DOI:10.15774/PPKE.ITK.2021.010

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

PhD Dissertation

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Budapest, 2021

DOI:10.15774/PPKE.ITK.2021.010

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LIST OF ABBREVIATIONS

2-AG	- 2-arachidonoylglycerol
AC	- adenylyl cyclase
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	-TRPV1 antagonist, (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
ARC	- arcuate nucleus of the hypothalamus
AVPV	- antero-ventral-periventricular nucleus
BBB	- blood-brain barrier
cAMP	- cyclic adenosine monophosphate
CB1	- cannabinoid receptor type 1
CB2	- cannabinoid receptor type 2
CPTIO	- 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
C_m	- membrane capacitance
DAG	- diacylglycerol
DGL	- diacylglycerol lipase
DPN	- diarylpropionitrile
DYN	- dynorphine
E2	- 17β-estradiol
EGTA	- ethylene-glycol-tetraacetic acid
ER	- estrogen receptor
ERα	- estrogen receptor alpha
ERβ	- estrogen receptor beta
FAAH	- fatty acid amide hydrolase
FSH	- follicle-stimulating hormone
GABA	- gamma-aminobutyric acid
$GABA_A$-R	- gamma-aminobutyric acid receptor type A
$GABA_B$-R	- gamma-aminobutyric acid receptor type B
GCs	-granulosa cells
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
GPR54	- G-protein-coupled receptor 54, also called Kiss1R
GT1-7	- immortalized gonadotropin-releasing hormone neuronal cell line
HPG axis	- hypothalamo-pituitary-gonadal axis
IGF-1	- insuline-like growth factor 1
KA	- kainate

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KCC2	- potassium-chloride cotransporter
Kiss1R	- kisspeptin receptor type 1
KO	- knockout
KP	- kisspeptin
KT5720	-protein kinase-A inhibitor, (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester
LH	- luteinizing hormone
LY294002	- 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
ME	- median eminence
mGluR	- metabotropic glutamate receptors
mPSC	- miniature postsynaptic current
NKB	- neurokinin B
NMDA	- *N*-methyl-d-aspartate
nNOS	- neuronal type nitric oxide synthase
NPLA	- nNOS inhibitor, N-propyl L-arginine hydrochloride
NO	- nitric oxide
NOS	- nitric oxide synthase
NPY	- neuropeptide Y
NTS	- nucleus tractus solitarii
OVLT	- organum vasculosum of the lamina terminalis
PCOS	- policystic ovary syndrome
POA	- preoptic area
PSC	- postsynaptic current
PVN	- paraventricular nucleus of the hypothalamus
R_{in}	- input resistance
RP3V	- rostral periventricular area of the third ventricle
sGC	- soluble guanylyl cyclase
SON	- supraoptic nucleus
sPSC	- spontaneous postsynaptic current
TRPV1	- transient receptor potential vanilloid 1
TTX	- tetrodotoxin
vGlut	- vesicular glutamate transporter
VIP	- vasoactive intestinal peptide
V_{rest}	- resting membrane potential

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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. Under certain environmental or physiological conditions, such as in anorexia nervosa, the suppression of reproductive functions is adaptive to survival [1]. Proper interaction of the two systems is, therefore, indispensable for the successful reproduction.

The hypothalamo-pituitary-gonadal axis

In mammals, the hypothalamo-pituitary-gonadal (HPG) axis, as a coherent system, regulates the reproductive functions. At the level of hypothalamus, the gonadotropin releasing hormone (GnRH) neurons are the primary regulators [2, 3]. The pulsatile secretion of GnRH triggers the secretion of gonadotropins (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) in the anterior pituitary, where GnRH binds to GnRH receptors expressed by gonadotropic cells **(Fig. 1.)**. The pulsatility of the GnRH secretion is important in two aspects. First, it helps to preserve the sensitivity of GnRH receptors to GnRH molecule and to prevent the downregulation of these receptors [4, 5]. This pulsatile secretion of GnRH is one mode to avoid desensitization, GnRH receptors are also more resistant to rapid desensitization upon stimulation [6, 7].

Secondly, pulsatility is also important in changing which hormone (LH vs. FSH) is released by the gonadotropic cells. Variations in GnRH pulse frequencies and amplitudes have differential effects on FSH and LH synthesis: FSH cells are activated at low GnRH pulse frequencies, while release of LH is stimulated at high GnRH pulse frequencies [8-10].

In males, gonadotropic hormones act on Leydig and Sertoli cells found in the testes. Leydig cells are in the interstitium adjacent to the seminiferous tubules. LH stimulates testosterone production in Leydig cells. Sertoli cells which are localized within seminiferous tubules, directly support spermatogenesis under the regulatory control of FSH and testosterone [11].

In females, FSH regulates granulosa cells (GCs) that surround the developing oocytes. FSH stimulates the growth and maturation of the immature follicle into Graafian follicle before ovulation. The GCs initially produce estrogen hormones needed for maturation of the developing follicles. Ovarian estrogens are formed by GCs through the aromatization of the androstenedione produced in theca cells of the follicle. After the LH surge, ovulation occurs, and the secondary oocyte is released. The remains of the Graafian follicle undergo transformation and lutein cells are formed from both the theca interna and granulosa cells.

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Gradually, LH transforms this structure into corpus luteum that secrets progesterone [12-14] **(Fig. 1.)**.

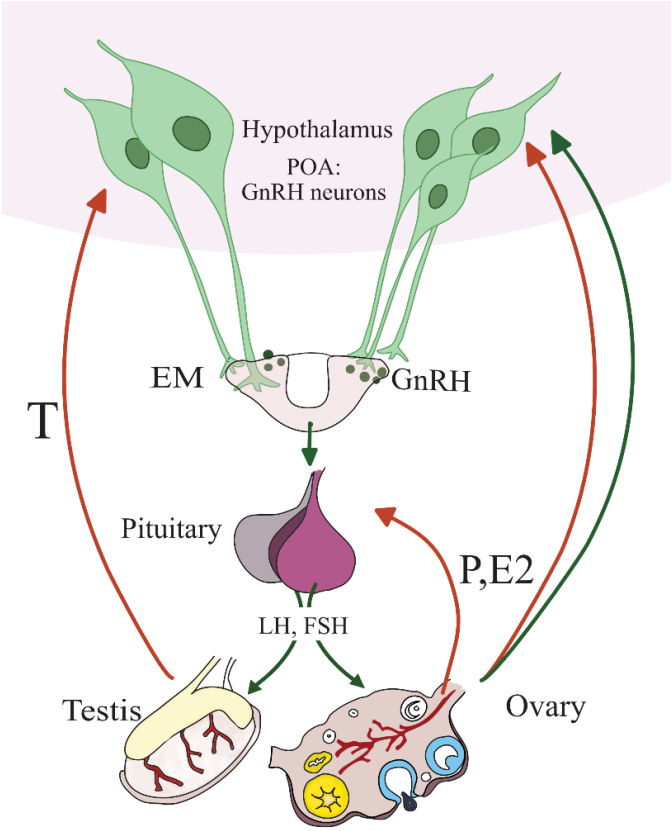

Figure 1.: The main characteristics of the HPG axis. Hypophysiotropic GnRH neurons release GnRH neurohormone into the portal circulation of the pituitary in median eminence (ME). GnRH reaches its target cells in the anterior pituitary and regulates the synthesis and secretion of the follicle-stimulating 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 estradiol (E2), progesterone (P), and testosterone (T) modulate- the hypothalamic and pituitary hormone secretions via feedback loops. Red arrows show inhibition, green arrows represent stimulation. POA: preoptic area

The steroid hormones (such as estrogens, androgens) released from the gonads can regulate LH and FSH secretion via various feedback loops. The indirect long loop feedback occurs at the level of the hypothalamus and influences the production and release of GnRH. The

DOI:10.15774/PPKE.ITK.2021.010

direct long loop feedback targets the pituitary where it modulates the function of gonadotropic cells. As a result, the response of gonadotropic cells to GnRH, and that of the gonads to FSH or LH changes during the estrous cycle in females [8]. In this process, the estrogen feedback is mostly negative, although estrogen can also exert a positive regulatory effect during proestrus on HPG axis preceding ovulation. In males, androgens exert negative feedback effects, both in the hypothalamus and pituitary, where they down-regulate GnRH and LH/FSH release **(Fig. 1., Fig. 3.)**.

Anatomical aspects of gonadotropin releasing hormone neurons

GnRH neurons migrate from the medial olfactory placode via the olfactory bulb and basal forebrain to their final destination, the medial septum, diagonal band of Broca region and the preoptic area [15] [16]. Disorders in the migration process can cause hypogonadotropic hypogonadism which results in the lack of hypothalamic GnRH neurons in the forebrain leading to reproductive deficiencies. The human disease is called Kallmann syndrome [17].

Hypophysiotropic GnRH axons project to the median eminence where they secrete the GnRH decapeptide into the portal circulation for the regulation of the pituitary-gonadal axis [3, 18] **(Fig. 2.)**. Most GnRH neurons have a bipolar morphology, with fusiform shape. The perikarya of these neurons are relatively small, and two processes arise from them which can be two dendrites or one dendrite and one axon. Sometimes the axon arises from one of the main dendrites [19, 20].

GnRH neurons form anatomical and functional networks. Adjacent GnRH neurons might be coupled by tight junctions. In addition, GnRH cells communicate with each other via axons and their collaterals [21] forming axo-somatic and axo-dendritic connections [22]. Intercellular bridges were also observed between neurons both in rat and monkey [23, 24]. These connections between GnRH neurons control the synchronicity of cell functions and the coordinated, pulsatile GnRH release from the neuronal network.

The axons of most hypophysiotropic GnRH neurons terminate in the organum vasculosum of laminae terminalis (OVLT) and median eminence (ME) [25, 26]. The ME is a secretory circumventricular organ, where various hypothalamic neurosecretory neurons release peptide hormones (releasing factors) into the hypophyseal portal vessels. Although in the ME there are no neurons [27] the axons of hypothalamic endocrine neurons terminate around the portal capillary loops. The portal microcirculation connects the hypothalamus with the anterior pituitary and transports the various releasing hormones to the adenohypophysis [8, 28, 29].

GnRH fibers also innervate the OVLT, which is another circumventricular organ of the brain. It is located around the ventral tip of the lamina terminalis [27, 30]. The OVLT, unlike

DOI:10.15774/PPKE.ITK.2021.010

ME, contains neurons and senses various signals which are present in the systemic circulation [27, 30].

It is worthy of mention that axon projections of GnRH neurons can also be found in many regions of the limbic system[3, 31-33].

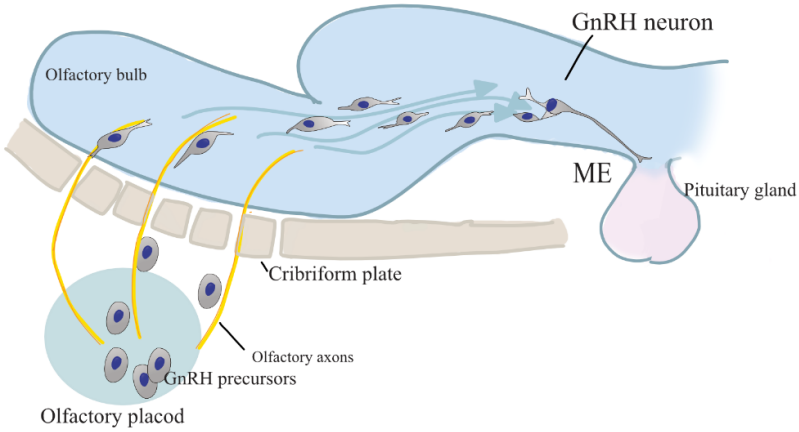

Figure 2.: The migration pathway of GnRH neurons. GnRH neurons migrate from the olfactory placod along with the vomeronasal or olfactory nerve into the brain through the cribriform plate. GnRH neurons settle down in the preoptic area and send axon projections to the median eminence to release GnRH for activation of pituitary gonadotropic cells. Figure is adapted from Sykiotis et al. [34].

The GnRH hormones and their receptors

The decapeptide GnRH - synthesized in GnRH cells - plays a key role in the regulation of reproduction [2, 3]. Various forms of GnRH molecule have been discovered in vertebrates during the decades [35]. The firstly discovered was GnRH-1 which is primarily responsible for the release of the gonadotropins from the anterior pituitary gland. The original name of the molecule was luteinizing hormone releasing hormone (LHRH) and it was first isolated by Schally's group from bovine hypothalami [36]. It is a ten amino acid peptide that is found in the brain of all vertebrate organisms [37].

The second GnRH molecule discovered was GnRH-2. It was first isolated from chicken hypothalamus [38], but since it has also been described in almost all vertebrate species [39]. GnRH2 has been discovered in the midbrain and limbic structures, suggesting a role in

DOI:10.15774/PPKE.ITK.2021.010

the modulation of reproductive behavior in mammals. In primates and humans, GnRH2 is present in the hippocampus, caudate nucleus and amygdala and has also been detected in the midbrain and hindbrain [40] .

The third GnRH molecule discovered till date is called GnRH-3 [37] and has only been identified in the telencephalon of teleosts.

GnRH binds to its specific receptor (GnRHR) which belongs to the G protein-coupled receptor family (GPCRs), with seven transmembrane domains [41, 42]. In vertebrates, three GnRH receptors or receptor-like sequences have been identified [43, 44]. In mammals Type I and Type II GnRH receptors have been characterized, and Type III was found only in fish, amphibians, and a few mammals [37, 43].

The Type I receptor is the one that is functional and predominant in the mammalian gonadotropic cells. Nevertheless, in some species, including humans, it is also expressed in reproductive tissues like breast, endometrium, ovary, and prostate [45].

Electrophysiological properties of GnRH neurons

In the past, the *in vitro* acute brain slice experiments with GnRH neurons were difficult, because only a couple of hundreds GnRH neurons are distributed in the mammalian hypothalamus. The efficacy of studying the electrophysiological properties of GnRH neurons in brain slices significantly increased after generation of the GnRH-GFP transgenic mice. In this transgenic animal model, green fluorescent protein is genetically expressed in GnRH neurons under the control of GnRH promoter gene. This structure allows the detection of GnRH neurons in slice preparations[46, 47]. Our experiments were performed in this type of transgenic mice.

The main electrophysiological properties, such as resting membrane potential, firing and passive parameters of GFP expressing GnRH neurons [46, 47], are similar to the unlabeled GnRH neurons using either brain slices, primary cell cultures [48, 49] or immortalized GnRH expressing neuronal cell line (GT1) [48, 50-52]. In the first experiments GnRH neurons in brain slices were identified after the recordings by single cell RT-PCR [48].

Resting membrane potential (V_{rest}) of GnRH-GFP neurons is usually between -55 to -65 mV. The input resistance (R_{in}) of GnRH neurons in physiological conditions is near 1 GΩ [47]. The high input resistance means that these cells have only a few channels open at the resting membrane potential. This is an important feature of GnRH neurons, as the conductance that contribute to intrinsic firing activity are relatively small [53].

Most GnRH neurons present intrinsic burst-type firing activity, although different inputs can modulate this pattern [53]. The amplitude of action potentials is around 60 mV [47].

DOI:10.15774/PPKE.ITK.2021.010

Immortalized GT1 neurons [54], and acutely dissociated GnRH neurons continue to fire in a burst pattern, therefore the synaptic connections are not substantial for this kind of firing of GnRH neurons [55]. This observation was confirmed in the experiments where firing of GnRH neurons in acute brain slices remained active after the selective blockade of ionotropic GABA and glutamate receptors, elements of the main fast synaptic transmission inputs to these cells [56].

In addition to burst firing, experiments in acute brain slices showed continuously firing and silent GnRH neurons [47, 48, 53, 57]. The three observed firing patterns are present in both gonadectomized and intact male and in female mice [48]. *In vivo* GnRH neurons show more variety in their firing frequency and these experiments showed that less GnRH neurons exhibit burst firing in mice compared to the *in vitro* experiments. The *in vivo* observed firing patterns were critically dependent upon $GABA_A$ receptor signaling [58].

Voltage-gated ion channels are expressed in the dendrites of GnRH neurons, and the dendrites are capable of action potential generation [59]. In mice, GnRH neurons unlike other neurons possess a single projection structure that functions simultaneously as a dendrite and axon, called dendron. It was shown that their projection to the median eminence to control pituitary hormone secretion possesses a spike initiation site and conducts action potentials while also exhibiting spines and synaptic appositions along its entire length [60].

A recent study has shown that the anatomically distinct dendritic compartments of GnRH neurons can generate pulse or surge modes [61]. *In vivo*, selective chemogenetic inhibition of the GnRH neuron distal dendrite abolishes the luteinizing hormone (LH) surge and dampens LH pulses [61]. In contrast, chemogenetic and optogenetic inhibition targeting the GnRH neuron soma-proximal dendritic zone abolishes the LH surge but have no effect upon LH pulsatility. These observations indicate that whereas electrical activity at the soma-proximal dendrites of the GnRH neuron are essential for the LH surge, meantime, the distal dendron represents an autonomous zone where synaptic integration drives pulsatile GnRH secretion [61].

