The modulatory effect of metabolic signals on the central regulation of reproduction

Theses of the PhD dissertation

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Introduction

Mammalian reproduction requires numerous precisely orchestrated events, for successful fertilization, and initiation of embryonic development. These processes can be readily modulated by the energy state of the body.

Since reproduction is a highly energy consuming process, therefore, it is vital for the body to be prepared for optimal circumstances when energy can be consumed for reproduction without any high risk.

Availability of food, thus the actual nutritional state can cause fluctuations in the level of metabolic hormones (such as leptin, IGF-1, ghrelin, etc.) having major effects upon reproduction [1]. Under certain environmental or physiological conditions, such as in anorexia nervosa, the suppression of reproductive functions is adaptive to survival [2]. Importance of examining the role of metabolic molecules in the process of reproduction is further emphasized by the extensive studies carried out about the high risk of infertility in case of metabolic problems.

Therefore, it is indispensable to reveal how various metabolic hormones act on hypophysiotropic gonadotropin releasing hormone (GnRH) neurons, the master cells in the central regulation of the reproductive process. In this Thesis, I will present two of them, the secretin, and the insulin-like growth factor 1 (IGF-1).

Secretin is an anorexigenic hormone [3], and it can serve as a signal molecule reporting level of the energy homeostasis. It was the first hormone discovered in 1902 [4]. It is released from the S-cells in the intestine when pylorus of the stomach opens to transfer food into the gut. In the periphery, secretin serves, as a local signal to pancreas for neutralizing the acidity of the chyme by secretion of bicarbonates [4]. It can cross the intact blood-brain barrier (BBB) [5, 6] and serve as a peripheral metabolic signal to neurons in numerous brain regions.

Only limited information has been available about the exact role of secretin in the regulation of reproduction so far [7]. There are a few reports indicating that it can be regarded as a putative regulator of the reproductive axis. In an early study, intracerebral (IC) injection of secretin into the hypothalamic preoptic region of rats resulted in a 10-fold elevation of luteinizing hormone (LH) concentration in the plasma [8], suggesting that GnRH neurons might be targeted by secretin. Therefore, it is highly conceivable that secretin, as one of the signal molecules of the homeostasis, also modulates function of GnRH neurons.

However, the exact cellular mechanism of the effect of secretin in the modulation of HPG axis has not been revealed, yet. In the present study, therefore, we carried out whole cell patch clamp recordings on GnRH-GFP neurons of male mice to elucidate the effect of secretin on firing and PSCs, and to uncover the second messenger cascade events occurring downstream to the secretin receptor in these neurons.

Insulin-like growth factor 1 (IGF-1) is one of the metabolic growth hormone molecules secreted primarily from the liver in adults [9, 10].

The concentration of IGF-1 in the serum decreases during fasting both in humans and rodents [11, 12] . The level of IGF-1 bindig protein-3 that primarily binds IGF-1, also elevates during fasting, which further reduces the free IGF-1 concentration [13].

During puberty, the IGF-1 concentration peaks in the plasma suggesting that the hormone shapes this process [14]. Indeed, high IGF-1 level accelerates the onset of puberty both in males and females [15]. In females, low IGF-1 concentration results in impaired estrous cycle [16]. Furthermore, its concentration in the serum is gonadal cycle dependent showing periodic oscillation during the estrus cycle [17, 18]. Since hypothalamic IGF-1 receptor (IgF1R) is the most abundant in proestrus, and E2 synergistically and mutually stimulates IGF-1 activity [15], these data indicate an essential role of IGF-1 in the central regulation of reproduction.

In this role it is of particular significance that IGF-1 can directly act on GnRH neurons. IGF-1R is expressed in GnRH neurons [19] and IGF-1 stimulates GnRH production and release [16]. IGF-1 of peripheral origin contributes to the initiation of female puberty by stimulating GnRH release from the hypothalamus, an effect that appears to be amplified by the increased presence of IGF-1Rs in the median eminence (ME) during first proestrus [20]. Mutation in IGF-1 in human patients [21] and GnRH specific deletion of IGF-1R in mice [22] resulted in a significantly delayed puberty providing further evidence for the important role of IGF-1 in puberty. More data suggested a long-term direct effect of IGF-1 on the GnRH expressing GT1 neuronal cell lines [23, 24]. However, the elements of the signaling pathway have not been fully understood, yet.

