Final report

Mechanisms of glucagon-like peptide 1 signaling upon GnRH-synthesizing neurons: role in hypothalamic regulation of reproduction

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The hypothalamo-hypophyseal system is in charge of regulating reproduction centrally. Infertility is a severe malfunction of the system that develops due to diverse pathological causes. Novel findings address the regulatory role of metabolic hormones (leptin, ghrelin, insulin) in the central control of reproduction. A major effort has been devoted to elucidation of the effects of the potent metabolic hormone, glucagon-like peptide-1 (GLP-1) upon GnRH neurons. Our working hypothesis has raised that regulation is executed via GLP-1 receptor and its corresponding signal transduction pathways in GnRH neurons resulting in activation of the neurosecretory neurons. We present beneath the main results of the project in four thematic work-packages (I-IV).

I. Modulation of the GnRH neuronal network by GLP-1

1. Studying the expression of GLP-1 receptor expression in GnRH-GFP neurons by quantitative real-time PCR technique.
2. Quantitative structural analysis of neuronal interaction sites established by GLP-1 immunoreactive (IR) axons projecting from the nucleus of the solitary tract (NTS) with hypophysiotropic GnRH neurons by means of immunocytochemical double labelling techniques.
3. Examining the biological effects of GLP-1 released from GLP-1 axon terminals neurons by optogenetic stimulation of the afferent peptidergic system of GnRH neurons using a triple transgene mouse, slice electrophysiology and photo stimulation.
4. Exploration of the effects of GLP-1 receptor (GLP-1R) agonists and antagonist modulating GnRH neurons and elucidation of the coupled signal transduction pathways using acute brain slice preparations and patch-clamp electrophysiology.

The completed studies have elucidated a new regulatory mechanism in the neuronal control of reproduction that acts via GLP-1 receptors and their associated signal transduction pathways in GnRH neurons. These biological processes modify the retrograde endocannabinoid and nitric oxide signaling mechanisms which converge to GABA-ergic afferents of GnRH neurons resulting in increased firing and frequency of miniature postsynaptic currents (mPSCs) via $GABA_A$ receptor mediation.

The grant support also allowed the widening of project's focus and to study additional metabolic hormone effects on GnRH neurons and to characterize novel hypothalamic targets innervated by GnRH neurons. These studies and their results include:

II. Exploration of the regulatory role of secretin on GnRH neurons in vitro. The studies have discovered a new excitatory hormone regulator of the GnRH neuron network, the gut-brain peptide, secretin, elucidated the contributing signal transduction pathway elements and identified both the generated retrograde signal molecule (NO) and the presynaptic target structure, the GABAergic afferent boutons.

III. In the field of estrogen hormone signaling, exploration of the effects of high (proestrus) and low (metestrus) estradiol (E2) levels on the gene expression of GnRH neurons and their major regulator, the medial preoptic area (MPOA).
Regarding the main results, the discovery of NO production and signaling in GnRH neurons and their contribution to the positive E2 feedback regulation of the system are original observations. Similar to that, our studies have also revealed first in the neuroendocrine field the molecular machinery of GnRH neurons operating under the negative E2 feedback regulation utilizing estrogen receptor beta and retrograde endocannabinoid signaling in the process.

Furthermore, the grant support has also served, in part, explorative studies that will be summarized as additional results under the following title:
IV. *Studies on extrahypothalamic tissues and organs regulated dominantly by estrogen signaling*

Summary of applied techniques

We applied a multidisciplinary approach exploiting modern structural and functional techniques as follows:

1. Molecular methods

Quantitative, real-time PCR, Affimetrix microarray, pathway analysis, bioinformatics

2. Structural, neuroanatomical techniques

Immunocytochemistry, multiple-immunolabeling. Confocal laser microscopy. Transmission immune-electron microscopy. 3DISCO immunolabeling. Neuron network reconstruction. Laser capture microdissection. Quantitative analysis.

3. Functional methods

Whole cell patch-clamp electrophysiology. Optogenetics. Transgenic animals.

Summary of results

I. *Modulation of the GnRH neuronal network by GLP-1*

1. GnRH neurons express GLP-1 receptor (GLP-1R)and nNOS1 mRNA

Expression of Glp1r mRNA was detected in pooled, patch pipette-harvested GnRH-GFP neuron cytoplasm samples at cycle threshold (Ct) 32.7 ± 0.4. The Ct value of Gapdh was 22.3 ± 0.1.