Properties of the GnRH neuronal system

Many reproductive disorders are associated with disruption of pulsatile secretion of GnRH. For instance, GnRH pulses that are faster than in normal conditions, have been observed in polycystic ovary syndrome (PCOS), a common disorder that affects almost 10% of the female population of reproductive age [9, 62].

Hypothalamic GnRH pulse frequency and amplitude is important for the physiological secretion of gonadotropins (LH, FSH). GnRH pulses occur every 30-90 minutes and both the proper frequency and amplitude are necessary for gonadotropin release [8]. Since the normal

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functioning and pulsatility of GnRH neurons is crucial in reproduction, in this section I will discuss the regulating mechanisms that modulate the function of GnRH neurons.

The kisspeptinergic afferents of GnRH neurons

Although GnRH neurons were first described in 1971, their afferent neuronal connections are still not fully understood [63]. Nevertheless, one of the most important neuronal inputs to GnRH neurons are kisspeptin/Neurokinin B/dynorphin (KNDy) expressing cells of the arcuate nucleus (ARC) in rodents [64]. This region is analogous to the infundibular nucleus in primates. The second population is the kisspeptin neurons of the rostral periventricular region of the third ventricle (RP3V) which forms a compact nucleus in the rodent brain, or in the POA in primates and sheep [65]. In young male humans, however, neurokinin B (NKB) expressing neurons do not synthesize detectable amount of kisspeptin (KP) or dynorphin (DYN), which shows that the functional importance of the three neuropeptides in reproductive regulation is different between sexes and species [66].

The receptor of kisspeptin is the G protein-coupled membrane receptor, GPR54 or as it is later called Kiss1r [67]. The Kiss1r receptor is expressed within the rodent hypothalamus predominantly in the ARC and in the POA mostly on GnRH neurons **(Fig. 3.)** [68-70]. Outside of the hypothalamus, Kiss1r can be found in the hippocampus [67, 69].

The key action of kisspeptin (KP) in the hypothalamo-pituitary-gonadal axis occurs directly at the level of GnRH neurons since GnRH neurons express GPR54 [71]. The direct effect of KP-producing neurons on GnRH neurons is supported by numerous observations. It was shown that KP axons innervate the perikarya and dendrites of GnRH neurons [72] and they respond to KP with increased neuronal activity [73]. Administration of KP results in an increase in serum gonadotropin levels via the stimulation of the secretory activity of GnRH neurons [71]. Selective optogenetic activation of KP neurons in rats, also increases the LH concentration in the serum [74]. Furthermore, kisspeptin depolarizes GnRH neurons [75] and increases GnRH neuron firing in vivo, too [58]. Significance of kisspeptin in the reproductive axis is further supported by the observation that in humans mutations in the KP gene or GPR54 can cause hypogonadotropic hypogonadism [76, 77], while overactivation of this system causes precocious puberty [78].

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The role of 17β-estradiol in the regulation of GnRH neurons

Estrogens in females are produced primarily by the ovaries. All sex steroids derive from cholesterol [79]. There are three important natural estrogen types: estrone (E1), estradiol (E2) and estriol (E3) The most potent one is 17β-estradiol (E2), which is produced by aromatase from testosterone or is converted from estrone at the end of the biosynthesis process [80]. The enzymes responsible for estrogen synthesis are also expressed in other neurons and astrocytes [79]. GnRH cells are regulated by E2, the main homeostatic feedback molecule operating between gonads and brain [81].

E2 is one of the most important substance modulating function of GnRH neurons. E2 can inhibit or stimulate GnRH release. This effect can be both indirect and/or direct and dependent on the estradiol concentration. Estradiol feedback mechanisms alter the synaptic transmission to GnRH neurons and their intrinsic excitability [82-84].

E2 exerts its effect through the classical, nuclear signaling or the fast, non-classical, membrane-initiated signaling [85-90]. In the nuclear (also called genomic) signaling pathway, E2 diffuses through the cell membrane and activates estrogen receptors (ERs) [91, 92]. This activation triggers receptor dimerization. The most known two receptor subtypes (ERα and ERβ) can form homo or heterodimers. The dimeric receptor form binds to the estrogen response element in the promoter regions of genes in the nucleus leading to activation or suppression [91, 92].

Indirect E2 effect is mediated among others via kisspeptin neurons which express ER receptors and mediate the effect of estradiol to GnRH neurons **(Fig. 3.)**. During negative feedback, low physiological concentration of estradiol suppresses gonadotropin secretion by inhibiting hypothalamic GnRH release [81, 84]. In females, the ERα expressing kisspeptin neurons of ARC nucleus are one of the key elements of this regulatory mechanism [86, 93].

In females, the RP3V population in the presence of high estradiol concentration is able to mediate the positive feedback of estradiol [86] in proestrus before the onset of ovulation [81, 94-96]. During positive feedback KP neurons in the RP3V release KP in response to E2 [86].

It has been long thought that estrogen feedback mechanisms regulate GnRH neurons only indirectly. Changes in the function of GnRH neurons were thought to be mediated exclusively by estradiol-sensitive afferent systems and the population of KP neurons located in arcuate nucleus was identified as the main regulator of estrogen negative feedback [86, 93] and that of the positive feedback via the KP neurons in the RP3V. This view was supported by the lack of ERα in GnRH neurons [93, 97-101].

The discovery of ERβ in GnRH neurons [99, 102-104] however, showed that estradiol can exert a direct effect on GnRH neurons, thus making this subtype of estrogen receptor an important player in the steroid feedback **(Fig. 3.)** [105]. ERβ is involved in both negative and

DOI:10.15774/PPKE.ITK.2021.010

positive feedback mechanisms. The direct negative feedback of E2 on GnRH neurons exerts tonic inhibition via the retrograde endocannabinoid system, regulated by ERβ [106]. This suppression decreases the excitatory GABAergic neurotransmission to GnRH neurons. In contrast, the stimulatory action in GnRH neurons during the positive feedback takes place via the activation of the ERβ-associated retrograde nitric oxide (NO) signaling to facilitate excitatory GABAergic and glutamatergic synaptic inputs to GnRH neurons [107].

Neurotransmitter regulation of GnRH neurons

The neuronal inputs of GnRH cells include a wide variety of neurotransmitters. Importance of neurotransmission in the function of GnRH neurons is emphasized by the observation that the pulsatile secretory activity of GnRH neurons is influenced by alterations in the synaptic inputs [81, 108]. Furthermore, Kisspeptin neurons are often coexpressing classical neurotransmitters, including γ -aminobutyric acid (GABA) and glutamate, which play a critical role in the control of GnRH neuron activity, particularly, in mediating steroid hormone feedback [105, 109-111].

The dendrites of GnRH neurons receive synapses from VGlut1 and VGlut2 containing neuronal fibers [112] suggesting presence of glutamatergic inputs. GnRH neurons indeed receive functional glutamatergic transmission via alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainic acid (KA) and N-methyl-D-aspartate (NMDA receptors [46, 113, 114][16,22,132][106]. Glutamate signaling is excitatory to GnRH neurons via these receptors [113] . AMPA-mediated currents in the proximal segments of dendrites of GnRH neuron can initiate action potential [20], whereas those ones received by the distal segments of the dendrite cannot. Numerous compelling evidences suggested that these inputs play role in the orchestration of estrous cycle. In young female rats, it was proved that glutamatergic synaptic appositions are increased, and GABAergic appositions are decreased in the proestrus stage of the cycle, but this kind of change disappeared with aging [108]. Glutamatergic inputs show gonadal cycle dependency in mice. During the preovulatory surge, the spine density increases in GnRH neurons [115], a morphological change typically associated with increased glutamatergic input in other neuronal systems.

Metabotropic glutamatergic neuromodulation is also present in GnRH neurons [113]. The family of metabotropic glutamatergic receptor (GluRs) consists of three groups (mGluRs I, II and III) [116]. These receptors are G-protein coupled receptors and are responsible for slower glutamatergic neuromodulation. The mGluRs in the first group (mGLUR I) are localized postsynaptically both in neurons or glial cells and their activation increases the intracellular Ca^{2+} level. The second and third groups (mGluR II and III) are located presynaptically and are

DOI:10.15774/PPKE.ITK.2021.010

responsible for feedback regulations [113]. Agonists of the presynaptic mGluR II and III decrease the frequency of GABAergic events in GnRH neurons [117].

The neurotransmitter GABA also plays a pivotal role in the afferent regulation of GnRH neurons [81]. GABAergic synapses on GnRH neurons were described too [118] and GnRH neurons express functional $GABA_A$, and $GABA_B$ receptors [46, 113, 114]. It is a well-known fact that GABA can trigger either excitation or inhibition, through $GABA_A$ receptor, depending on the intracellular chloride concentration of GnRH neuron, whereas only inhibition is evoked via $GABA_B$-R [82, 119, 120]. Mature GnRH neurons maintain high intracellular chloride concentration which can result in excitation through $GABA_A$-R activation in adult mice [82] [121] and rats [122, 123] [124, 125]. Although in the past it has been raised that GABA inhibits GnRH neurons via $GABA_A$-R, the current view is rather in favor of its stimulatory effect [82, 119, 122, 126-128].

The level of putative innervation to GnRH neurons from presynaptic GABA and glutamatergic terminals is not different between males and females in the diestrus stage of the cycle, demonstrating no apparent sexual dimorphism in innervation patterns in this hormonal state. However, the estrogen positive feedback results in increased GABAergic input to GnRH neurons, suggesting the steroid hormone-induced plasticity of GABAergic terminals communicating with GnRH neurons [129].

As mentioned above, GnRH neurons express the metabotropic $GABA_B$ receptor [130, 131]. $GABA_B$ receptor is mainly linked to potassium channels which means that $GABA_B$ receptors are responsible for inhibitory postsynaptic currents (iPSCs) in GnRH neurons [131-133]. In addition, $GABA_B$-R might be able to reduce activity of calcium channels which also contributes to its inhibitory action.

It is worthy of note that GnRH neurons themselves express mRNA of the vesicular glutamate transporter vGlut2 in rats [134], indicating that GnRH neurons could release glutamate – in addition to GnRH -from their nerve terminals. Recently, it has also been demonstrated that certain GnRH neurons are GABAergic in the mouse brain showing the heterogeneity of hypothalamic GnRH neuron population [135].

DOI:10.15774/PPKE.ITK.2021.010

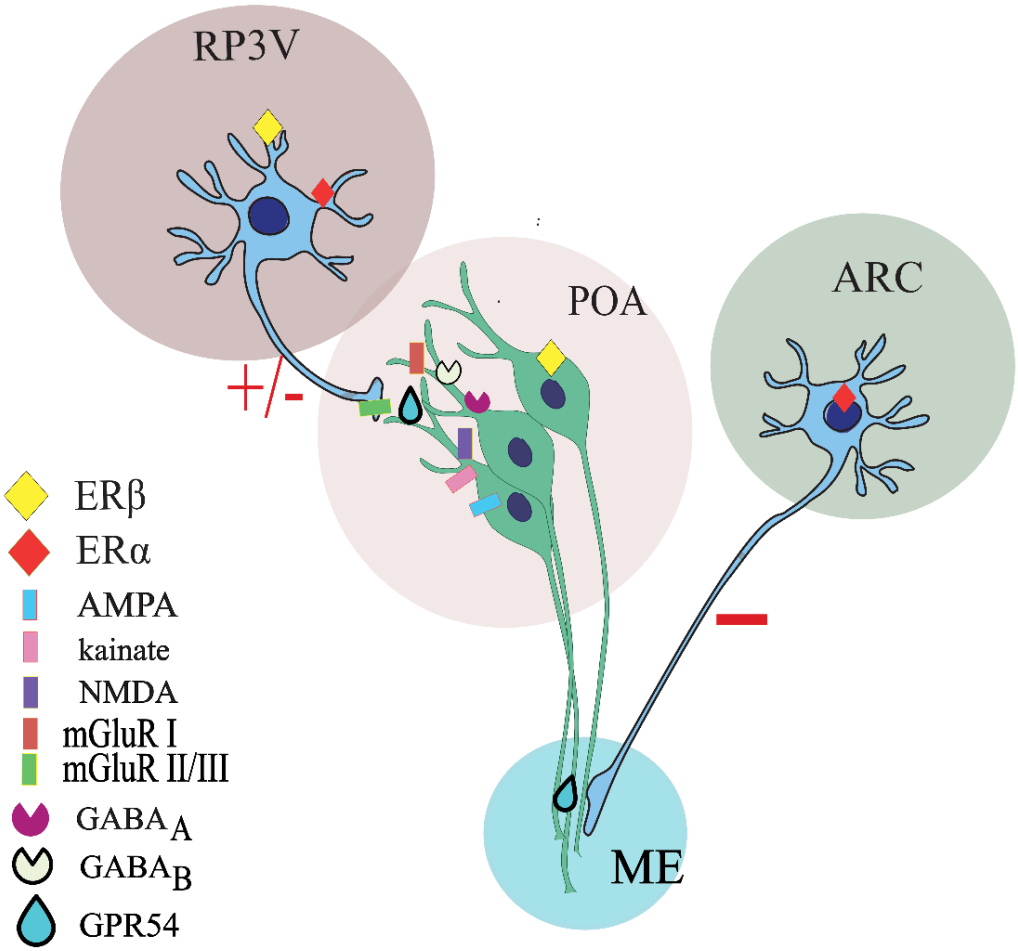

Figure 3.: The main regulatory inputs of GnRH neurons.
GnRH neurons express AMPA, kainate, NMDA, $GABA_A$ and $GABA_B$ receptors. GnRH neurons also express GPR54 receptor, which is the receptor of kisspeptin. In males, both ARC and RP3V populations of kisspeptin neurons modulate the negative feedback. In females, the RP3V population of kisspeptin neurons in the presence of high estradiol concentration mediates the positive feedback prior to ovulation. Abbreviations: ERα: estrogen receptor alpha, ERβ: estrogen receptor beta, AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, NMDA: *N*-methyl-d-aspartate, $GABA_A$: gamma-aminobutyric acid receptor type A, $GABA_B$: gamma-aminobutyric acid receptor type B

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Retrograde signaling pathways

One of the important mechanisms modulating glutamatergic and/or GABAergic synaptic inputs to neurons is the retrograde signaling. Retrograde signaling from the postsynaptic cell to the presynaptic axon terminals is critical in the formation and plasticity of synaptic connections [136].

Retrograde machinery means, that a retrograde messenger is released by the postsynaptic cell and binds to its receptor in the presynaptic axon terminals where it modulates neurotransmitter release. Retrograde messengers, similarly to the conventional neurotransmitters, need to meet several criteria to be considered as a retrograde signal [137] :

1. The machinery for synthetizing and releasing retrograde messenger needs to be located in the postsynaptic neuron.
2. Inhibition of the synthesis in the postsynaptic neuron stops the retrograde mechanism.
3. The retrograde messenger must act at the presynaptic terminal.
4. Disruption of the machinery in the presynaptic terminals eliminates the retrograde signaling.
5. Exposure of the presynaptic receptors to the retrograde substance should mimic the physiological retrograde signaling process.

The retrograde messengers discussed in this dissertation are the retrograde nitric oxide (NO) and retrograde endocannabinoid ones. In GnRH neurons both signaling pathways were described as an important regulatory mechanism, which can influence reproduction [106, 138-140].

One of the important features of these retrograde mechanisms is that in contrast to classical neurotransmitters, both NO and endocannabinoids are not synthesized ahead of time and are not stored in synaptic vesicles.

Nitric oxide

Nitric oxide (NO) was originally found to be released from endothelial cells and it was called endothelium-derived relaxing factor mediating vascular relaxation [141] [142]. NO is a gas molecule, synthesized by the enzyme NO synthase (NOS) from L-arginine [143]. Three types of NOS have been described so far: neuronal-type NOS I (nNOS), endothelial-type (eNOS), and inducible NOS (iNOS) form [144]. After its synthesis NO diffuses across the cell membranes and binds to its receptor [145, 146] the soluble guanylyl cyclase (sGC) which is located in the cytoplasm [142]. nNOS immunoreactivity is present in most areas of the peripheral and central nervous system [147], with a more dense labelling in the cerebellar cortex, hippocampus, striatum, olfactory bulb and the hypothalamus [148].

DOI:10.15774/PPKE.ITK.2021.010

The regulating effect of NO in the central nervous system (CNS) has been extensively studied, and the important role as a retrograde signaling molecule was first mentioned in connection with the NMDA-mediated long-term potentiation (LTP) [147, 149]. The NO is released into the extracellular space and facilitates LTP and spontaneous presynaptic neurotransmitter release in the hippocampus [150], in the paraventricular nucleus in the hypothalamus (PVN) [151] and in the cerebellum [152]. *In vitro* and *in vivo* studies demonstrated, that in the PVN, NO has a pivotal role in the NPY-related modulation of food intake [153]. The metabolic factor leptin also has an NO mediated effect in the preoptic area of the hypothalamus [154].

In the POA, GnRH neurons express nNOS, and therefore, can generate NO. The effect of estradiol on GnRH neurons at proestrus afternoon is mediated by NO and this gaseous transmitter accelerates GABA and glutamate transmission to GnRH neurons [107]. These results strengthen the view that retrograde NO signaling is involved in the central regulation of ovulation.