Therefore, using *in vitro* electrophysiology, we investigated the electric response of GnRH neurons to IGF-1 administration and the molecular pathways acting downstream to IgF-1 receptor. According to our earlier studies, various hormones trigger retrograde signaling pathways in GnRH neurons [25-27] suggesting strongly that this machinery might also be involved in the signal transduction downstream to the IGF-1R. In addition, GABA with excitatory role is the main neurotransmitter to GnRH neurons and the retrogradely released endocannabinoid and/or NO target the GABAergic presynaptic axon terminals [26], providing strong rationale to examine the role of retrograde signaling to GABAergic afferents in the action of IGF-1.

Specific aims

The purpose of my doctoral thesis was to gain more accurate information about signaling pathways related to metabolic signals in GnRH neurons using electrophysiological methods. In the first project, described in this dissertation, I investigated the effect of secretin on GnRH neurons, via whole cell patch clamp experiments. I was in search of the answers for the following questions:

1. Can secretin modulate the electrophysiological properties of GnRH neurons?
2. Is this modulatory effect direct on GnRH neurons via secretin receptor?
3. Are retrograde signaling pathways involved in this mechanism?
4. What signaling pathway is activated in the modulatory effect of secretin?

In the second project, I present my results about the regulatory role of the insulin-like growth hormone-1.

I attempted to answer these questions:

1. Can IGF-1 modulate the electrical parameters of GnRH neurons?
2. Is this modulatory effect direct in GnRH neurons via IGF-1 receptor?
3. Which molecular pathways act downstream to the IGF-1 receptor in GnRH neurons?
4. Are retrograde signaling pathways involved in this machinery?

Experimental procedures

Adult, pubertal (50 days) and prepubertal (23-29 days) male GnRH-green fluorescent protein (GnRH-GFP) transgenic mice bred on a C57Bl/6J genetic background were used for electrophysiological experiments [28].

Brain slice preparation and whole cell patch clamp experiments

Brain slice preparation was carried out based on our earlier experiments [26]. Two hundred fifty μm-thick coronal slices were prepared from the medial preoptic area (POA). During whole-cell patch clamp experiments spontaneous and miniature postsynaptic currents, action potentials and membrane potentials were measured either in voltage- or current clamp mode.

Whole-cell patch-clamp measurements started with a control recording (5 min), then secretin or IGF-1 was pipetted into the aCSF-filled measurement chamber containing the brain slice in a single bolus and the recording continued for further 10 minutes. Pretreatment with

extracellularly used antagonists started 10 minutes before adding the agonist. The antagonists were continuously present in the aCSF during the electrophysiological recording. Intracellularly applied drugs were added to the intracellular pipette solution and after achieving whole-cell patch clamp configuration, we waited 15 min to reach equilibrium in the intracellular milieu before starting recording. Each neuron served as its own control when drug effects were evaluated.

Reagents and chemicals

Extracellularly used drugs				
Name	**Purpose**	**Concentration**	**Producer**	**references**
Secretin	Secretin receptor agonist	30 nM- 1 µM	Tocris, UK	Dose-response curve
Secretin antagonist	Secretin receptor antagonist	3 µM	Distribio-Genecust-Labbx, Luxembourg	[29]
picrotoxin	GABA-A-R blocker	100 µM	Sigma, US	[30, 31]
IGF-1	IGF-1 receptor agonist	1-66 nM	Sigma	[32]
JB-1	IGF-1 receptor antagonist	800 nM	Bachem, DE	
AM251	CB1 endocannabinoid receptor inverse agonist	1 µM	Sigma, US	[26, 27]
TTX	Tetrodotoxin, voltage-gated sodium channel blocker	660 nM	Tocris, UK	[26, 27]
Intracellularly used drugs				
GDP-β-S	G-protein inhibitor (membrane impermeable)	2 mM	Sigma, US	[33-35]
NPLA	neuronal nitric oxide synthase inhibitor	1 µM	Tocris, UK	[36-38]
KT5720	protein kinase-A inhibitor	2 µM	Sigma, US	[39, 40]
AMG9810	transient receptor potential vanilloid 1 antagonist	10 µM	Sigma, US	[41-43]
LY294002	phosphoinositol-3-kinase inhibitor	50 µM	Sigma, US	[44]

Results I

Thesis 1.: Secretin modulates the electrophysiological properties of GnRH neurons

At 100 nM concentration secretin significantly increased the firing rate and the frequency of spontaneous and miniature postsynaptic currents of GnRH neurons in adult male mice. Secretin also depolarized the membrane potential of GnRH neurons. Secretin acted in a dose dependent manner. These results demonstrate that secretin has an excitatory effect on GnRH neurons.