Expression of Gnrh1 mRNAs was also verified in each patch pipette-harvested individual GnRH-GFP cytoplasm sample at Ct value of 24.5 ± 0.8. Samples with detectable Gfap mRNA were omitted from the study. Amplification curves showed that GnRH neurons expressed Nos1 mRNA (Ct 29.5 ± 1.5) encoded by the neuronal nitric oxide synthase gene. The cycle threshold value of the housekeeping gene Gapdh was 23.3 ± 0.3.

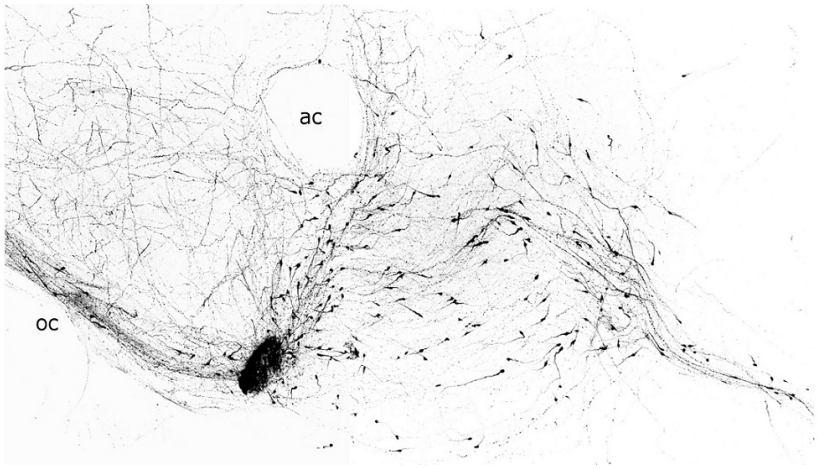
2. GnRH neurons receive GLP-1 immunoreactive (IR) innervation

2.1. Conventional double immunofluorescent double labeling

The putative involvement of the brain-born GLP-1 in the central regulation of reproduction was studied by the simultaneous detection of the GnRH and GLP-1-immunoreactive (IR) neuronal systems. In mice, the vascular organ of lamina terminalis (OVLT) and the preoptic region contained rich GLP-1-IR fiber networks and also individual GnRH-synthesizing neurons scattered within the network of GLP-1-IR axons. GLP-1-IR axons approached and established single or occasionally, multiple contacts with one tenth of GnRH neurons. Distribution of the contacts was rather inhomogeneous and exhibited the highest density in the proximity of the OVLT. GLP-1 inputs often targeted the dendritic compartment of GnRH cells. Both the smooth surfaced and the rough surfaced subtypes of GnRH neurons received GLP-1-IR axons. These results suggest the cooperation of GLP-1 and GnRH neuronal systems and identifies GLP-1 as a putative regulator of GnRH neuron cell functions.

2.2. Studies utilizing the 3DISCO immunocytochemical technique

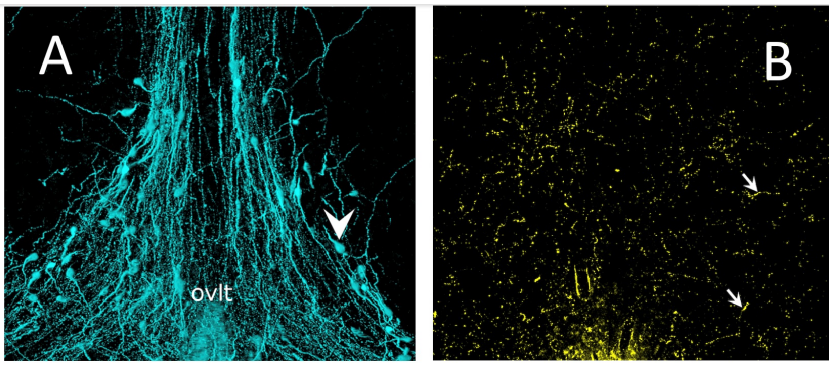
The morphological connections established between GLP-1 axons and GnRH neurons were also examined in 1 mm thick slices using the 3DISCO-based double fluorescent immunocytochemical technique that allows a deep penetration of the used antibodies and exploration of the interaction in 3D. Using this approach, a few (2-4) 1 mm thick brain slices through the preoptic area contained the overwhelming majority of GnRH neurons and in case of sagittal slices even the embryonic migratory path of GnRH neurons from the olfactory bulb toward the basal forebrain became visible.