Endocannabinoids

The active component of cannabis, Δ9-tetrahydrocannabinol [155] binds to specific receptors in the brain [156]. The firstly discovered cannabinoid receptor was CB1, and it is usually referred as the neuronal cannabinoid receptor in the CNS. The CB2 was discovered later [157] and originally thought to be important only in immunological mechanisms. Later, the CB2 distribution has been shown in multiple areas in the CNS in neurons [158-162], and also in glial cells [163]. Both CB1 and CB2 are G-protein coupled receptors, connected to G_i or G_0 proteins inhibiting adenylyl cyclases (AC) [164] and thus blocking various intracellular pathways.

Endogenous ligands of CB1 and CB2 receptors are referred as endocannabinoids. The two most known endocannabinoids are anandamide (AEA) [165] and 2-arachidonoylglycerol (2-AG) [166]. 2-AG is a more potent activator of CB1 and CB2 than anandamide [167-169]. Precursors of endocannabinoids are present in every cell membrane. They are released in one or two steps by various triggers, such as the depolarization of the neuron [170] [171]. Both 2-AG and AEA are lipid molecules, containing arachidonic acid. Both are generated from membrane glycerophospholipids in different synthesizing pathways [172].

After the depolarization of a neuron, phospholipase C generates diacylglycerol (DAG) from the membrane lipid phosphatidylinositol 4,5-bisphosphate. DAG lipase then converts it into 2-arachidonoylglycerol [137]. Anandamide can be generated from its membrane precursor N-arachidonoil phosphatidylethanolamine by phospholipase D [172].

CB1 receptor is localized in various presynaptic axons, in both GABA and glutamatergic terminals, and therefore, this machinery renders endocannabinoids an important

DOI:10.15774/PPKE.ITK.2021.010

retrograde regulator of neural circuits [173-178]. CB1 was also found in most hypothalamic nuclei [176] indicating that endocannabinoid signaling mechanisms modulate the function of various hypothalamic neuronal networks [179].

Relationship between metabolic signals and GnRH neurons

Reproduction is a highly energy consuming process. It is therefore vital for the body to be prepared for optimal conditions when energy can be consumed for reproduction without any high risk. Availability of food, thus nutritional state can cause fluctuations in the level of metabolic hormones (such as leptin, IGF1, ghrelin, etc.) having major effects upon reproduction [180]. Importance of examining the role of metabolic molecules in reproduction is emphasized by extensive studies carried out in relation to the high risk of infertility in case of metabolic diseases.

Hormone production and neurohormone release of GnRH neurons are regulated by diverse neuronal circuits of the brain [181, 182] and by various endocrine hormones and metabolic signals arriving from the periphery [139, 140, 183-187]. Our group showed earlier that various metabolic factors, such as ghrelin and GLP-1 can indeed act directly on GnRH neurons [139, 140]. It is indisputable by now that GnRH neurons can sense the status of energy homeostasis via action of various metabolic molecules affecting function of GnRH neurons.

Therefore, it is indispensable to reveal how various metabolic factors act on GnRH neurons. In this Thesis I will present two of them, secretin, and insulin-like growth factor 1 (IGF-1).

Secretin

Secretin is an anorexigenic hormone [188], and it can serve as a signal molecule reporting about the energy homeostasis. It was the first hormone discovered in 1902 [189]. It is released from the S-cells in the intestine **(Fig. 4.)** and is secreted in the gut when pylorus of the stomach opens to transfer food into this locus. It is produced in response to the acid milieu to stimulate bicarbonate secretion from the pancreas to neutralize gastric chyme acidity. In the periphery, secretin serves, therefore, as a local signal to pancreas [189]. Those features clearly indicate that secretin can indeed be considered as a signal molecule of the high energy status of the body. Furthermore, it can cross the intact blood-brain barrier (BBB) [190, 191] and serve as a peripheral metabolic signal to neurons in numerous brain regions.

Secretin is synthesized not only peripherally but also in several brain areas. The most intensive secretin immunoreactivity was detected in the Purkinje cells of the cerebellum and in some of the neurons of the deep cerebellar nuclei. Secretin immunoreactivity was also observed

DOI:10.15774/PPKE.ITK.2021.010

in a subpopulation of neurons in the primary sensory ganglia [192]. Within the hypothalamus, secretin synthesis was described in the supraoptic nucleus (SON) and in the magnocellular compartment of the paraventricular nucleus (PVN) [193].

The G-protein-coupled secretin receptor has a similar structure and thus belongs to the same receptor subfamily as the vasoactive intestinal peptide (VIP) receptor, and GLP1 receptor [194]. This family also called the secretin receptor family or the B1 GPCR receptor family [195].

Specific binding of secretin to its receptor was demonstrated in various brain areas such as the cerebellum, cortex, thalamus, hippocampus, and hypothalamus [196]. Secretin receptor mRNA showed wide distribution in the CNS. It was detected in numerous brain regions including the area postrema, cerebellum, central amygdala, hippocampus, thalamus, in the cortex, and in the nucleus tractus solitarii (NTS) [197, 198]. Secretin receptor was also observed in the latero-dorsal nucleus of the thalamus and in the hypothalamus [198].

Intracerebroventricular injection of secretin increased the expression of c-Fos in several brain regions including the area postrema, medial region of the NTS, paraventricular hypothalamus, and various cortical areas indicating a central action of the hormone in rats. In contrast, in other areas secretin attenuated c-Fos immunoreactivity [199]. In the hypothalamus, intracerebroventricular administration of secretin stimulated vasopressin expression and release, indicating that it had a role in regulating the water homeostasis by modulating the hypothalamo-neurohypophysial axis [200].

Electrophysiological effect of secretin was examined first in the rat cerebellar cortex, where secretin facilitated the evoked, spontaneous, and miniature GABAergic inhibitory postsynaptic currents (IPSCs) recorded in Purkinje cells. Secretin mRNA was found in the Purkinje cells, and secretin receptor was present in both Purkinje cells and GABAergic interneurons, suggesting an autocrine regulation [201]. In other electrophysiological experiments secretin depolarized neurons of the NTS via nonselective cation channels [202], while in the PVN it modulated the firing rate of the neurons *in vivo* [203].

Although limited information has been available about the exact role of secretin in the regulation of reproduction so far [204], there have been a couple of experimental facts indicating its regulator role in the reproductive axis. In an early study, intracerebral (IC) injection of secretin into the preoptic region of rats resulted in 10-fold elevation of luteinizing hormone (LH) concentration in the plasma [205] suggesting that GnRH neurons might be targeted by secretin. This is in line with our earlier results revealing that GnRH neurons residing in the preoptic area can sense the energy status of the body via various homeostatic signaling molecules such as ghrelin and GLP-1 [139, 140]. Therefore, it is highly conceivable that

DOI:10.15774/PPKE.ITK.2021.010

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 elucidated, yet. 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 secondary messenger cascade events occurring downstream to the secretin receptor in these neurons.

IGF-1

Insulin-like growth factor 1 (IGF-1) is one of the metabolic growth hormone molecules secreted primarily by the liver in adults [206, 207]. It belongs to the insulin/IGF/relaxin hormone family due to its insulin-like tertiary molecular structure and amino-acid sequence [206].

The secretion of growth hormone (GH) from the anterior pituitary into the peripheral circulation stimulates the production of peripheral IGF-1 from the liver **(Fig. 4.)**, or from secondary targets such as lung, kidney, thymus, spleen, heart, muscle, and gonads [208, 209].

The concentration of IGF-1 in the serum decreases during fasting both in humans and rodents [210, 211] . 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 [212].

In the CNS, IGF-1 is secreted in the brain including the olfactory bulb, cerebral cortex, brainstem, and cerebellum [206, 209]. IGF-1 is also expressed in the hypothalamus involving the arcuate, paraventricular, supraoptic, medial preoptic nuclei and the dorsal and lateral hypothalamic areas [213-216], regions known to communicate with GnRH neurons, suggesting various putative actions of IGF-1 on GnRH neurons. In addition to the circumventricular organs, IGF-1 can also enter the brain by crossing the blood-brain-barrier (BBB) [217-219]. Therefore, GnRH neurons may gain excess to both liver and brain-derived IGF-1.

IGF-1 receptor (IGF-1R) belongs to the family of tyrosine kinase receptors [220]. IGF-1R is expressed in various areas of the brain [209], suggesting that neuronal networks in the central nervous system (CNS) are affected by this hormone. The receptor is especially enriched in the circumventricular organs, choroid plexus, hypothalamus, cerebellum, and olfactory bulb [221]. IGF-1R is also highly expressed in the median eminence (ME) [222-224] where hypophysiotropic neurosecretory axons, including GnRH fibers, terminate.

During puberty, the IGF-1 concentration peaks in the plasma suggesting that the hormone shapes this process [225]. Indeed, high IGF-1 level accelerates the onset of puberty both in males and females [209]. In females, low IGF-1 concentration results in impaired estrous cycle [226]. Furthermore, its concentration in the serum is gonadal cycle dependent showing periodic oscillation during the cycle [227, 228]. Since hypothalamic IGF-1R is the

DOI:10.15774/PPKE.ITK.2021.010

most abundant in proestrus, and E2 synergistically and mutually stimulates IGF-1 activity [209], these data indicate an essential role of IGF-1 in the central regulation of reproduction.

In this task it is of particular significance that IGF-1 can directly act on GnRH neurons. IGF-1R is expressed in GnRH neurons [229] and IGF-1 stimulates GnRH production and release [226]. 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 ME during first proestrus [230]. In an *in vivo* experiment, it was also shown in prepubertal female rats that IGF-1 increases GnRH release [231]. In contrast, cell-specific lack of IGF-1R in GnRH neurons delayed puberty [226] and blockade of IGF-1R decreased the number of GnRH neurons expressing c-Fos in young females in proestrus [232]. Mutation in IGF-1 in human patients [233] and GnRH specific deletion of IGF-1R in mice [234] resulted in a significantly delayed puberty providing further evidence for the important role of IGF-1 in puberty. Further data suggested a long-term direct effect of IGF-1 in the GnRH expressing GT1 neuronal cell lines [235, 236]. However, the elements of the signaling pathway has not been fully understood, yet.

Therefore, using *in vitro* electrophysiology we investigated the electric response of GnRH neurons to the IGF-1 administration and the molecular pathways acting downstream to the IGF-1 receptor. According to our earlier papers, various hormones trigger retrograde signaling pathways in GnRH neurons [106, 138, 140] 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 targets the GABAergic presynaptic axon terminals [138], providing strong rationale to examine the role of retrograde signaling to GABAergic afferents in the action of IGF-1.

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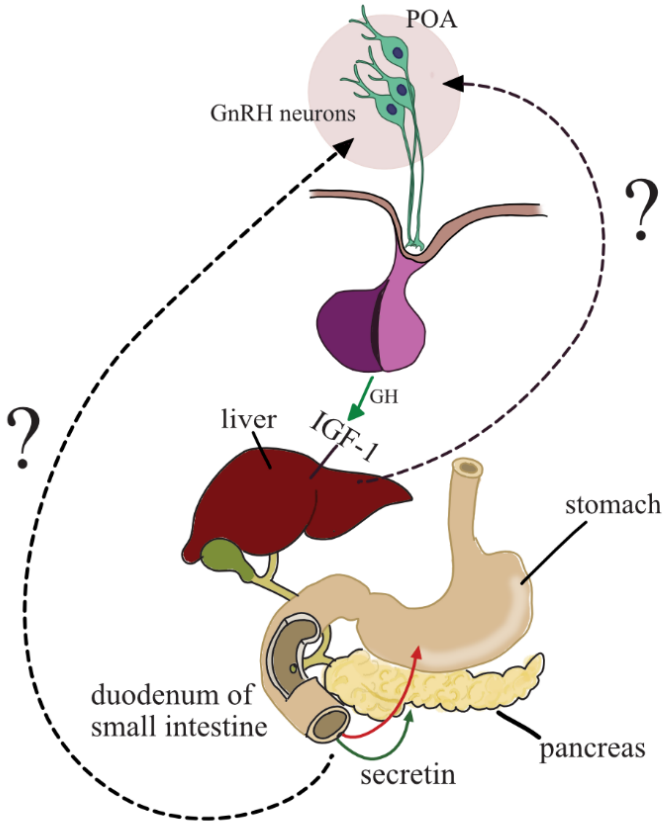

Figure 4.: Schematic figure of the relationship between the peripheral signals and the central regulation of reproduction. Secretin is secreted into the peripheral circulation in the duodenum, and after entering the central nervous system, it can affect GnRH neurons and reproduction. IGF-1 is produced in the liver and also can reach GnRH neurons in the hypothalamus. In our studies, the main focus was on the direct regulation of GnRH neurons by secretin and IGF1. Abbreviations: POA: preoptic area; IGF-1: insulin-like growth factor 1; GnRH: gonadotropin-releasing hormone; GH: growth hormone.

DOI:10.15774/PPKE.ITK.2021.010

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 following questions:

1. Can secretin modulate the electrophysiological properties of GnRH neurons?
2. Is this modulatory effect direct in GnRH neurons via secretin receptor?
3. What sort of signaling mechanisms are involved in this modulatory effect of secretin?

In the second project, I present my results about the regulatory role of insulin-like growth factor 1 (IGF-1) upon GnRH neurons.

I raised the following questions:

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

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MATERIALS AND METHODS

Ethics statement

All animal studies were carried out with permissions from the Animal Welfare Committee of the IEM Hungarian Academy of Sciences (Permission Number: A5769-01) and in accordance with legal requirements of the European Community (Directive 2010/63/EU). All animal experiments described below are designed in accord with accepted standards of animal care and all efforts were made to minimize animal suffering. We carried out sacrifice of animals by decapitation in deep anesthesia by Isoflurane inhalation.

Animals

In the secretin project adult male mice, and in the IGF-1 project pubertal and prepubertal male mice were used. All experimental animals were bred at the Medical Gene Technology Unit of the Institute of Experimental Medicine Hungarian Academy of Sciences (IEM). They were housed in light (12:12 light-dark cycle, lights on at 06:00 h) — and a temperature-controlled environment (22 ± 2°C), with free access to standard food and water. GnRH-green fluorescent protein (GnRH-GFP) transgenic mice bred on a C57Bl/6J genetic background were used for electrophysiological experiments. In this animal model, a GnRH promoter segment drives selective GFP expression in the GnRH neurons **(Fig. 5.)** [47].

Brain slice preparation

Brain slice preparation was carried out as follows [138]. After decapitation, the heads were immersed in ice-cold low-Na cutting solution, continuously bubbled with carbogen, a mixture of 95% O_2 and 5% CO_2, and the brains were removed rapidly from the skull. The cutting solution contained the following (in mM): saccharose 205, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 5, NaH_2PO_4 1.25, $CaCl_2$ 1, glucose 10. Hypothalamic blocks were dissected, and 250 μm-thick coronal slices were prepared from the medial preoptic area (POA) with a VT-1000S vibratome (Leica Microsystems, Wetzlar, Germany) in the ice-cold low-Na, oxygenated cutting solution. The slices containing POA were transferred into artificial cerebrospinal fluid (aCSF) (in mM): NaCl 130, KCl 3.5, $NaHCO_3$ 26, $MgSO_4$ 1.2, NaH_2PO_4 1.25, $CaCl_2$ 2.5, glucose 10, bubbled with carbogen and left in it for 1 hour to equilibrate. Equilibration started at 33°C and it was let to cool down to room temperature.

Recordings were carried out in carbogenated aCSF at 33°C. Axopatch-200B patch-clamp amplifier, Digidata-1322A data acquisition system, and pCLAMP 10.4 software (Molecular Devices Co., Silicon Valley, CA, USA) were used for recording. Neurons were visualized with

DOI:10.15774/PPKE.ITK.2021.010

a BX51WI IR-DIC microscope (Olympus Co., Tokyo, Japan). The patch electrodes (OD = 1.5 mm, thin wall; WPI, Worcester, MA, USA) were pulled with a Flaming-Brown P-97 puller (Sutter Instrument Co., Novato, CA, USA).

GnRH-GFP neurons in the proximity of the vascular organ of lamina terminalis (OVLT; Bregma 0.49–0.85 mm) were identified by brief illumination at 470 nm using an epifluorescent filter set, based on their green fluorescence, characteristic topography and fusiform shape [47].