Thesis 2.: The modulatory effect is direct through secretin receptor

Electrophysiological experiments demonstrated that secretin receptor is mandatory for the observed effect of secretin on GnRH neurons, because in the presence of the specific secretin receptor antagonist secretin could not increase the frequency of miniature postsynaptic currents.

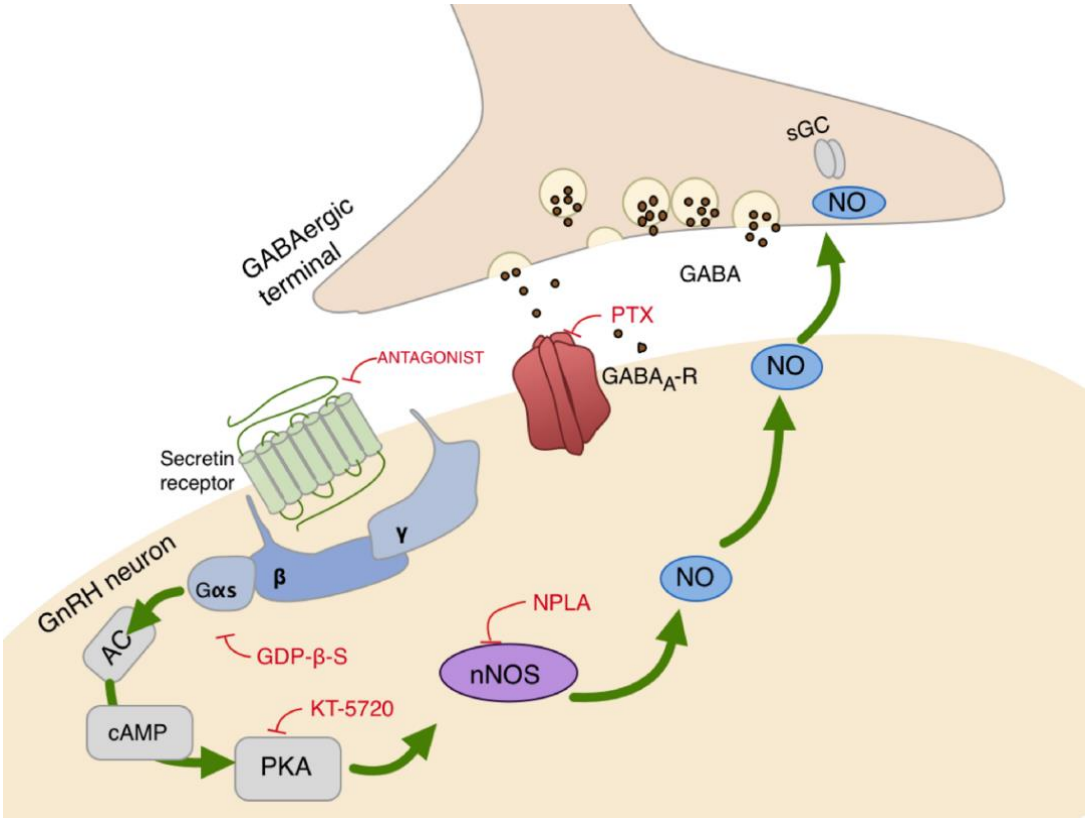
Intracellular blockade of the G-protein coupled receptors by GDP-β-S also prevented the frequency-elevating effect of secretin. Since secretin receptor is a G-protein coupled receptor, this experiment proved, that secretin receptor is active in GnRH neurons.

Thesis 3: Secretin activates the retrograde nitric oxide signaling pathway

Electrophysiological results revealed the involvement of nitric oxide (NO) retrograde signaling in the effect of secretin, In the presence of nitric oxide synthase blocker (NPLA), secretin was unable to elevate the frequency of the miniature postsynaptic currents.

Thesis 4. The retrograde nitric oxide pathway can be regulated by phosphokinase A in GnRH neurons.

We showed that the presence of selective PKA blocker KT5720 in the intracellular solution abolished the frequency-increasing effect of secretin on mPSCs of GnRH neurons.