Visualization of immunolabeled GnRH neurons in 1mm thick, para-median sagittal slice of the mouse brain. The cell population is located between the anterior commissure (ac) and the vascular organ of lamina terminalis (OVLT) and anteriorly it stretches toward the olfactory bulb. oc: optic chiasm.

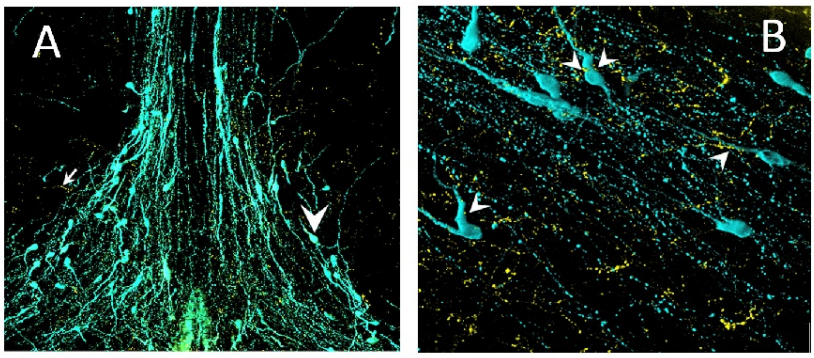
Similar to GnRH neurons, a rich GLP-1 immunoreactive axon network was also detectable in the medial septum-OVLT region. The confocal microscopic analysis of the double labeled thick

slices showed that the applied antibodies penetrated the entire thickness of the slices and properly visualized both neuronal systems. Accordingly, the 3DISCO immunocytochemical technique appeared suitable for studying the interaction of immunolabeled neuronal systems and carrying out the quantitative analysis of the communicating profiles



GnRH (A) and GLP-1 (B) immunoreactive structure detected in the same thick, frontal slice. Arrowhead point to an individual GnRH neuron, arrows mark GLP-1 axons. Ovlt: vascular organ of lamina terminalis.

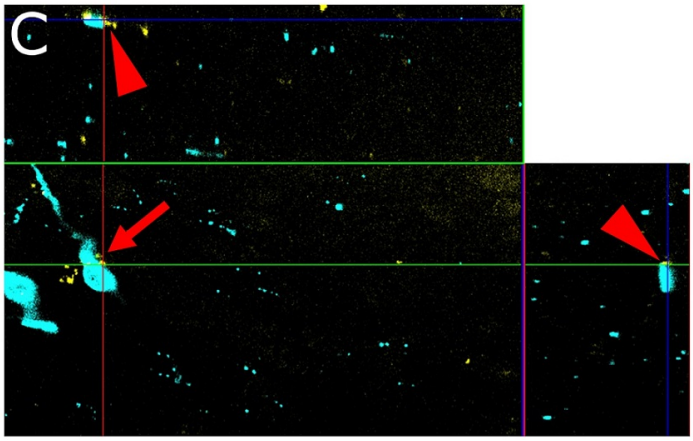
The simultaneous visualization of the two immunolabeled systems in thick slices allowed the detection of interaction sites. Using high power (40x) and orthogonal view, the real juxtapositions became distinguishable in the thick slice. The comprehensive analysis of such interneuronal communication sites served the goal of quantification of interactions.



Simultaneous detection of GnRH (blue) and GLP-1 (gold) immunoreactivities in low (A) and medium(B) power micrographs obtained from a I mm thick slice. In Fig. ***A,*** *one of the GnRH neurons is marked by arrowhead, while a single GLP-1-IR axon is labeled by arrow. In Fig.* ***B,*** *arrowheads indicate GLP-1 axons contacting GnRH neurons.*

2.3. Quantification of GLP-1-IR innervation of GnRH neurons

The analysis was carried out in 6 adult male mice using thick slices (0.5-1 mm) of the medial septum-OVLT region. Immunofluorescent double labelling was carried out in the framework of the 3DISCO technique. A juxtaposition of GLP-1 axon and GnRH-IR profile was defined as interaction if the juxtaposed profiles possessed the characteristic structural features and the contact was discernable in 1 micrometer thick optical slice in both orthogonal views (X-Y, Z).



GLP-1 axon (yellow) contacts (arrow) a GnRH neuron (blue) in a 1 micrometer thick optical slice. Arrowheads point to the same interaction site in the corresponding orthogonal view (upper and right-side panels).