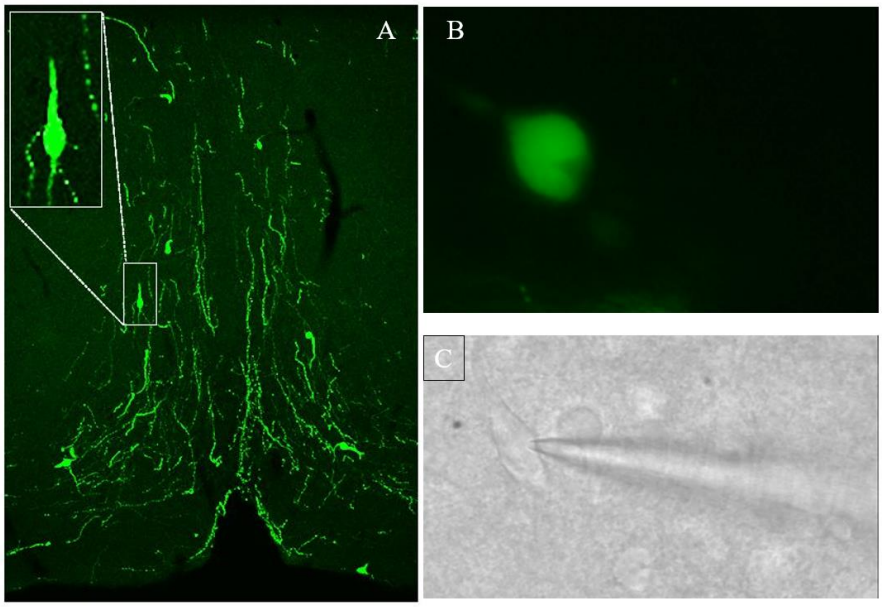

Figure 5: GnRH-GFP neurons and fibers in the organum vasculosum of the lamina terminalis. **A**: Immunocytochemical visualization of GnRH neurons in the OVLT region. **B**: Visualization of an individual GnRH neuron for electrophysiological recordings **C**: Catching a GnRH neuron under visual control. Courtesy of Dr. Imre Farkas and Dr. Csaba Vastagh, IEM Laboratory of Endocrine Neurobiology

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. After the drug application the recording continued for further 10 minutes. Pretreatment with extracellularly used antagonists started 10 minutes before adding the secretin and the antagonists were continuously present in the aCSF during the electrophysiological recordings. 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.

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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	[237]
picrotoxin	GABAA-R blocker	100 µM	Sigma, US	[238, 239]
IGF-1	IGF-1 receptor agonist	1-66 nM	Sigma	[240]
JB-1	IGF-1 receptor antagonist	800 nM	Bachem, DE	[241]
AM251	CB1 endocannabinoid receptor inverse agonist	1 µM	Sigma, US	[138, 140]
TTX	Tetrodotoxin, Voltage gated sodium channel blocker	660 nM	Tocris, UK	[138, 140]
Intracellularly used drugs				
GDP-β-S	G-protein inhibitor (membrane impermeable)	2 mM	Sigma, US	[242-244]
NPLA	neuronal nitric oxide synthase inhibitor	1 µM	Tocris, UK	[245-247]
KT5720	protein kinase-A inhibitor	2 µM	Sigma, US	[248, 249]
AMG9810	transient receptor potential vanilloid 1 antagonist	10 µM	Sigma, US	[250-252]
LY294002	phosphoinositol-3-kinase inhibitor	50 µM	Sigma, US	[253]

Table 1. Chemicals, agonists, and antagonists used in the experiments.

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Patch clamp technique

Patch clamp technique was developed by Sakmann and Neher in the 70s [254] and this work was awarded with the Nobel prize in 1991. It is used to study ionic currents in living individual cells, in brain slices, cell cultures or living animals.

During whole-cell patch clamp measurements the glass pipette is connected to the cell membrane tightly, which can reduce electrical noises and it gives the opportunity to see very small ion current and voltage changes in the cells.

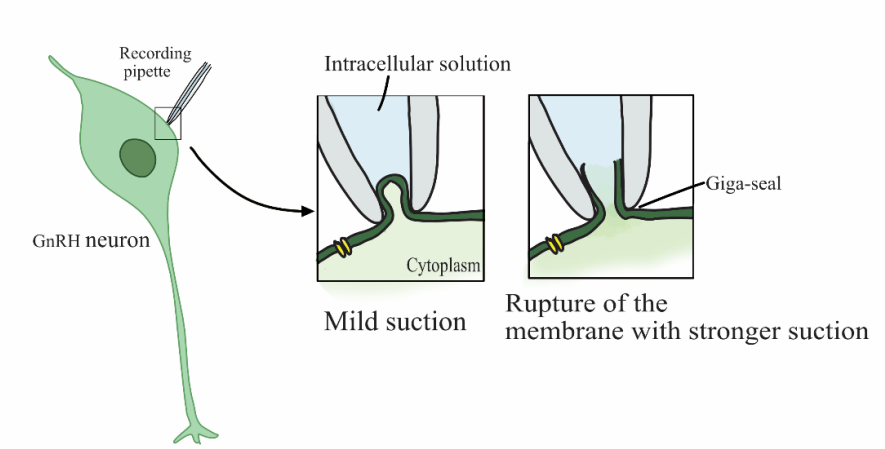

Figure 6.: Whole cell configuration. Whole-cell measurements involve the usage of a single microelectrode. In this method, a glass micropipette electrode with an approximately 1 μm diameter, is pressed against the membrane of a cell. With a slight suction to the inside of the pipette, high-resistance seal forms between the pipette tip and the cell membrane. The piece of sealed membrane is called a patch, and the pipette is called a patch pipette**.** Application of a stronger suction causes the patch to rupture, creating a continuous pathway between the cytoplasm of the cell and the intracellular solution in the pipette.
The figure is based on Edward G. Moczydlowski's figure [255].

The formation of a high-resistance seal between the pipette and the membrane usually causes an omega-shaped deformation of the cell surface. For whole cell patch clamp measurements a stronger suction is applied to break the membrane while maintaining the "giga-ohm" seal **(Fig.6.)** [256]. This small opening on the cell surface makes it possible to monitor multiple currents, and to use different drugs and inhibitors intracellularly **(Fig.6.)** [257]. To avoid dilution of the intracellular components that are important for ion channels during the measurements, it is always indispensable to use an intracellular solution as close to the original cell cytoplasm as possible.

Patch clamping can be performed using voltage clamp or current clamp technique.

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- In current clamp, the injected current is commanded during the recordings and the resulting membrane potential changes are monitored [53]. In our experiments I used this configuration to record action potentials or resting membrane potentials.
- In voltage clamp, membrane potential of the cell is controlled by the experimenter. Channel inhibitors and various membrane potential values can be used to isolate and record individual currents, such as various voltage-gated ion currents and post-synaptic currents (PSCs).

  In our voltage clamp recordings two types of PSC measurements were undertaken [53]. During the first type I measured synaptic events that occur spontaneously in the recorded neuron. These are the spontaneous postsynaptic currents (sPSCs), where the synaptic events represent responses to the spontaneous release of neurotransmitters at synapses on the recorded cell. These can occur due to either the action potential (AP)-dependent neurotransmitter secretion or in a stochastic fashion. In the second type, the AP-dependent events can be isolated from the stochastic ones using the voltage-activated sodium channel inhibitor tetrodotoxin (TTX) to block AP generation and therefore AP-triggered PSCs. The remaining stochastical neurotransmitter release evokes the so-called miniature PSCs (mPSCs).

Whole cell patch clamp experiments

The spontaneous postsynaptic currents (sPSCs) and miniature postsynaptic currents (mPSCs) in GnRH neurons were measured as follows [138]. The neurons were voltage-clamped at –70 mV holding potential. Intracellular pipette solution contained (in mM): HEPES 10, KCl 140, EGTA 5, $CaCl_2$ 0.1, Mg-ATP 4, Na-GTP 0.4 (pH = 7.3 with NaOH). The resistance of the patch electrodes was 2–3 MΩ. Spike-mediated transmitter release was blocked in all mPSC experiments by adding the voltage-sensitive Na-channel inhibitor tetrodotoxin (TTX, 660 nM, Tocris) to the aCSF 10 min before mPSCs were recorded. The mPSCs recorded under the conditions used in our experiments were related to $GABA_A$-R activation [138, 258]. This GABAergic input was validated in our measurements by the $GABA_A$-R inhibitor picrotoxin (100 μM, Tocris). GABAergic input via $GABA_A$-R is excitatory to GnRH cells [119, 121, 123]. Time distribution graphs of frequencies were generated by using 1-min time bins to show time courses of effect of secretin.

Resting membrane potential (V_{rest}) was recorded in current-clamp mode with 0 pA holding current in the presence of TTX.

To show action of secretin on the firing, GnRH neurons of male mice, current clamp measurements were recorded. Three 900-ms-long current steps were applied (-25, 0, and +25 pA). Firing was analyzed during the depolarizing step. After control recording, secretin was pipetted into the measurement chamber and 1, 3, 5, and 10 min later the three current steps were repeated.

DOI:10.15774/PPKE.ITK.2021.010

Only cells with low holding current (<50 pA) and stable baseline were used. Input resistance (R_{in}), and membrane capacitance (C_m) were also calculated from the current step recordings. R_{in} measures cell membrane conductivity, and it is in relationship with the open ion channels at resting condition and indirectly with the size of the cells. If the electrode resistance does not change, the input resistance is a true measure of the membrane resistance. Total membrane capacitance is directly proportional to the membrane surface area, it is an important cellular feature which shows how quickly a cell can respond to current changes [53]. The input resistance was determined from the voltage response to a 1 sec application of hyperpolarizing current. The time constant ($\tau=R_{in}$ x C_m) was the time required to reach 63% of the maximum voltage response to hyperpolarizing current [46]. The C_m was then calculated by dividing the time constant by the R_{in}.

To ensure consistent recording qualities, we monitored the R_{in} together with the access resistance. Access resistance (R_a) is the sum of the pipette resistance and the ruptured patch. The value of R_a is usually around 5 MΩ and it is compensated during the recordings.

Only cells with R_{in} > 500 MΩ, and C_m > 10 pF were accepted. Consistency in the passive parameters shows that the effect of secretin application was not the result of insufficient recording quality.

Spontaneous firing activity of GnRH neurons was recorded in whole-cell current clamp mode at 0 pA holding current.

Statistical analysis

Recordings were stored and analyzed off-line. Event detection was performed using the Clampfit module of the PClamp 10.4 software (Molecular Devices Co., Silicon Valley, CA, USA).

Firing rate, spontaneous postsynaptic current (sPSC) and miniature postsynaptic current (mPSC) frequencies were calculated as number of action potentials (APs) or PSCs, respectively, divided by the length of the corresponding time period. Mean values of the control and treated part of the recording were calculated from these frequency values. All the experiments were self-controlled in each neuron: percentage changes in the firing rate or parameters of the PSCs were calculated by dividing the value of the parameter in the treated period with that of the control period.

Group data were expressed as mean ± standard error of mean (SEM). Two-tailed Student's *t* test were applied for comparison of groups and the differences were considered as significant at $p < 0.05$. Cumulative probabilities of interevent-intervals of neurons were analyzed by using Kolmogorov–Smirnov test ($p < 0.05$) to show statistical differences between the interevent-intervals of the control and secretin treated periods. The analysis of frequency

DOI:10.15774/PPKE.ITK.2021.010

changes in case of the action potentials or PSC groups was carried out by One-way ANOVA with repeated measurements followed by Dunnett's test.

DOI:10.15774/PPKE.ITK.2021.010

RESULTS I.-The electrophysiological investigation of the effect of secretin on GnRH neurons

In this section I describe the results of the secretin experiments.

Secretin increases the frequency of spontaneous postsynaptic currents and depolarizes the membrane potential in GnRH neurons of male mice

Administration of 30 nM secretin revealed no significant change neither in frequency **(Fig.7.)** nor in amplitude parameters of spontaneous postsynaptic currents (sPSC). Rise and decay τ of sPSCs also presented no significant change **(Tables 2, 3, 6)**. Frequency of sPSCs after 100 nM secretin administration resulted in a significant increase up to 118.0 ± 2.64% of the control values (3.244 ± 0.8151 Hz, n=8, Student's t-test, *p=0.0005*) **(Fig. 7a-b Table 2)**. The increase in frequency of the sPSCs started approximately 2 minutes after the administration of secretin, as shown by the distribution graph under the recording (**Fig. 7a**). In contrast, values of amplitude, rise, and decay τ of the sPSCs presented no significant change **(Fig. 7a, Tables 3, 6.)**. Administration of 1 μM secretin also significantly increased the frequency of sPSCs to 124.3 ± 9.404% (control value: 1.914 ± 0.519 Hz, Student's t-test, n=7, *p=0.049*) **(Fig. 7b, Table 2)**. The bar graph shows the percentage changes in the frequency of sPSCs resulted from secretin application, demonstrating the dose dependency of the effect of secretin **(Fig. 7b)**. Values of amplitude, rise, and decay τ of the sPSCs after 1μM secretin administration presented no significant change **(Tables 3, 6.)**.

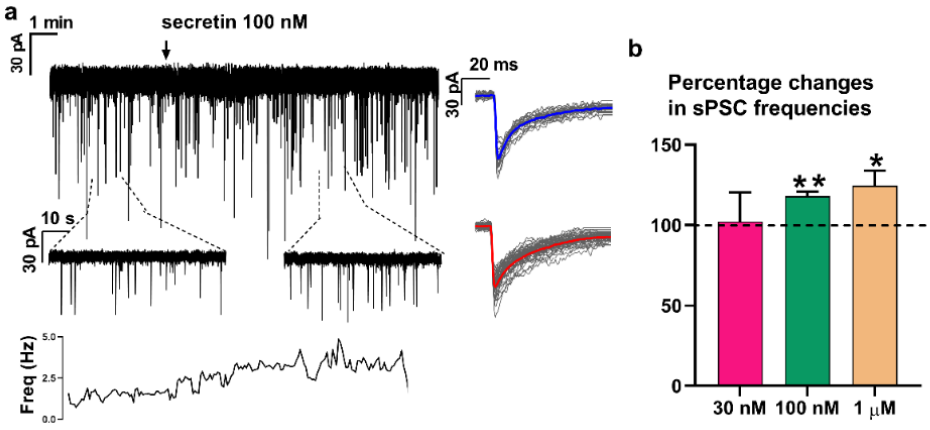

Figure 7. Secretin increases the frequency of sPSCs in GnRH neurons.
a: Secretin increased the frequency of the sPSCs with no change in the average amplitude. Average sPSCs next to the recording represent no change in the shape of events after secretin treatment. The inserts below the 15 min recordings are 1-1 min zoomed periods from the recordings before and after secretin administration. The frequency distribution graph under the inserts also reveals that secretin elevated the sPSC frequency.

DOI:10.15774/PPKE.ITK.2021.010

b: The bar graph shows that effect of secretin on the frequency is dose dependent (Student's t-test *p <0.05; ***p<0.001*).

		Frequency of control period in Hz	Average percentage changes after secretin administration	n/N
sPSC	**30 nM secretin**	1.348±0.442	102.10±4.33	9/4
	100 nM secretin	3.244±0.8155	***118±2.64	8/3
	1 µM secretin	1.914±0.509	*124.3±9.404	7/3
mPSC	**100 nM secretin**	1.367±0.315	*147.6±19.19	16/7
	secretin receptor antagonist +100nM secretin	0.7229±0.2358	92.88±8.949	8/4
	GDP-β-S +100 nM secretin (G protein blocker)	0.632±0.124	102.1±0.957	10/4
	NPLA + 100 nM secretin (nNos blocker)	1.045±0.2297	90.38±4.60	10/5
	KT5720+ 100 nM secretin (PKA blocker)	2.016±0.7367	97±5.987	13/6

Table 2. Changes in the frequency of PSCs after secretin treatment. The first column shows the frequency of the control period in Hz. Second column shows frequency change after secretin treatments in %, the third column shows the number of neurons (n) and animals (N) used for the experiments. (Student's t-test; **p<0.05*; ****p<0.001*)

		Amplitude of control period in pA	Average percentage changes after secretin administration	n/N
sPSC	**30 nM secretin**	31.98±3.628	96.78±3.166	9/4
	100 nM secretin	54.04±7.366	97.38±1.209	8/3
	1 µM secretin	37.91±2.727	99.71±3.160	8/3
mPSC	**100 nM secretin**	41.77±3.061	101.3±2.406	16/7
	secretin receptor antagonist +100nM secretin	47.75±3.034	100.3±2.295	10/5
	GDP-β-S + 100 nM secretin (G protein blocker)	76.56±9.424	101.0±1.483	10/4
	NPLA + 100 nM secretin (nNos blocker)	38.95±3.347	104.1±4.037	10/5
	KT5720 + 100 nM secretin (PKA blocker)	54.04±9.231	99.0±1.665	13/6

Table 3. Changes in the amplitude of PSCs upon secretin treatment.

DOI:10.15774/PPKE.ITK.2021.010

The first column shows the amplitude of the PSCs (pA) in control period. Second column shows changes in amplitudes in %, the third column shows the number of neurons (n) and animals (N) used for the experiments. (Student's t-test)

Secretin increases the frequency of evoked action potentials in GnRH neurons of male mice

The number of evoked action potentials (APs) increased significantly after secretin administration (100 nM) when measured in current clamp mode at 1- and 3-minutes time points. The frequency increased after 1 minute to 144.3 ± 10.8 % (*p=0.0005)* and after 3 minutes up to 138.2 ± 11.24 % compared to the control value (11.56 ± 1.819 Hz (*p=0.0023*)). Firing rate showed no significant changes at other time points **(Fig. 8. a and c, Table 4)** (n=7; One-way ANOVA with repeated measurements).

The rheobase, which shows the strength of the current required to activate at least a *single* action potential, decreased in 6 neurons out of 7, after application of secretin. Firing in 3 neurons of 7 started at 0 pA current injection, suggesting that secretin could increase the spontaneous activity of these neurons. Passive membrane parameters, such as input resistance (R_{in}) and membrane capacitance (C_m) showed no significant changes **(Table 4).** The current step measurements revealed that resting membrane potential (V_{rest}) depolarized significantly at 1 and 3 min (11.92±4.487 mV, $p<0.0451$; 13.82±4.986 mV, $p<0.0392$, **Table 4**) suggesting that elevation in the firing rate resulted from this change in the V_{rest}.

