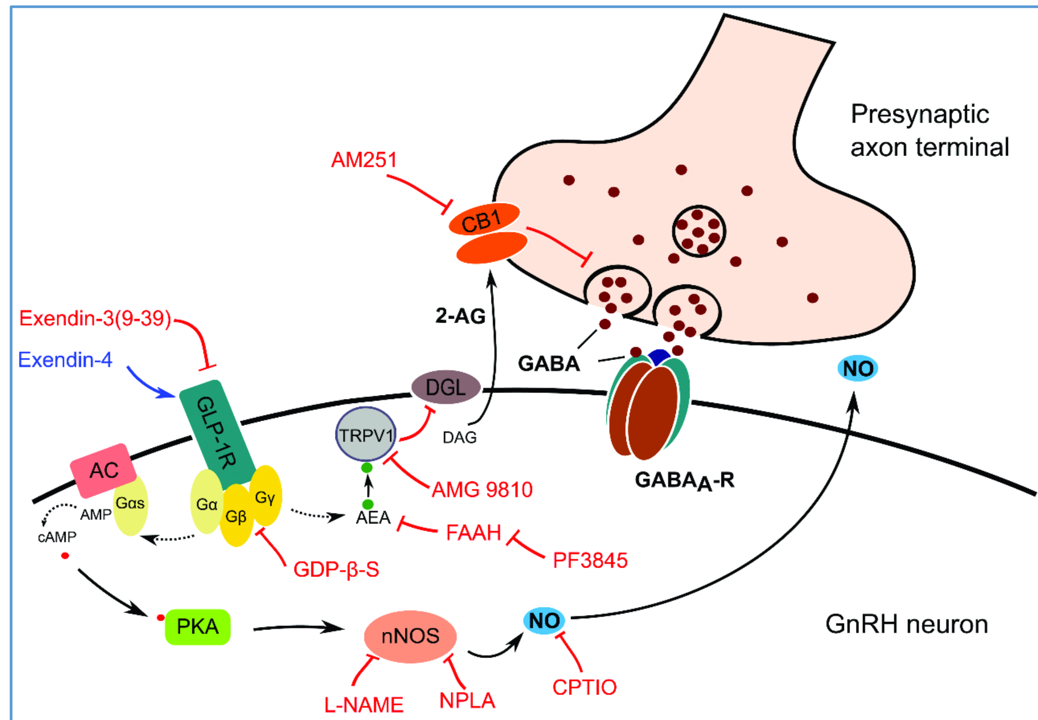

Figure 20. Schematic illustration of the proposed action of GLP-1 receptor signaling in GnRH neurons Effect of GLP-1R agonist (Exendin-4) is mediated by G-protein complexes which activate two retrograde signaling systems. The first one involves activation of nNOS, which leads to an increased NO production. 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).

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NEW SCIENTIFIC 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 inverse agonist and by arresting 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 under low physiological concentration of estradiol is used.

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 antagonize these effects. Furthermore, 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.

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List of publications underlying the thesis

Bálint, F., Liposits, Z., Farkas, I. 'Estrogen receptor beta and 2-arachydonoylglycerol mediate the suppressive effects of estradiol on frequency of postsynaptic currents in gonadotropin-releasing hormone neurons of metestrous mice: an acute slice electrophysiological study'. *Frontiers in Cellular Neuroscience*, vol. 10, March 2016, doi: 10.3389/fncel.2016.00214. Impact Factor: 4.555

Farkas I., Vastagh C., Farkas E., Bálint F., Skrapits K., Hrabovszky E., Fekete C., Liposits Z. 'Glucagon-Like Peptide-1 Excites Firing and Increases GABAergic Miniature Postsynaptic Currents (mPSCs) in Gonadotropin-Releasing Hormone (GnRH) Neurons of the Male Mice via Activation of Nitric Oxide (NO) and Suppression of Endocannabinoid Signaling Pathways' *Frontiers in Cellular Neuroscience*, vol. 10, September 2016, doi: 10.3389/fncel.2016.00214. Impact Factor: 4.555

List of publications not related to the subject of the thesis

Farkas I.*, Bálint F.*, Farkas E., Vastagh C., Fekete C., Liposits Z. 'Estradiol Increases Glutamate and GABA Neurotransmission into GnRH Neurons via Retrograde NO-Signaling in Proestrous Mice during the Positive Estradiol Feedback Period' *eNeuro*, 17 July 2018, ENEURO.0057-18.2018; doi: 10.1523/ENEURO.0057-18.2018

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