Schematic illustration of secretin receptor signaling in GnRH neurons. Secretin activates cAMP/PKA/nNOS pathway and generates NO that binds to its presynaptic receptor, sGC, located in the GABAergic terminals. This signaling process increases the release of GABA, therefore, facilitates the synaptic inputs to GnRH neurons via $GABA_A$-receptor. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; Gαs, Gβ, Gγ, G-protein subunits; $GABA_A$-R, $GABA_A$-receptor; PTX, picrotoxin, selective $GABA_A$-receptor blocker; PKA, protein kinase A; KT5720, protein kinase A inhibitor; nNOS, neuronal nitric oxide synthase; NPLA, nNOS inhibitor; GDP-β-S, G-protein inhibitor; sGC, soluble guanylyl cyclase, NO receptor. Red lines depict inhibitory actions, green arrows refer to the signal transduction pathway resulting in excitatory action of NO.

Results II.

Thesis 5: IGF-1 modulates the GnRH neurons of prepubertal and pubertal male mice

IGF-1 significantly elevated the frequency of spontaneous postsynaptic currents, action potential and miniature postsynaptic currents of GnRH neurons in approximately half of the measured GnRH neurons in prepubertal male mice. This stimulatory effect was dose dependent.

We also demonstrated that IGF-1 increases the frequency of mPSCs in half of the GnRH neurons of pubertal male mice too.

Thesis 6: IGF-1 modulates the GnRH neurons directly via IGF-1 receptor

The frequency-increasing effect of IGF-1 on the mPSCs was prevented by the specific IGF-1 receptor antagonist (JB1). This suggests the functional role of the IGF-1R expressed in GnRH neurons

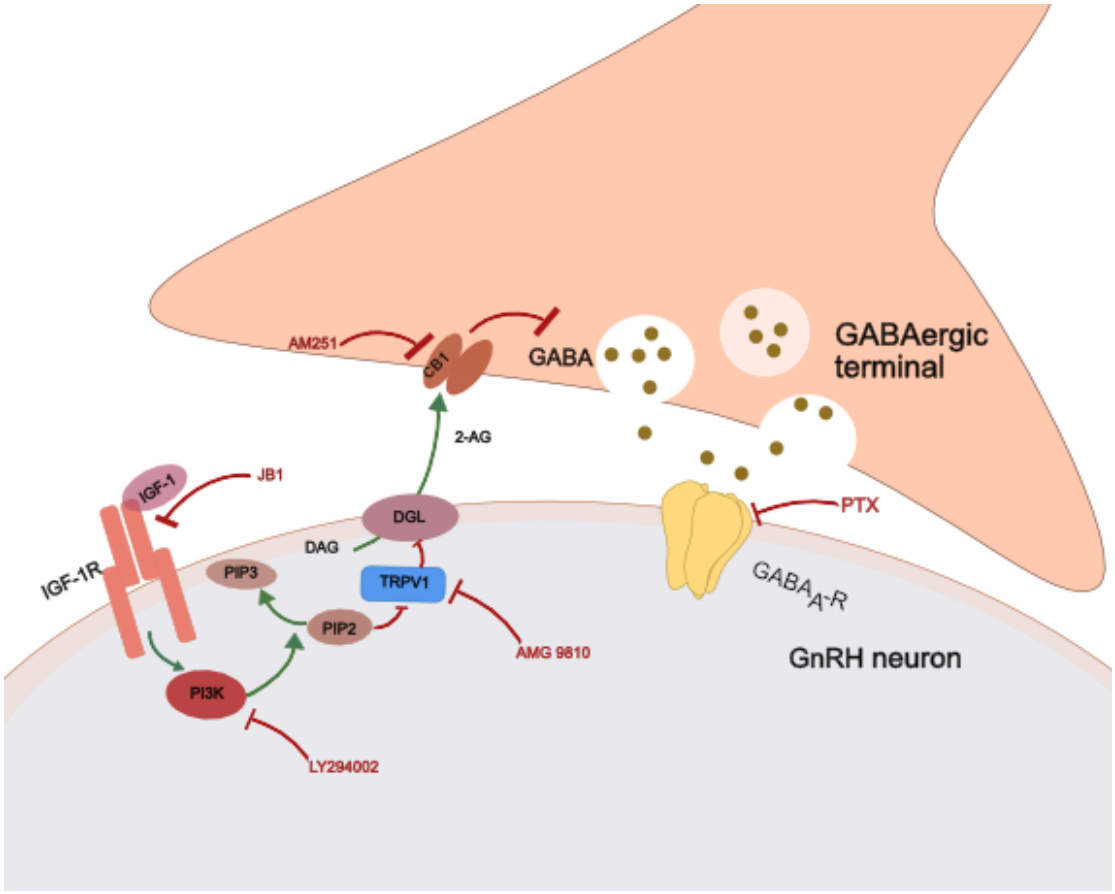
Thesis 7: Retrograde endocannabinoid signaling pathway is involved in the effect of IGF-1.