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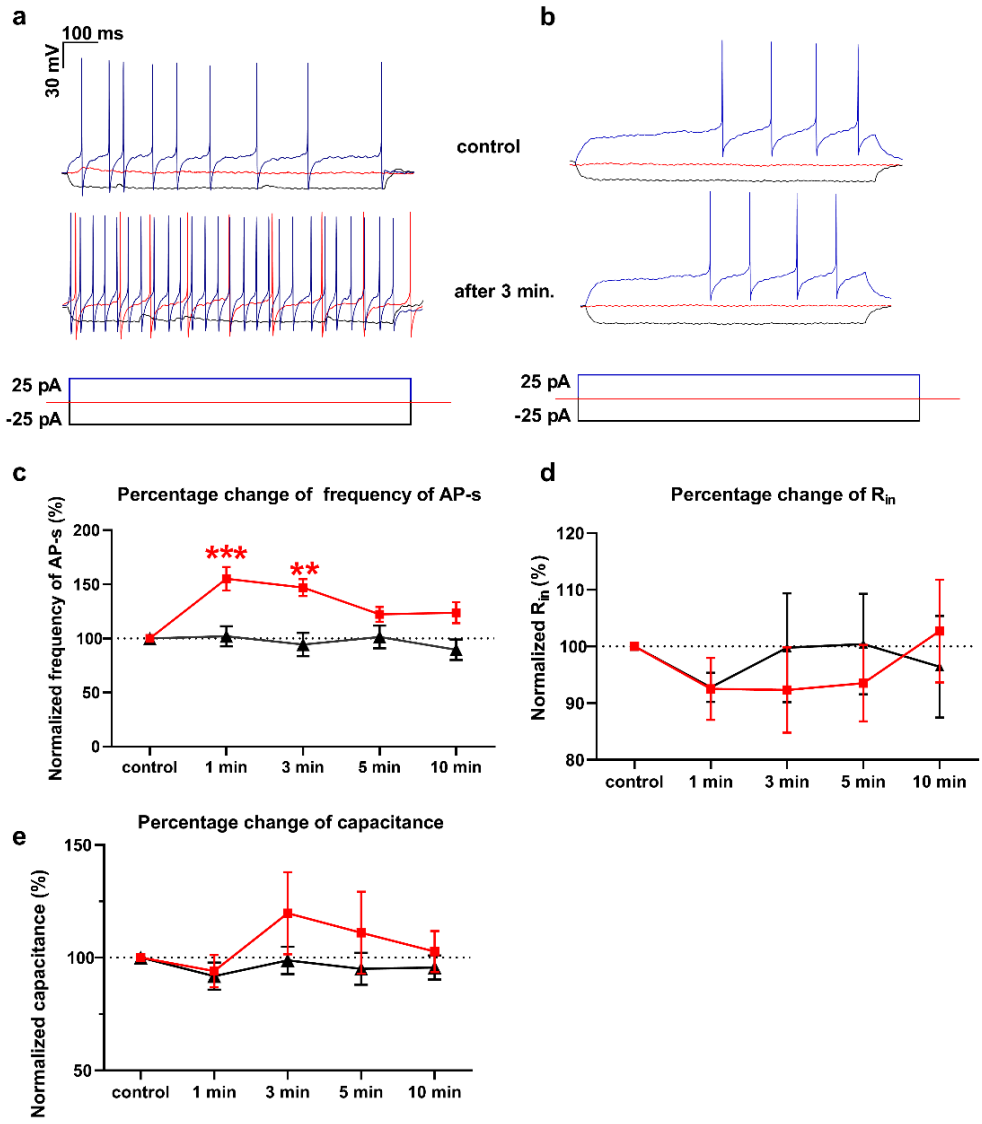

Figure 8.: Secretin (100 nM) increases the frequency of the evoked APs.
a: Representative recording shows that frequency of APs evoked by depolarizing current steps elevated 3 min after secretin administration. Also, the rheobase of APs decreased after secretin treatment. There was no change in the average amplitudes of APs. **b:** Representative recording of the effect of secretin in the presence of picrotoxin **c:** Secretin results in a significant rise in the frequency of Aps after 1 and 3 min of its administration (marked by red ■). In the presence of picrotoxin, however, there was no significant change (marked by ▲). **d:** Changes in the R_{in} represented no significant alteration. **(e)** Membrane capacitance also showed no significant change. (**$p < 0.01$; ***$p < 0.001$).

DOI:10.15774/PPKE.ITK.2021.010

	Control	Average percentage or delta changes after secretin administration				n/N
		1 min	3 min	5 min	10 min	
frequency of APs	11.56± 1.819 Hz	***144.3± 10.8 %	**138.2± 11.24 %	114.7± 9,45 %	123.7± 9.76 %	7/3
capacitance	22.66± 3.93 pF	97.57± 6.09 %	100.6± 7.387 %	106.4± 6.244 %	109.6± 7.422 %	7/3
R_{in}	972± 96.13 MΩ	92.5± 5.476 %	92.33± 7.54 %	93.5± 6.756 %	102.7± 9.087 %	7/3
V rest	70.61± 4.498 mV	11.92±4.487 mV **	13.82±4.986 mV **	10.37± 8.052 mV *	8.540± 6.795 mV	7/3

Table 4. Effect of secretin on the evoked action potentials, the passive membrane properties and V_{rest}.
The first column shows the control values of the frequency, capacitance, input resistance (R_{in}) and the V_{rest}. The next four columns demonstrate the changes in the measured parameters after 1, 3, 5 and 10 minutes. The last column contains the number of neurons (n) and animals (N) used for the experiments. (One-way ANOVA with repeated measurements followed by Dunnett's test; *p<0.05 ***p<0.01*; *** *p<0.001*)

	Control	Average percentage or delta changes after secretin administration in the presence of picrotoxin				
		1 min	3 min	5 min	10 min	n/N
frequency of APs	15.18± 6.878 Hz	102± 9.295%	94.40± 10.79%	101.4± 10.50%	89.60± 9.51%	6/3
capacitance	18.87± 0.8304 pF	91.80± 6.012%	98.80± 6.094%	95.0± 7.162%	95.60± 5.354%	6/3
R_{in}	1048± 116.6 MΩ	92.80± 2.296%	99.80± 9.604%	100.4± 8.875%	96.40± 8.976%	6/3
V rest	65.14±5.042 mV	0.5075± 0.4413 mV	0.3663± 0.3472 mV	0.8924± 0.2314 mV	1.5970± 0.7586 mV	6/3

Table 5. Effect of secretin on the evoked action potentials, the passive membrane properties and V_{rest} in the presence of picrotoxin.
The first column shows the control values of the frequency, capacitance, input resistance (R_{in}) and V_{rest}. The next four columns demonstrate the changes in the measured parameters after 1, 3, 5 and 10 minutes. The last column contains the number of neurons (n) and animals (N) used for the experiments. (One-way ANOVA with repeated measurements followed by Dunnett's test.)

DOI:10.15774/PPKE.ITK.2021.010

Secretin modulates GABAergic synaptic transmission

Earlier studies showed that in GnRH neurons of adult mice the mPSCs are exclusively excitatory via $GABA_A$ receptor [119, 121, 138, 258, 259]. We eliminated all the mPSCs by application of selective $GABA_A$ receptor blocker picrotoxin and after secretin administration no new PSCs could be observed (not shown) suggesting that the recorded mPSCs in these experiments were $GABA_A$ receptor-mediated currents.

We hypothesized that $GABA_A$ receptor plays an exclusive role in the effect of secretin on the firing of GnRH neurons. The $GABA_A$-R blocker picrotoxin totally eliminated the effect of secretin on the evoked APs of GnRH neurons, there was no residual change **(Fig. 8b-c)**. This fact indicates that effect of secretin on the firing rate correlates with the action of secretin on the GABAergic PSC frequency, and the elevation in the firing rate (i.e., the increased excitability) results from the elevated frequency of the GABAergic PSCs. Other passive membrane parameters, such as input resistance (R_{in}) and membrane capacitance (C_m) also showed no significant changes. **(Fig. 8d-e, Table 5.)**.

The input resistance is the sum of the membrane resistance and the electrode resistance [260]. Supposing that electrode resistance does not change during the measurement, the input resistance is a true measure of the membrane resistance. Therefore, if the input resistance shows no change, it indicates that membrane resistance presents no change too. The current step measurements also showed, that in the presence of picrotoxin V_{rest} calculated from the current steps presented no significant change at any time point (**Table 5.**) indicating that GABAergic neurotransmission plays role in the membrane depolarization which eventually results in the elevation in the firing rate.

Secretin acts directly on GnRH neurons via secretin receptor

In order to demonstrate the direct action of secretin on GnRH neurons, miniature postsynaptic currents (mPSCs) were recorded in the presence of TTX. The administration of secretin (100 nM) resulted in a significant increase in the mean mPSC frequency reaching 147.6 ± 19.19% of control values (1.367 ± 0.315 Hz, n = 14; Student's t-test; p =0.0274) (**Fig. 9a, Table 2, 3, 6)**. Elevation of the mPSC frequency started 1-3 min after administration of secretin. Values of amplitudes rise τ, and decay τ of the mPSCs presented no significant change **(Fig. 9a, Table 6,)**.

DOI:10.15774/PPKE.ITK.2021.010

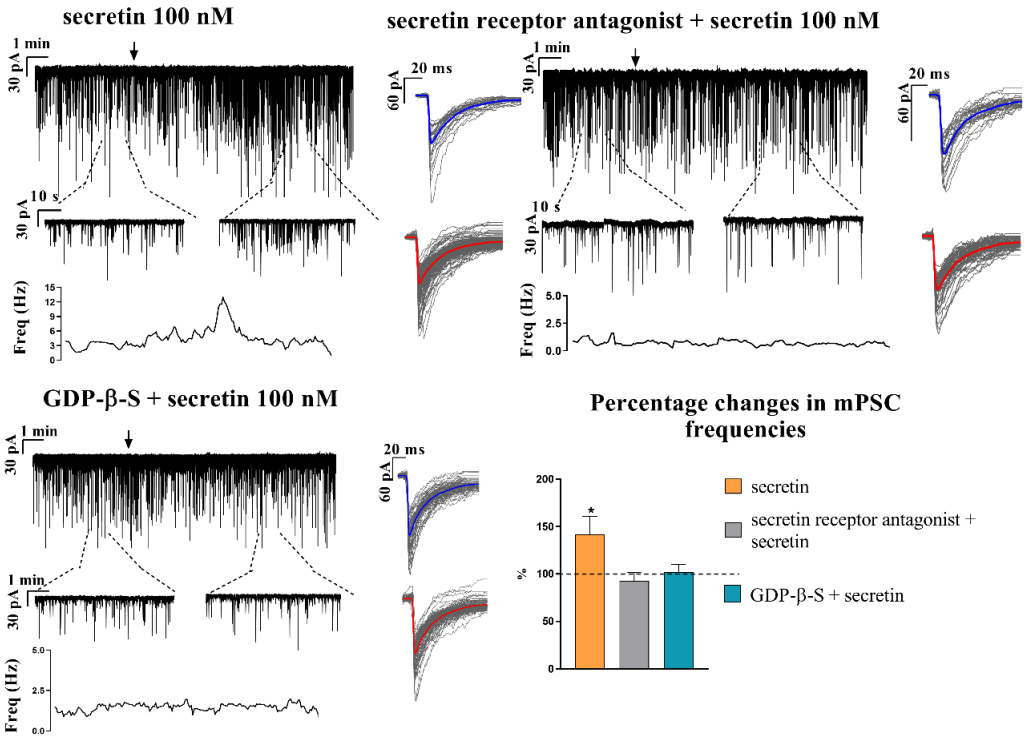

Figure 9.: Secretin elevates the frequency of mPSCs of GnRH neurons directly via secretin receptor. (a) Secretin (100 nM) increased the frequency of mPSCs in GnRH neurons, as shown in a representative recording, the 1 min zoomed periods and in the frequency distribution graph. There was no change in the average amplitude or in the shape of the events representing the individual PSCs next to the recording. **(b)** Pretreatment of the brain slice with secretin receptor antagonist (Secretin 5–27) eliminated the effect of secretin on GnRH neurons. **(c)** Intracellular application of G-protein blocker, GDP-β-S also abolished the effect of secretin. **(d)** Bar graph shows that the effect of secretin was mediated via the G-protein coupled secretin receptor. The inserts below the 15 min recordings are 1-1 min zoomed periods from the recordings before and after secretin administration. The frequency distribution is also presented under each recording. Average mPSCs next to the recording represent no change in the shape of events after secretin treatment. Arrow shows the administration of secretin (*$p < 0.05$).

Pretreatment of the slices with secretin receptor antagonist (secretin 5-27; 3 μM) 15 minutes before the application of secretin (100 nM), eliminated the stimulatory action of secretin on the mean frequency of mPSCs (100.3 ± 2.295%) **(Fig. 9b, Table 2)**.

To prove the direct action of secretin in GnRH neurons, its effect on the mPSCs was further examined in the intracellular presence of the membrane impermeable G-protein blocker GDP-β-S (2 mM). The blockade of G-proteins in GnRH neurons eliminated the observed effect of secretin on mPSCs (101.0 ± 1.483%) **(Fig. 9c, Table 2)**. Values of amplitude and shape of the PSCs also presented no significant change **(Fig. 9c, Tables 3, 6)**.

DOI:10.15774/PPKE.ITK.2021.010

Bar graph summarizes the effect of secretin on the mean frequency of the mPSCs and full inhibition of the secretin-triggered action by antagonizing secretin receptor and the intracellular blockade of G-proteins in GnRH neurons. **(Fig. 9d, Table 6)**.

Current clamp measurements revealed that secretin (100 nM) triggered membrane depolarization in GnRH neurons in the presence of TTX. The mean of the changes was 12.74 ± 4.539 mV (Student's t-test, n=6, *p=0.0186*) (**Fig. 10**). Depolarization usually occurred 1 min after secretin application and as the figure shows it returned to the baseline after a short time. In the presence of secretin receptor antagonist, the observed stimulating effect did not occur, there was no significant change in the membrane potential (n=5) **(Fig. 10)**.

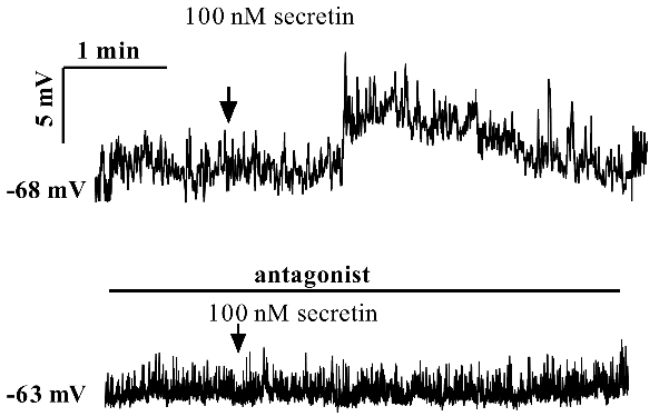

Figure 10: Depolarization in the membrane potential is demonstrated in a 4 min period. Arrow shows application of secretin. The bottom recording reveals no significant change after the administration of secretin (100 nM) in the presence of secretin receptor antagonist.

Involvement of PKA and retrograde NO signaling mechanisms in the effect of secretin

Changes in the frequency of mPSCs but not in the amplitude suggested changes in the presynaptic site after application of secretin. Previous studies, however, have demonstrated that activation of the retrograde NO signaling pathway in GnRH neurons results in an increased mPSC frequency [139].

To test the hypothesis that retrograde NO signaling mediates the effect of secretin on the GABAergic synaptic input of GnRH neurons NPLA (1 μM), an nNOS blocker, was applied intracellularly into the recorded GnRH neurons 15 min before adding secretin (100 nM). Intracellular application of NPLA alone did not alter basal frequency or amplitude of mPSCs in GnRH neurons [139] **(Fig. 11 a, b, Table 2, 3, 6)**. NPLA treatment fully eliminated the action

DOI:10.15774/PPKE.ITK.2021.010

of secretin (90.38 ± 4.60%, Student's t-test, *p=0.0746).* Values of amplitude, rise and decay τ of the PSCs also presented no significant changes **(Fig. 11 a, Table 3, 6)**.