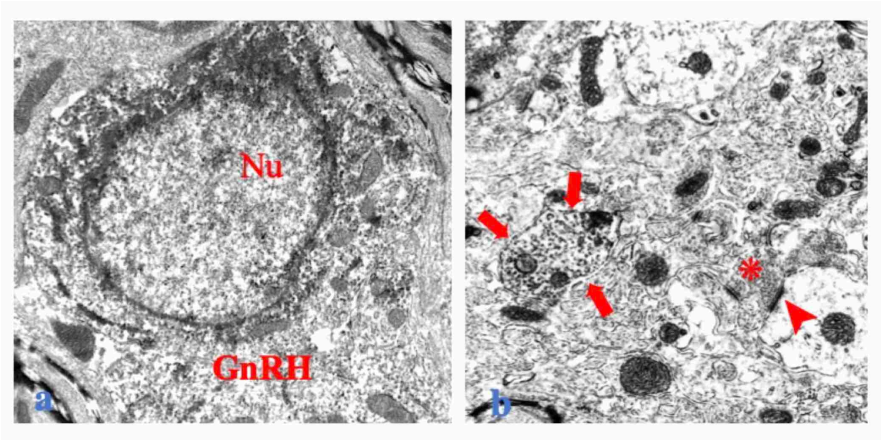
Altogether, 435 GnRH were analyzed, 72.5±11.7 neurons as average per animal. Almost a quarter of neurons (23±1.4%) received GLP-1-IR innervation. The GnRH neuron dendrites received the majority of innervating GLP-1 axons (65 %), the perikarya were reached by the rest (35 %). Both single and multiple contacts were detected. The highest incidence of innervation was noted in case of GnRH neurons distributed in the OVLT region.

2.4. The ultrastructural correlates of innervation

The observed juxtapositions of GnRH and GLP-1 profiles were studied further by means of ultrastructural analysis. To achieve this goal, we used a highly sensitive immunoelectron microscopic double labelling technique that had been elaborated previously by our group.
The presynaptic axonal profiles are identified by silver-gold-intensified diaminobenzidine (DAB) chromogen, while the postsynaptic elements are judged by the presence of the deposited DAB label. For the specific detection of the two neurohormones, previously characterized, highly specific antibodies were used. The biological brain samples were taken from either wild-type or transgenic mice. The genetically modified animals expressed channelrhodopsin-2 in their pre-pro-glucagon-synthesizing neurons. The yellow fluorescent protein (eYFP) added to the C-terminus of the ChR2 allowed the immunocytochemical detection of the expressed fusion protein. The elaboration and characterization of the transgenic mice is described in section 3.1. The use of transgene animals allowed the identification of GLP-1 IR axons based on the ChR2-eYFP content of the their axolemma.

2.4.1 Ultrastructural properties of GnRH neurons

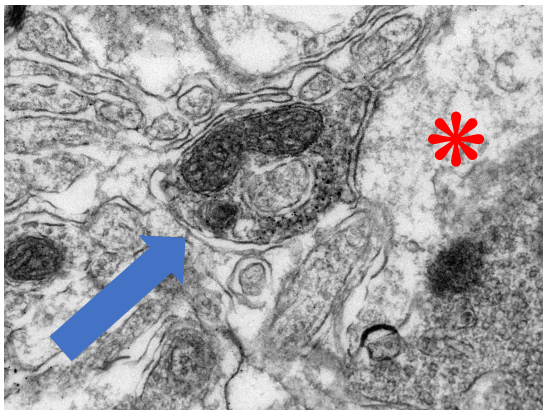
In accordance with our previously published observations, the DAB label is associated with ribosomes, the Golgi system and neurosecretory granules of 80-120 nm diameter. From the cell bodies, two dendritic processes arise. The perikarya exhibit a fusiform shape. Longitudinally or cross sectioned dendritic processes were also detectable due to the deposited electron dense DAB chromogen.