The relationship between IGF-1 and endocannabinoid systems was confirmed when IGF-1 was not effective during the blockade of cannabinoid receptor type 1 (CB1). The role of transient receptor potential cation channel subfamily V member 1 (TRPV1) in the signaling mechanism was also demonstrated in our experiments. Intracellular blockade of TRPV1 eliminated the effect of IGF-1 on the mPSCs.

Blockade of CB1 and the intracellular blockade of TRPV1 supported the view that 2-arachidonoylglycerol is synthetized in GnRH neurons and involved in the effect of signals modulating GnRH neuron activity.

Thesis 8: The activation of the retrograde endocannabinoid pathway includes phosphoinositol-3-kinase (PI3K).

PI3K has a major role in the activation of the retrograde endocannabinoid pathway by IGF-1. The intracellular specific blockade of PI3K abolished the frequency elevation triggered by IGF-1.



Schematic illustration of the IGF-1 receptor signaling in GnRH neurons. IGF-1 activates PI3K which leads to the phosphorylation of PIP_2 to PIP_3. In cells, TRPV1 is inactivated by its binding to PIP_2, and after the activation of PI3K, TRPV1 receptor will be released from the PIP_2 blockade. Activation of TRPV1 leads to the blockade of DGL and decreases the postsynaptic production and release of 2-AG resulting in the suppression of inhibition of the presynaptic excitatory GABA release.

Abbreviations: IGF-1R: Insulin-like growth factor 1 receptor; JB1: IGF-1R antagonist; PI3K: Phosphoinositide-3 kinase; LY294002: PI3K blocker; PIP2: Phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5 trisphosphate; DAG: Diacylglycerol; DGL: Diacylglycerol lipase; TRPV1: transient receptor potential cation channel subfamily V member 1; AMG9810: TRPV1 antagonist; 2-AG: 2-Arachidonoylglycerol; CB1: Cannabinoid receptor type 1; AM251: CB1 receptor antagonist; $GABA_A$-R: GABA-A receptor; PTX: picrotoxin.

POTENTIAL APPLICATIONS OF THE RESULTS

The process of reproduction requires energy availability in access. Chronic energy deficiency, usually resulted from reduced food intake, overexercise or stress, can disturb the hypothalamic-pituitary-gonadal (HPG) axis resulting in anovulation mainly due to improper metabolic hormone levels. Nevertheless, it does not necessarily mean that only serious metabolic disorders or energy deficiency might cause problems in reproduction. Dietary changes can also initiate modulation of the metabolic signals in the serum affecting the reproductive process. Hence, it is critical to understand the central control of reproduction for new possible treatments in infertility caused by metabolic disturbances and the scientific fact-based promotion of the importance of balanced diet.

Fluctuations in the metabolic hormone levels are even able to impair the HPG axis orchestrated by GnRH neurons and lead to infertility in humans. Anorexia nervosa, diabetes, and obesity, for example, might be related to anovulatory syndromes. The novel regulatory mechanisms whereby secretin and IGF-1 act on GnRH neurons described in this thesis call attention for the fact that the new drugs developed as obesity and diabetes therapy might also affect fertility. Furthermore, high serum concentration of IGF-1 is detrimental because it is thought to play a role in the pathophysiology of the polycystic ovary syndrome (PCOS). This syndrome is one of the highest incidence disorders causing infertility in women impacting 5-10 % of them. Medication of these patients consumes tremendous amount of energy with huge medical cost. The exploration of IGF-1R related signaling pathways in GnRH neurons provides new insights into the mechanisms operating in these kinds of infertility problems.

My results showed the direct regulatory action of the metabolic signal molecules secretin and IGF-1 on GnRH neurons and elucidated the molecular mechanisms in the downstream actions of these hormones. Our results further support the relevance of dietary changes in reproductive disorders such as PCOS, anorexia, obesity, and diabetes.

The interaction between the metabolic and reproductive systems possesses a significant pathophysiological relevance. The cellular and molecular mechanisms that link energy balance and central regulation of reproduction are still not well understood. By clarifying the effects of secretin and IGF-1 in the central regulation of reproduction, we have contributed to a better understanding of the relation between nutritional status and gonadal function.

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Co-first authorship

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List of publications related to the subject of the thesis

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