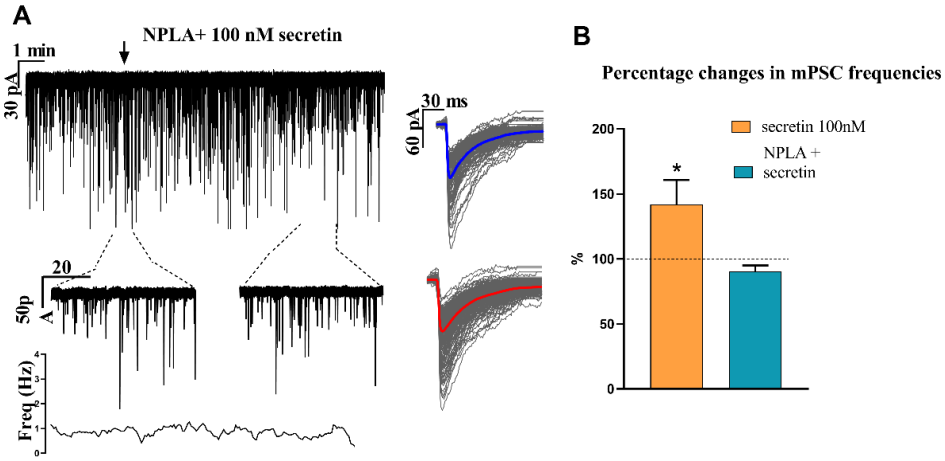

Figure 11. Investigation of the signaling pathway
(A) Intracellular application of the nNOS blocker NPLA extinguished the effect of secretin**. (B)** Bar graph shows that secretin triggers a retrograde NO-coupled signaling mechanism. The inserts below the 15 min recordings are 1-1 min zoomed periods from the recordings before and after secretin administration. The frequency distribution is also presented under each recording. Average mPSCs next to each recording showed no change in the shape or amplitudes of events after secretin treatment. Arrow shows the administration of secretin. (∗p < 0.05)

Nitric oxide activation can be induced via different intracellular signaling pathways. Earlier studies showed that one of the main pathways activated by secretin receptor is the cAMP/PKA pathway [261]. Therefore, the selective PKA blocker KT5720 was applied intracellularly into GnRH neurons. The presence of KT5720 in the intracellular solution abolished the frequency increasing effect of secretin on mPSCs of GnRH neurons (97 ± 5.987%) **(Fig. 12 a, b, Table 2)**.

Values of amplitude, the rise, and the decay τ of mPSCs also presented no significant changes **(Fig. 12a, Table 2, 5)**. Bar graph depicts the full inhibition of the secretin-triggered action on mPSCs by intracellularly applied NPLA and KT5720 **(Fig. 11b and 12b, Table 2)**.

In summary, these results demonstrate that secretin acts directly on GnRH neurons via secretin receptors and activates the cAMP/PKA/nNOS pathway which enables the generation of NO in the recorded GnRH neurons in male mice. The retrograde messenger can reach the GABAergic synaptic boutons and increases the release of GABA by enhancing frequency of GABAergic mPSCs of GnRH neurons as seen on the schematic illustration **(Fig. 9, 11, 12, 18)**.

DOI:10.15774/PPKE.ITK.2021.010

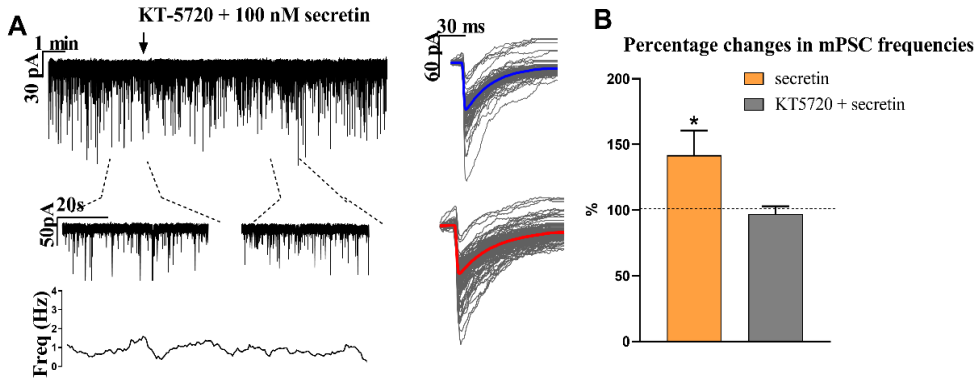

Figure 12. Investigation of the signaling pathway with intracellular blockade of PKA. **A:** The PKA inhibitor KT5720 applied intracellularly eliminated the effect of secretin. **B:** Bar grah shows that administration of secretin utilizes PKA. The inserts below the 15 min recordings are 1-1 min zoomed periods from the recordings before and after secretin administration. The frequency distribution is also presented under each recording Average mPSCs beside each recording showed no change in the shape or amplitudes of events after secretin treatment. Arrow shows the administration of secretin ($*p < 0.05$)

DOI:10.15774/PPKE.ITK.2021.010

		Rise tau (ms)	Average percentage changes after secretin administration	Decay tau (ms)	Average percentage changes after secretin administration	n/N
sPSC	**30 nM secretin**	3.716± 0.440	103.9± 4.808	22.57± 1.431	102.8± 4.160	9/4
	100 nM secretin	4.460± 0.6166	101.6± 7.926	23.05± 1.711	97.88± 3.182	8/3
	1 µM secretin	4.244± 0.3608	98.38± 5.867	26.17± 1.833	101.9± 1.903	7/3
mPSC	**100 nM secretin**	4.866± 0.6075	101.7± 12,96	19.07± 2.140	104.6± 12.18	16/7
	secretin receptor antagonist +100nM secretin	4.081± 0.8153	102.8± 7.947	32.78± 10.22	97.30± 10.82	8/4
	GDP-β-S +100 nM secretin (G protein blocker)	3.940± 0.4759	100.03± 10.29	22.96± 4.250	91.20± 7.297	10/4
	NPLA + 100 nM secretin (nNOS blocker)	3.281± 0.453	106.3± 8.593	21.51± 4.044	102.4± 6.608	10/5
	KT5720+ 100 nM secretin (PKA blocker)	4.475± 0.4489	101.8± 10.61	20.26± 1.258	112.5± 10.02	13/6

Table 6.: **Changes in the rise τ and decay τ after 100 nM secretin.** The first column shows the rise tau in the control period. The second column shows changes in the rise time in %. Third column shows the decay tau in the control period, the fourth column shows changes in the decay time in %. The fifth column shows the number of neurons (n) and animals (N) used for the experiments. (Student's t-test)

DOI:10.15774/PPKE.ITK.2021.010

RESULTS II.- The electrophysiological investigation of the effect of IGF-1 on GnRH neurons

In this chapter I discuss the results related to the effect of IGF-1 on GnRH neurons.

IGF-1 significantly increases the frequency of spontaneous postsynaptic currents (sPSCs) in GnRH neurons of prepubertal male mice

To investigate the effect of IGF-1 in GnRH neurons, spontaneous postsynaptic currents (sPSCs) were recorded to demonstrate action of IGF-1 using whole-cell patch clamp method. Low dose of IGF-1 (1.3 nM) triggered no change in frequency of the sPSCs in any of the neurons (n=8) measured. Higher concentration (6.5 nM), however, evoked a significant elevation in the frequency of sPSCs in 7/13 neurons (up to 135.7 ± 7.70% of the basal frequency 0.87 ± 0.374 Hz, *p=0.0036*, Student's t-test). Administration of 13 nM resulted in a significant increase in the sPSC frequency in 12 of 21 examined GnRH neurons (up to 165.8 ± 11.02% of the baseline value 0.48 ± 0.071 Hz; *p=0.001*, Student's t-test) (**Fig. 13A and C, D Tables 7,8,9**), and 66 nM also caused increase in the frequency (9/17 neurons, 180 ± 26 % of the basal value 0.40 ± 0.135 Hz, *p=0.037*, Student's t-test) revealing that IGF-1 modulates synaptic transmission to GnRH neurons. The zoomed 1 min-long recordings and the frequency distribution graph under the 15 min-long recording demonstrate the elevation in the frequency data. Considering the dose-response graph (**Fig. 13C**), the 13 nM IGF-1 concentration was chosen for the subsequent experiments. Individual PSCs and their average shape next to the recording (blue: control, red: IGF-1 treated) show that the amplitude **(Table 8)** and decay of the sPSCs exhibited no change (**Fig. 13A**) suggesting role of a presynaptic process. IGF-1 increased the frequency of sPSCs within 1-2 minutes.

IGF-1 increases firing rate in GnRH neurons in prepubertal mice

Postsynaptic currents and firing activity of GnRH neurons positively correlate with each other [83, 117, 140], therefore, the increased sPSC frequency suggested an elevated firing rate. Thus, we investigated effect of IGF-1 on spontaneous firing rate of GnRH neurons. Administration of IGF-1 significantly elevated firing rate (up to 209.5±34.88% of the baseline value 0.65 ± 0.169 Hz in 8/14 neurons; *p=0.0164*, Student's t-test) (**Fig. 13B and D, Tables 7, 8 and 9**).

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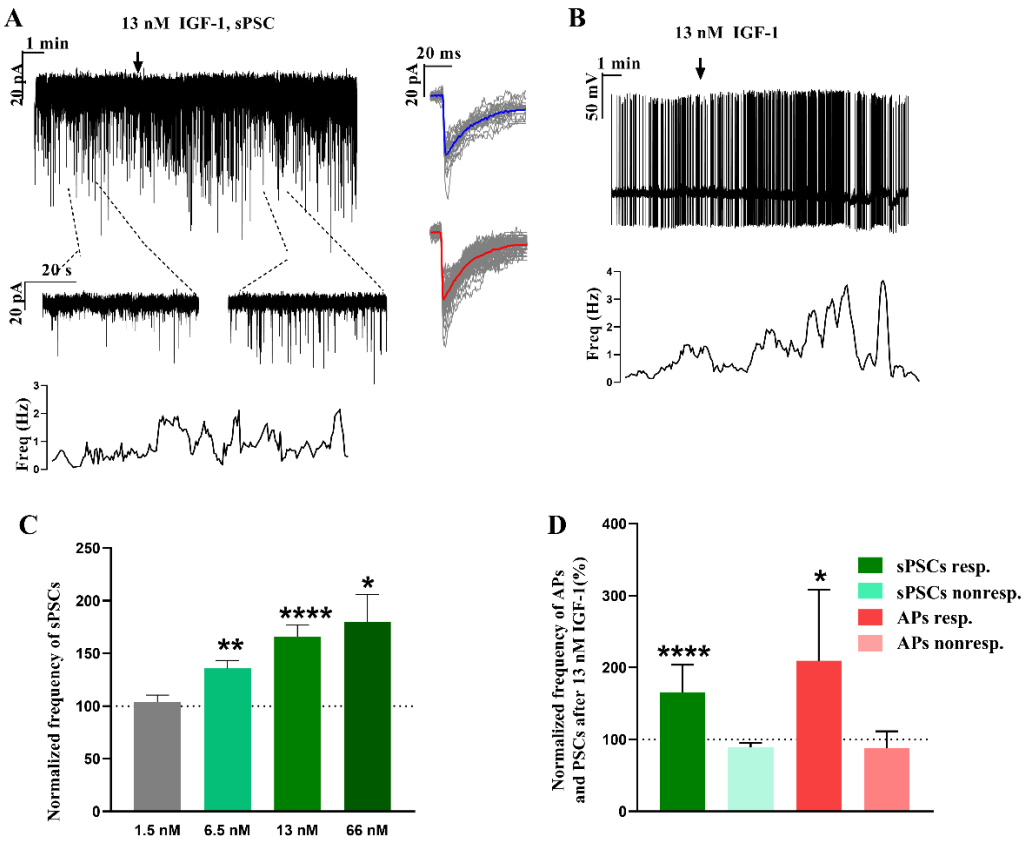

Figure 13.: Effect of IGF-1 on the postsynaptic currents (PSCs) and firing of GnRH neurons.

A. IGF-1 significantly increased the frequency of sPSCs without any change in the average amplitude and shape. Average sPSCs are presented next to the recording (blue: before IGF-1; red: after IGF-1). The inserts below the 15 min recordings and frequency distribution graph also show the frequency elevating effect of IGF-1. **B.** Effect of 13 nM IGF-1 on the firing of GnRH neurons. **C.** Dose response curve of IGF-1 on spontaneous postsynaptic currents. **D.** Bar graph reveals that IGF-1 significantly elevated the frequency of PSCs and APs in some of the measured neurons (Student's t-test $*p<0.05$; $**p<0.01$; $***p<0.001$). Arrow shows the IGF-1 administration. Average PSCs before the administration are shown by blue and by red after the administration.

DOI:10.15774/PPKE.ITK.2021.010

Effect of IGF-1 is direct via IGF-1R in prepubertal mice

In order to examine whether IGF-1 acts directly in GnRH neurons, the peptide was administered in the continuous presence of tetrodotoxin (TTX) in the aCSF and then miniature PSCs (mPSCs) were recorded. Application of IGF-1 (13 nM) resulted in a significant elevation of the mPSC frequency in 7 of the 16 measured neurons (145.7±13.60% of the baseline value 0.269 ± 0.06 Hz; *p=0.015*, Student's t-test) (**Fig. 14A, B, Table 7**), suggesting that IGF-1 influences spontaneous release of neurotransmitters. The zoomed figures and the frequency distribution graph confirm this observation. Shape of the mPSCs presented no change indicating that amplitude and decay parameters were not affected by the application of IGF-1. To test whether these currents are $GABA_A$-R mediated ones, we used PTX, a selective $GABA_A$-R blocker. In the presence of PTX no mPSCs were observed and IGF-1 application evoked no further mPSCs (data not shown).

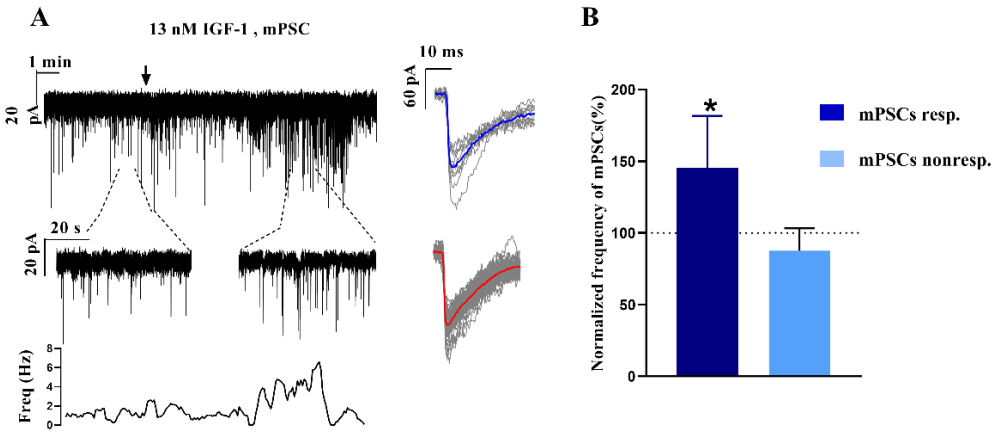

Figure 14.: Direct effect of IGF-1 on GnRH neurons of prepubertal male mice
A: IGF-1 increased the frequency of the mPSCs with no change in the average amplitude. The one-minute-long periods under the recording, average mPSCs before and after the IGF-1 treatment, and the frequency distribution graph demonstrate the same observation. Approximately half of the measured neurons responded to IGF-1. **B**: Bar graph shows the responding and nonresponding GnRH neurons after IGF-1 treatment. **p<0.05*

The action of IGF-1 on mPSCs of GnRH neurons was blocked by the selective IGF-1R antagonist JB-1 (1 µM) (93.33 ± 2.21%, *p=0.257*, Student's t-test, **Fig. 15A and B, Table 7**) suggesting role of the IGF-1R expressed in GnRH neurons (n=7).

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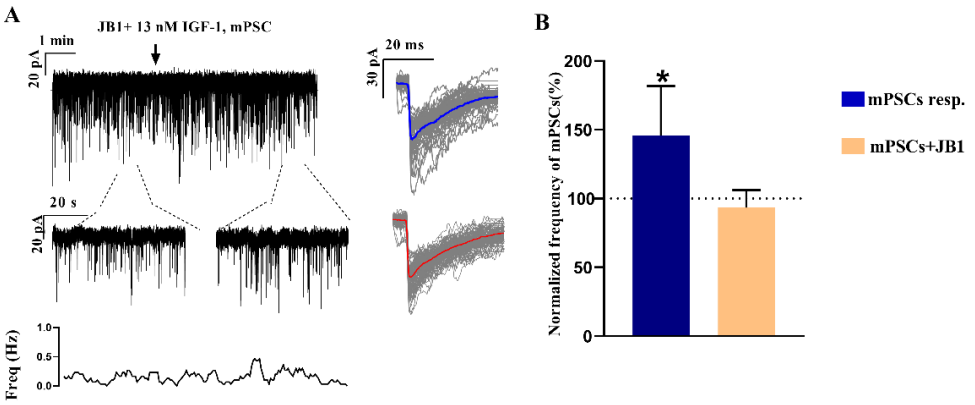

Figure 15.: IGF-1 acts via IGF-1 receptor.

A: Pretreatment of the brain slice with IGF-1 receptor antagonist JB-1 prevented the frequency elevating effect of IGF-1. The one-minute-long periods under the recording, average mPSCs before and after the IGF-1 treatment, and the frequency distribution graph demonstrate the same observation. **B:** Bar graph demonstrates, that after JB1 treatment, IGF-1 could not elevate the frequency of mPSCs

IGF-1 increases frequency of mPSCs in GnRH neurons in pubertal mice

Effect of IGF-1 was examined in pubertal mice, too. Application of IGF-1 (13 nM) increased frequency of mPSCs in 5/10 neurons significantly (142±7.9% of the baseline value, *p=0.015*, Student's t-test) with no change in the amplitude or in the shape of mPSCs (**Fig. 16A and B, 7,8 and 9**), similarly to that of prepubertal mice.