*Ultrastructural appearance of DAB-labeled GnRH perikaryon (**a**) and dendritic process (arrows, **b**). Asterisk labels an axon terminal that forms an asymmetric synapse (arrowhead) with an unlabeled dendrite. Nu: nucleus.*

2.4.2. The fine structure of GLP-1 axons juxtaposed to GnRH profiles

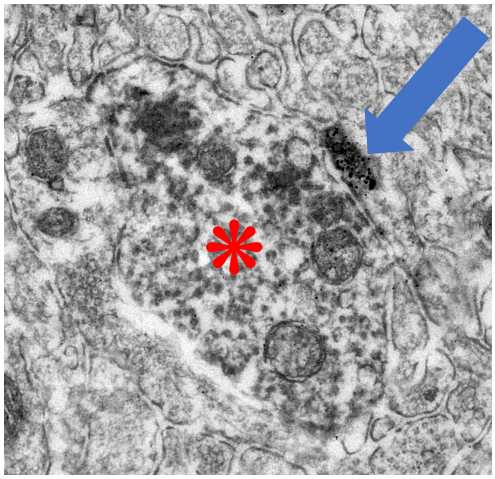
The highly electron dense silver-gold grains being deposited into the DAB chromogen unequivocally identified immunolabeled GLP-1-IR axons in the medial septum-OVLT region. The peptidergic boutons were filled with electron-lucent synaptic vesicles, contained neurosecretory granules and mitochondria. They were juxtaposed to both non-labeled and GnRH immunoreactive neuronal profiles.



Cross-sectioned GLP-1 axon (arrow) juxtaposed to a non-labeled dendrite (asterisk) in the MPOA.

2.4.3. Juxtaposition of GLP-1 axons and GnRH neurons

Tracing the GLP-1/ChR2 axons juxtaposed to perikarya and dendrites of GnRH neurons in series of ultrathin sections, resulted in the verification of the close contact – juxtaposition - seen at the light microscopic level. The ultrastructural analysis is going to be continued in order to elucidate whether the juxtaposed profiles form state of the art synapses.

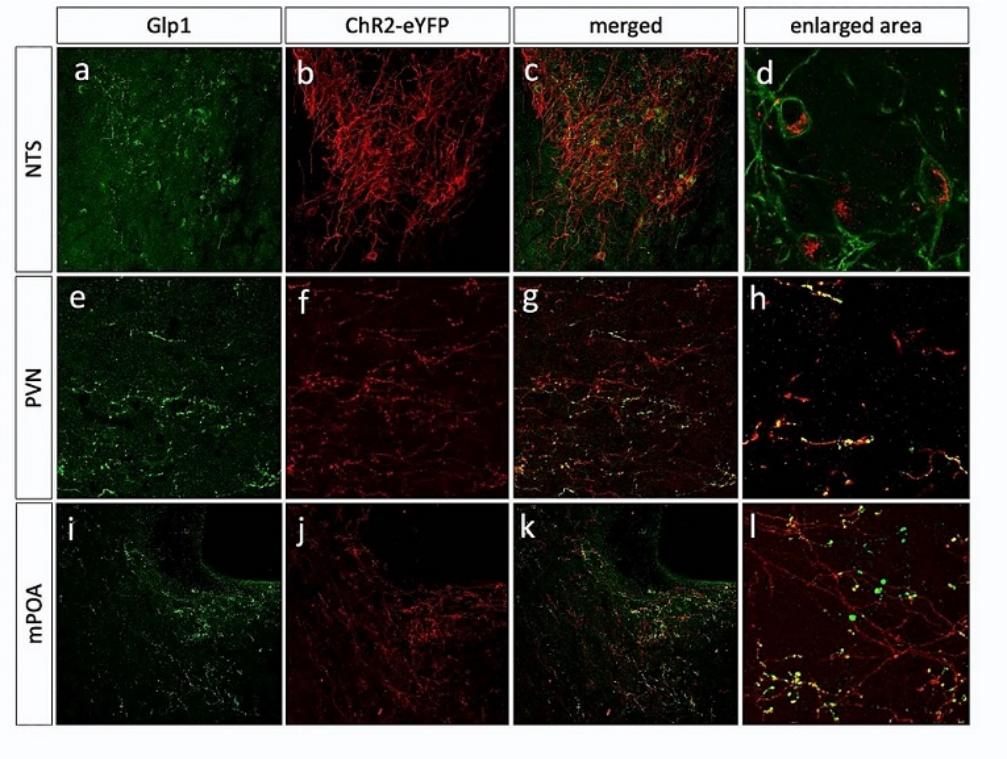


Silver-gold intensified DAB chromogen-labeled GLP-1/ChR2 axon (arrow) is in contact with a GnRH immunoreactive neuronal profile (asterisk).