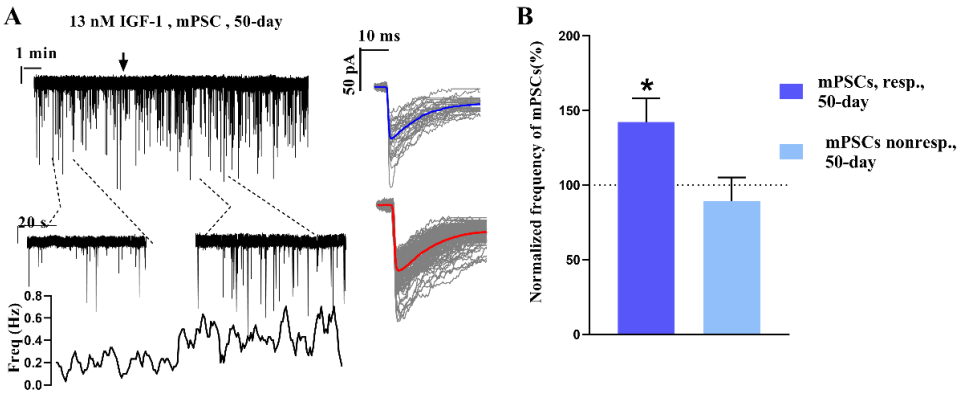

Figure 16.: IGF-1 is also effective in pubertal mice.

A. IGF-1 elevates frequency of mPSCs of GnRH neurons in pubertal mice.
The one-minute-long periods under the recording, average mPSCs before and after the IGF-1 treatment, and the frequency distribution graph demonstrate the same observation.

DOI:10.15774/PPKE.ITK.2021.010

B. Bar graph shows the difference between the responding and nonresponding group of neurons in 50-day-old mice.

Effect of IGF-1 is mediated via PI3K, TRPV1 and tonic endocannabinoid release in prepubertal mice

Increase in the frequency of the GABAergic mPSCs could be evoked by activation of the retrograde nitric-oxide (NO) machinery in hypothalamic neurons, including GnRH cells [107, 139]. In this mechanism, NO generated in GnRH neurons retrogradely affects GABA release from the presynaptic terminal. Therefore, we examined whether this mechanism was involved in the elevation of mPSC frequency after IGF-1 application. To block nitric-oxide synthase (NOS), the slices were pretreated with NPLA (1 μM). Recording of the mPSCs showed, however, that in the presence of NPLA, IGF-1 was still able to increase the frequency of mPSCs to 150.7 ± 16.87% in 5/10 neurons (**Fig. 17A and E, Tables 7,8 and 9**). This value presented no significant difference (*p=0.9974*, Dunnet's post-hoc test) comparing to the change measured in GnRH neurons in the absence of NPLA suggesting that NO-machinery is not involved in the action. Beside the retrograde NO-signaling mechanism, inhibition of the 2-AG endocannabinoid released tonically from GnRH neurons could also result in an increase of the frequency of mPSCs [139]. This tonic 2-AG pathway, involving the regulator transient receptor potential cation channel subfamily V member 1 (TRPV1), forms another retrograde machinery resulting in inhibition of GABAergic neurotransmission. Therefore, the endocannabinoid receptor 1 (CB1) antagonist AM251 (1 μM) was applied extracellularly. In the presence of AM251 administration of IGF-1 presented no effect on the mPSCs recorded in GnRH neurons. Measurements revealed that neither frequency (98.83 ± 9.203%, **Fig. 17B and E, Table 7**) nor shape of the mPSCs altered upon IGF-1 application, indicating that suppression of retrograde 2-AG endocannabinoid signaling mediates function of IGF-1. Literature data indicate that activation of PI3K stimulates function of TRPV1 [262] eventually inhibiting this tonic 2-AG release [139, 263]. Therefore, role of PI3K was investigated by applying IGF-1 in the intracellular presence of its blocker LY294002 (50 μM). The pretreatment eliminated action of IGF-1 (101.5 ± 8.01%, **Fig. 17C and E, Table 7**). The zoomed figures and the frequency distribution graph confirm this observation, with no change in the shape of the mPSCs. In addition, the representative recording and its zoomed parts with the frequency distribution graph demonstrate that blockade of TRPV1 by its specific inhibitor AMG9810 (1 μM) also abolished effect of IGF-1 (101.1 ± 4.80%, **Fig. 17D and E, Table 7**). Shape of the mPSCs showed no alteration, either **(Table 8.)**. ANOVA analysis on mPSC data together with the Dunnet's post hoc test revealed that the inhibitors JB1, LY294002, AMG9810 and AM251 abolished effect of IGF-1 as compared with the blocker-free action of IGF-1 (**Table 9.**)

DOI:10.15774/PPKE.ITK.2021.010

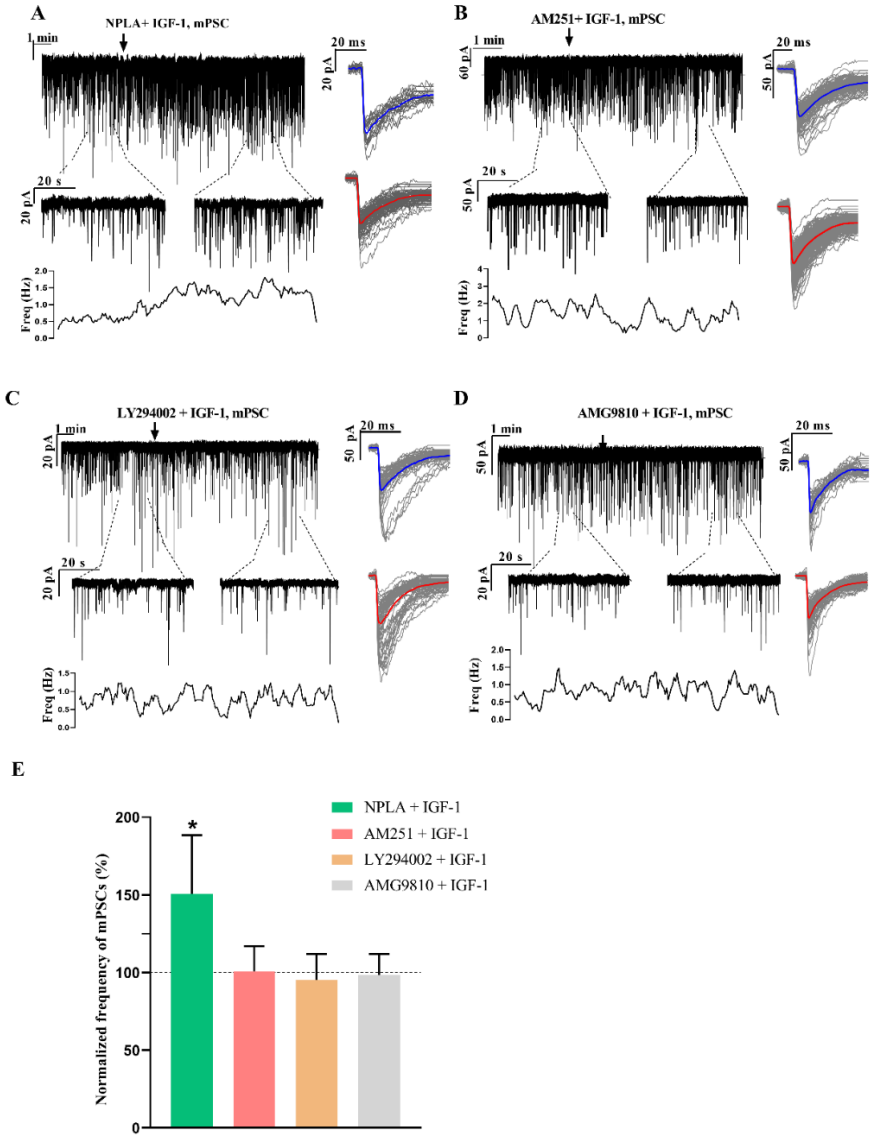

Figure 17.: Investigation of the activated signaling pathway

A. Blockade of the retrograde NO pathway by NPLA resulted in no change in the action of IGF-1. **B.** CB1 receptor blocker AM251 prevented the frequency elevating effect of IGF-1. **C.** Intracellular application of PI3K blocker LY294002 extinguished the effect of IGF-1. **D.** TRPV1 receptor blocker AMG9810 also eliminated the effect of IGF-1 on GnRH neurons. **E.** Bar graph shows that PI3K, TRPV1 and CB1 have a major role in the effect of IGF-1. The inserts below the 15 min recordings are 1-1 min zoomed periods from the recordings before and after IGF-1 administration. The frequency distribution is also presented under each recording. Average PSCs next to the recordings show that there was no change in the amplitude nor in the shape of the PSCs after IGF-1 treatment (blue shows the control period, red shows the treated period). Arrows show the administration of IGF-1. *p<0.05

DOI:10.15774/PPKE.ITK.2021.010

		Frequency of control peroid (Hz)	Average changes after IGF-1 administration in % compared to the normalized value of control	p value	n	N
sPSC	1.5 nM responding	0.491±1.18	104±6.76	0.458	8	3
	6.5 nM responding	0.874±0.374	**135.7±7.701	0.0036	7	3
	13.1 nM responding	0.4851±0.071	****165.8±11.02	<0.0001	12	7
	13.1 nM nonresponding	0.699±0.140	88.81	0.4589	9	
APs	66 nM responding	0.409±0.135	*180±26	0.037	5	2
	responding	0.655±0.169	*209.5±34.88	0.0164	8	4
mPSC	nonresponding	0.8976±0.1	88.40±10.26	0.321	6	
	responding	0.269±0.06	*146.5±13.84	0.0202	7	5
	nonresponding	0.649±0.22	90.02±10.77	0.3759	9	
	50-day, responding	0.6781±0.2934	*142.3±7.941	0.013	5	3
	50-day nonresponding	0.6490±0.03523	89.40±7.038	0.2065	5	
	JB1 (IGF_1 receptor antagonist)	0.567±0.12	96.14±0.488	0.4883	7	2
	LY294002 (PI3K blocker)	1.086±0.2205	101.5±8.016	0.855	10	3
	NPLA, resp. (nNOS blocker)	0.5775±0.1175	150.7±16.87	0.0398	6	3
	AMG9810 (TRPV1 blocker)	0.671±0.090	101.1±4.806	0.8241	10	4
	AM251 (CB1 blocker)	0.631±0.167	98.83±9.203	0.9041	6	3

Table 7. Student's t-test of sPSC, action potential (AP) and mPSC frequencies. Changes with various significance are marked with increasing number of asterisks. n=number of neurons, N=number of mice

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		Amplitude of control period	Average changes after IGF-1 administration in % compared to the normalized value of control	p value	n	N
sPSC	1.5 nM responding	46.68±10.16 pA	102.2±4.868	0.7308	8	3
	6.5 nM responding	54.57±8.188 pA	96.63±2.283	0.1706	7	3
	13.1 nM responding	30.91±1.121 pA	101.1±2.313	0.6371	12	7
	13.1 nM nonresponding	38.28±6.543 pA	101.8±2.694	0.5317	9	
	66 nM responding	46.95±6.543 pA	100.6±2.389	0.8102	5	2
APs	responding	108.1±2.040 mV	100.4±0.58	0.4655	8	4
	nonresponding	108.5±2.040 mV	99.83±0.6009	0.7926	6	
mPSC	responding	28.22±2.379 pA	103.3±1.820	0.1067	7	5
	nonresponding	33.04±2.379 pA	99.78±0.9594	0.8272	9	
	50-day, responding	26.97±2.963 pA	97±2.887	0.4078	5	3
	50-day nonresponding	36.46±8.204 pA	100.7±2.963	0.8429	5	
	JB1 (IGF-1 receptor antagonist)	34.38±20.36 pA	90.83±0.8272	0.3616	7	2
	LY294002 (PI3K blocker)	52.21±8.263 pA	95.30±13.68	0.3055	10	3
	NPLA (nNOS blocker)	30.35±2.984 pA	96.63±4.987	0.5294	6	3
	AMG9810 (TRPV1 blocker)	40.11±5.209 pA	98.90±1.767	0.549	10	4
	AM251 (CB1 blocker)	37.35±4.56 pA	100.6±8.69	0.43	10	3

Table 8. Student's t-test of amplitude of sPSCs, action potentials (AP) and mPSCs
n=number of neurons, N=number of mice

DOI:10.15774/PPKE.ITK.2021.010

	F (5,42)	p (ANOVA)		p (Dunnett's post-hoc test)
Frequency	9.176	0.0001****	responding vs. nonresponding	0.0001****
			responding vs. JB-1 (IGF-1 receptor antagonist)	0.0001****
			responding vs. LY294002 (PI3K)	0.0001****
			responding vs. AM251 (CB1 receptor blocker)	0.0002****
			responding vs. AMG9810 (TRPV1 blocker)	0.0001****
			responding vs. NPLA (nNOS blocker)	0.9974

Table 9. ANOVA and Dunnet's post-hoc analysis on mPSC frequency data concerning blocker-free action of IGF-1 and in the presence of JB1, LY294002, AM251, AMG9810, and NPLA. ****$p<0.0001$*

DOI:10.15774/PPKE.ITK.2021.010

DISCUSSION

Secretin and IGF-1 have been shown to modulate the HPG axis; however, the site/s of action have not been explored so far [205]. Our results provided electrophysiological evidence for a direct action of secretin and IGF-1 on GnRH neurons.

Accordingly:

1. Secretin activated the secretin receptors expressed in GnRH neurons and increased the frequency of their APs and GABAergic PSCs.
2. Downstream signaling of secretin receptor involved the activation of protein kinase A (PKA) and neuronal nitric oxide synthase (nNOS) that in turn, led to activation of the retrograde NO signaling pathway. The release of NO enhances the release of GABA from the presynaptic sites which has an excitatory effect on GnRH neurons via $GABA_A$-R.
3. IGF-1 increases firing activity and frequency of postsynaptic currents via IGF-1 receptor.
4. This signaling suppresses the tonic 2-arachidonoylglycerol (2-AG) production in GnRH neuron resulting in an elevated excitatory GABA release from the presynaptic terminals.
5. The activated signaling pathway by IGF-1 involves phosphoinositide 3-kinase (PI3K) and transient receptor potential cation channel subfamily V member 1 (TRPV1).

Secretin is excitatory on GnRH neurons via secretin receptor

The present findings demonstrate that secretin exerts excitatory effects on GnRH neurons. Frequency of PSCs and APs increased, the rheobase of evoked APs decreased, and the membrane potential depolarized upon secretin administration. These data are in line with other studies reporting the stimulatory effect of secretin in different brain areas and different cell types. In the PVN for example secretin elevated the firing rate *in vivo,* and in the nucleus of the solitary tract (NTS) it depolarized neurons via nonselective cationic channels [202, 203]. Both central and peripheral administration of secretin induced c-Fos expression in the PVN and the arcuate nucleus, suggesting the activation of these hypothalamic nuclei [264].

Both the GABAergic mPSC frequency and the firing rate were increased in GnRH neurons after secretin administration. Since GABA has a special excitatory effect on GnRH neurons via $GABA_A$-R [138, 258], the elevation detected in mPSC frequency and the increased firing rate correlate well.

Although the recorded mPSCs of GnRH neurons in male mice are GABAergic under basal conditions, we could not exclude the theoretical possibility of the additional effects of glutamate [119, 121, 123, 138, 265]. The selective inhibition of $GABA_A$ receptors with picrotoxin, however, totally abolished the effect of secretin on PSCs, indicating the exclusive role of GABAergic inputs in the effects of secretin.

DOI:10.15774/PPKE.ITK.2021.010

We proved that the effect of secretin is specific to secretin receptor using secretin receptor antagonist. In addition, the intracellular blockade of secretin receptor by the membrane impermeable G-protein blocker, GDP-β-S, also abolished the secretin-evoked changes in the mPSC frequency, proving that secretin action occurs postsynaptically on GnRH neurons.

A previous study has reported that secretin augmented plasma luteinizing hormone (LH) concentration following its stereotaxic delivery into medial preoptic area (mPOA) suggesting that the effect of secretin on LH cells and LH production was indirect, and presumably the activation occurred at the level of GnRH neurons [205]. Our findings confirm that secretin is capable of centrally regulating the HPG axis via a direct activation of GnRH neurons.

Changes in the frequency of GABAergic mPSCs but not in their amplitude suggest that alterations take place at the presynaptic site. This might indicate that secretin has a direct effect at the presynaptic site via secretin receptors as it was discovered in an earlier work from the Purkinje cells of cerebellum [201]. In our study, the intracellular blockade of the G-proteins and the NO pathway in the postsynaptic GnRH neurons eliminated the effect of secretin excluding this opportunity.

The hyperpolarizing current step measurements showed that input resistance (and therefore, membrane resistance) has not changed upon secretin application and the subsequent GABA release. This seems to be in contrast with the data revealing a secretin-dependent depolarization of V_{rest} when the current step is zero. One of the putative explanations for this observation is that response to GABA can be voltage-dependent, provided presence of $GABA_B$-R in the neuron [266]. It is well known that GnRH neurons bear $GABA_B$-R [119, 267] suggesting a putative voltage-dependency of GABA response in GnRH neurons. This idea, however, requires further elaboration.

Secretin activates the retrograde NO signaling pathway via upregulation of cAMP/PKA

Our results indicate that secretin acts directly on GnRH neurons via secretin receptors whose activation triggers a downstream cascade event leading to activation of retrograde NO signaling in GnRH neurons. The mechanism of secretin's effect is often linked to NO production at the periphery [268-270]. Within the brain, increase in the frequency of the GABAergic mPSCs could be evoked by activation of the NO machinery in hypothalamic neurons [271]. Previously, the presence and the activity of nNOS have been demonstrated by our group in GnRH neurons of mice. In these studies, the activation of nNOS increased the frequency of GABAergic mPSCs in GnRH neurons [107, 139]. Stimulatory effect of retrograde NO signaling on GABAergic currents was described earlier in the PVN, where NO excited the GABAergic mPSCs in the neurons of PVN [272]. NO also activated the GABAergic inputs of oxytocin- and

DOI:10.15774/PPKE.ITK.2021.010

vasopressin-containing neurons in the supraoptic nucleus [273]. The stimulatory effect of retrograde NO signaling was also proved in case of glutamatergic neural circuits, where NO released from the postsynaptic neurons could increase the probability of glutamate release from presynaptic glutamatergic axon terminals in different brain areas [150, 274-276].

All members of the secretin-like family of G-protein coupled receptors (B1 family) such as, VIP, secretin, and pituitary adenylate cyclase-activating peptide (PACAP) stimulate the adenylate cyclase via stimulatory G-protein (G_s) [277-279] resulting in PKA activation [261]. The presence of PKA in GnRH secreting neurons was found earlier [280], and we also revealed that the application of PKA blocker totally inhibited the effect of secretin, providing further evidence that the underlying mechanism of secretin requires PKA activation.

The present findings demonstrate that secretin acted postsynaptically and resulted in a change of mPSC frequency, suggesting that secretin receptor activates the cAMP/PKA/nNOS pathway and generates NO that binds to its presynaptic receptor, soluble guanylyl cyclase (sGC), located in the GABAergic axon terminals **(Fig. 18.)**. The expression of sGC has been recently detected in GABAergic and glutamatergic presynaptic boutons of GnRH neurons [107, 139]. sGC activates cGMP dependent protein kinase G, which can phosphorilate synaptophysin, the main molecule responsible for vesicle fusion. This mechanism has a facilitatory effect on the neurotransmitter release [281]. This retrograde signaling process increases the release of GABA, therefore, facilitates the synaptic inputs to GnRH neurons via $GABA_A$-receptor. PACAP and VIP can also activate nNOS and the production of NO via the cAMP/PKA/nNOS pathway in PC12 cells [282].

These current results support further the concept that GnRH neurons can sense the metabolic state of the organism and strengthen the view that secretin exerts a central regulatory role on the HPG axis via acting upon hypophysiotropic GnRH neurons.

DOI:10.15774/PPKE.ITK.2021.010

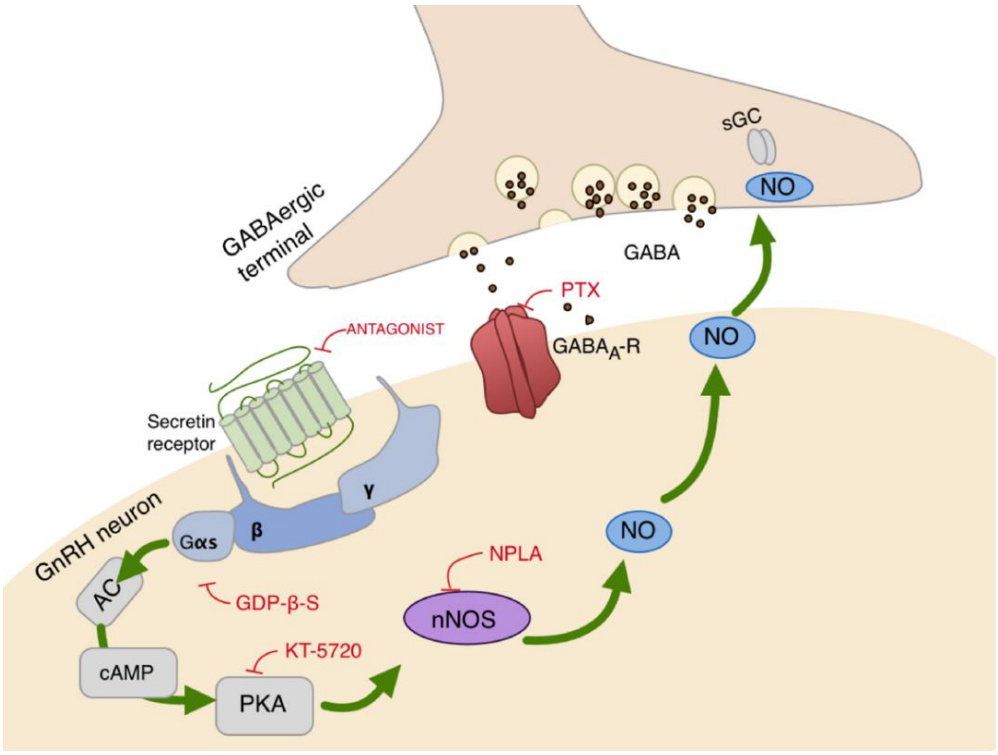

Figure 18.: **Schematic illustration of secretin receptor signaling in GnRH neurons.** Secretin activates cAMP/PKA/nNOS pathway and generates NO that binds to its presynaptic receptor, sGC, located in the GABAergic terminals. This signaling process increases the release of GABA, therefore, facilitates 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.

DOI:10.15774/PPKE.ITK.2021.010

IGF-1 exerts direct excitatory effect on GnRH neuron via IGF-1R

Our study clearly showed that IGF-1 is excitatory on GnRH neurons. It elevated frequency of both action potentials and postsynaptic currents, confirming the general concept that firing rate and frequency of the PSCs mostly correlate in these neurons [138, 258].

Half of the GnRH neurons responded to IGF-1 administration. This ratio is in accordance with earlier reports demonstrating the presence of IGF-1R in approximately 60% of GnRH neurons of peripubertal (P30) male mice [209, 229] indicating that not all GnRH neurons express IGF-1R. Nevertheless, these literature data confirm further our current finding that in the IGF-1R antagonist pre-treated slices IGF-1 could not exert any significant alteration in the firing rate and the frequency of PSCs. Our mPSC-related measurements supported further the view that IGF-1 can act directly via IGF-1R in GnRH neurons since in the presence of the voltage-gated Na-channel blocker TTX, this hormone could still alter frequency of mPSCs.

Our current results presented excitatory effect of IGF-1 in these neurons. This observation is supported by earlier studies revealing stimulatory action of IGF-1 on the reproductive axis including function of GnRH neurons [226, 230, 235, 283-285].

Retrograde endocannabinoid but not nitric-oxide pathway is involved in the effect of IGF-1

Earlier findings showed that at least two distinct retrograde signaling pathways can exert excitatory modulation on GnRH neurons and consequently, stimulation of reproductive functions. One of them is triggering the NO-machinery and the other one is suppressing the tonic endocannabinoid release [107, 139, 286-288]. Therefore, first we studied putative regulatory role of NO in the effect of IGF-1. However, blockade of nNOS in GnRH neurons resulted in no significant diminution in the evoked change of frequency. Thus, we next examined whether the tonic retrograde endocannabinoid pathway operating in GnRH neurons [138] was suppressed in this action. In this scenario, the endocannabinoid 2-AG, tonically secreted from the GnRH neuron, decreases the excitatory GABA release from the presynaptic axon terminals and thus, suppression of the endocannabinoid production results in an increased GABA release. Indeed, when CB1 receptors were antagonised in our present experiment, this pre-treatment abolished the effect of IGF-1 demonstrating indispensable role of the endocannabinoid machinery in the observed result.

DOI:10.15774/PPKE.ITK.2021.010

The IGF-1 activated downstream signaling pathway involves PI3K and TRPV1

Our measurements revealed that TRPV1 was involved in the modulation of endocannabinoid production. Indeed, suppression of 2-AG production can be achieved by activating TRPV-1 [263]. TRPV1 regulates local calcium milieu beneath the postsynaptic membrane [289]. Since activity of the 2-AG producing enzyme, diacylglycerol lipase (DAGL) and therefore, 2-AG production are calcium dependent, the low calcium concentration eventually alters GABAergic neurotransmission [290, 291]. Importance of TRPV1 in the function of GnRH neurons has also been demonstrated [292]. In addition, relevance of the relationship between TRPV1 and the 2-AG production was revealed in GnRH neurons earlier [139] supporting our present results showing significance of this functional link in the execution of the effect of IGF-1.

The experiments presented here demonstrated the importance of PI3K as another downstream element of the IGF-1R activated pathway in GnRH neurons. Literature data revealed that indeed IGF-1R can phosphorylate the insulin receptor substrate-1 subsequently resulting in the phosphorylation of PI3K [285, 293]. PI3K, in turn, can convert phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) [293, 294]. Numerous experiments revealed that PIP2 can inhibit TRPV1 activity, and when PI3K converts PIP2 to PIP3 the relief from this inhibition may contribute to activation of TRPV1 [295-298] leading to the suppression of tonic 2-AG production which, in turn, increases the excitatory GABAergic neurotransmission as the schematic figure indicates (**Fig. 19**).

Mutation in IGF-1 in human patients [233] and GnRH specific deletion of IGF-1R in mice [234] result in a significantly delayed puberty, strongly supporting our present data that IGF-1 excites GnRH neurons at prepubertal and pubertal age indicating a regulatory role of this action in puberty. In addition, role of IGF-1 in puberty has also been suggested by demonstrating a significant change in IGF-1 level around puberty [230, 299-301]. Our present data shed light on the mechanism by which IGF-1 acts in GnRH neurons at these ages.

Our current results presented here strengthen the concept that hypophysiotropic GnRH neurons can sense the metabolic state of the organism. Furthermore, these data provide compelling evidence that IGF-1 exerts a central regulatory role on the HPG axis via acting upon these GnRH neurons.

DOI:10.15774/PPKE.ITK.2021.010

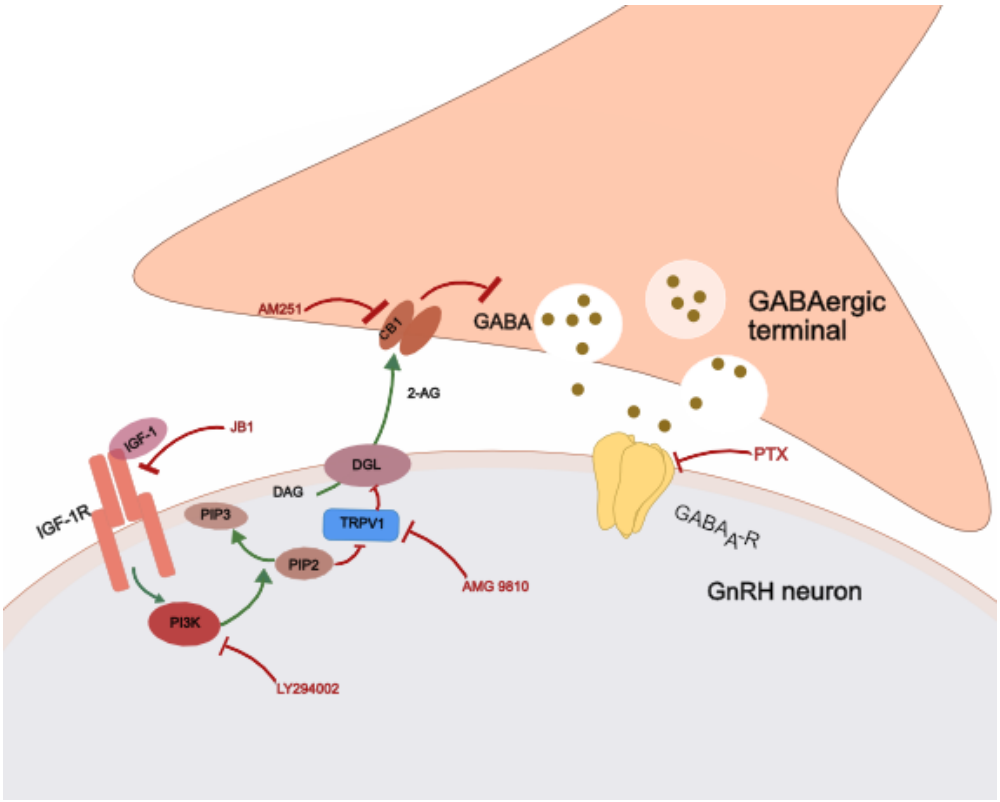

Figure 19.: Schematic illustration of the IGF-1 receptor signaling in GnRH neurons. IGF-1 activates PI3K which leads to the phosphorylation of PIP_2 to PIP_3. In cells, TRPV1 is inactivated by its binding to PIP_2, and after the activation of PI3K, TRPV1 receptor will be released from the PIP_2 blockade. 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.

DOI:10.15774/PPKE.ITK.2021.010

NEW SCIENTIFIC RESULTS

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. The effect was dose dependent. Secretin also depolarized the membrane potential of GnRH neurons. 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 also a G-protein coupled receptor, these experiments 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 protein kinase 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.

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

DOI:10.15774/PPKE.ITK.2021.010

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.

DOI:10.15774/PPKE.ITK.2021.010

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.

DOI:10.15774/PPKE.ITK.2021.010

ACKNOWLEDGEMENTS

Foremost, I am deeply thankful to *Dr. Imre Farkas* who was my tutor during my undergraduate and Ph.D. studies. I got extraordinary patience and attention while he tried to teach me the basics of the scientific thinking and the electrophysiology. I got a lot of good advice that will lead me on my way in life.

I am very grateful to *Prof. Zsolt Liposits* for his support, and for giving me the opportunity to belong to his group. I learned a lot in this group.

Thanks to my former and current colleagues: *Dr. Flóra Bálint, Dr. Zoltán Péterfi, Dr. Anett Stiftné Szilvássy Szabó, Andrea Juhász, Dr. Andrea Kádár, Balázs Göcz, Balázs Kovács, Dr. Csaba Vastagh, Dr. Csilla Molnár, Dóra Kővári, Dr. Edina Varga, Dr. Erzsébet Hakkel, Dr. Erik Hrabovszky, Dr. Imre Kalló, Dr. Katalin Skrapits, Krisztina Nagy, Márta Turek, Dr. Miklós Sárvári, Dr. Petra Mohácsik, Dr. Szabolcs Takács, Sarolta Thrin, Tamás Wilheim, Viktória Váczi, Yvette Ruska, Dr. Zsuzsanna Bardóczi, Zsuzsanna Beliczai*
for their help, and for the friendly community in the lab. I have many good memories.

I am very thankful to the Doctoral School, especially to *Prof. Gábor Szederkényi* and *Prof. Péter Szolgay* and *Prof. Pongor Sándor* for the opportunity to participate in the doctoral program, and *Dr. Tivadarné Vida* for her unlimited patience and kind help during my doctoral studies.

Last but certainly not least, I will be forever grateful to my *family* who supported me in my whole life. I don't think that I can ever give back even a small percentage of the emotional and financial support I got.
I am especially grateful to *my husband Benedek Varga* who helped me with his love, patience and emotional support in the past few years, from the moment I met him.

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DOI:10.15774/PPKE.ITK.2021.010

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List of publications underlying the thesis

First authorship:

V. Csillag, C. Vastagh, Z. Liposits, and I. Farkas, "**Secretin Regulates Excitatory GABAergic Neurotransmission to GnRH Neurons via Retrograde NO Signaling Pathway in Mice,**" (in eng), *Frontiers in Cellular Neuroscience,* Original Research vol. 13, no. 371, 2019. August 23. 2019, doi: 10.3389/fncel.2019.00371.

https://pubmed.ncbi.nlm.nih.gov/31507377/

Impact factor: 3.921

Co-first authorship

F. Balint, V. Csillag, C. Vastagh, Z. Liposits, and I. Farkas, "**Insulin-like growth factor 1 (IGF-1) increases GABAergic neurotransmission to GnRH neurons via suppressing the retrograde tonic endocannabinoid signaling pathway in mice,**" (in eng), *Neuroendocrinology,* Dec 24 2020, doi: 10.1159/000514043.

https://pubmed.ncbi.nlm.nih.gov/33361699/

Impact factor: 4.271

List of publications related to the subject of the thesis

Co-first authorship

C. Vastagh, V. Csillag, N. Solymosi, I. Farkas, and Z. Liposits, "**Gonadal Cycle-Dependent Expression of Genes Encoding Peptide-, Growth Factor-, and Orphan G-Protein-Coupled Receptors in Gonadotropin- Releasing Hormone Neurons of Mice,**" (in eng), *Frontiers in Molecular Neuroscience,* vol. 13, p. 594119, 2021, doi: 10.3389/fnmol.2020.594119.

https://pubmed.ncbi.nlm.nih.gov/33551743/

Impact factor: 5.639

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Imoact factor: 6.167