3. Effects of optogenetic stimulation of GLP-1/ChR2 axons upon the electrophysiology of target structures in vitro

3.1. Elaboration and characterization of pre-pro-glucagon (PPG)-channelrhodopsin 2 (ChR2) double transgene mouse strain

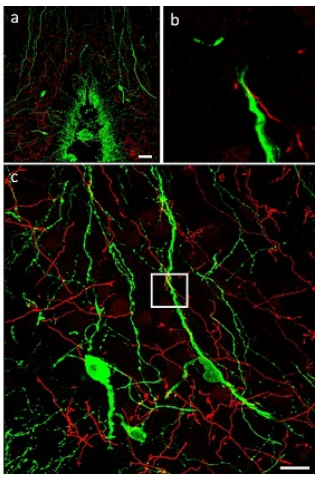
We have received the pre-pro-glucagon-cre mouse from the laboratory of M.M. Scott (University of Virginia School of Medicine, Charlottesville, Virginia, USA). The strain underwent rederivation in our Gene Technology Center. The newly re-established strain was also genotyped. Animals showing cre expression were crossed with floxed-channelrhodopsin-2 (ChR2) reporter mice. The PPG-cre-ChR2-eYFP double transgene mice were genotyped and characterized by immunocytochemical methods. ChR2-eYFP expression was found in neurons within the nucleus of the solitary tract and the reticular nucleus. These neuron populations were also immunoreactive for GLP-1. The colocalization rate was 95 %. GLP-1/ChR2 axons were detectable in many loci of the neuroaxis, including the hypothalamic paraventricular nucleus (PVN) and the medial preoptic area (mPOA) is. Similar to the cell bodies, the ChR2-eYFP-IR axons also showed GLP-1 immunoreactivity.



*Immunocytochemical characterization of PPG-cre-ChR2 transgene animal. Neurons of the nucleus of the solitary tract (NTS) are immunoreactive for GLP-1 (**a**) and also express channelrhodopsin-2. (**b**). The two distinct immunoreactivities are in colocalization (**c, d**). GLP-1/ChR2 axons innervate the paraventricular nucleus (PVN) (**e-h**) and the medial preoptic area (mPOA) (**i-l**).*

3.2 Elaboration and characterization of pre-pro-glucagon (PPG)-channelrhodopsin 2 (ChR2)-GnRH-GFP triple transgenic mouse strain.

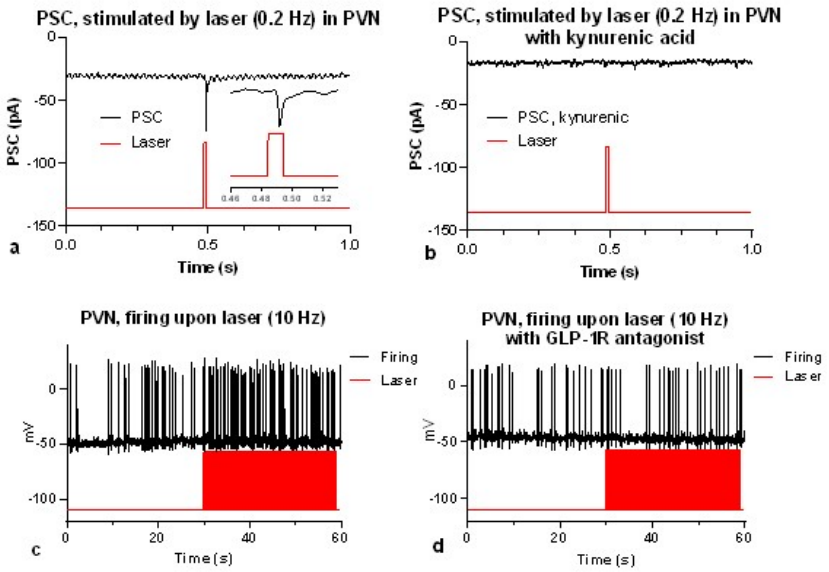
The characterized PPG-cre-ChR2 strain was crossed the GnRH-GFP mouse line, provided kindly by S. Moenter. The newly elaborated mouse strain was genotyped and characterized by immunocytochemical methods. In the preoptic area of these brains, GnRH neurons showing GFP expression were detectable which received juxtaposing GLP-1/ChR2 immunoreactive axons. This animal model made possible examining the biological effects of GLP-1 released from axon terminals on the electrophysiological performance of GnRH neurons *in vitro*.



Simultaneous visualization of GnRH neurons and GLP1/ChR2 immunoreactive axons in the MPOA of the triple transgenic mouse.
*In double labeled sections (**a-c**), both GnRH neurons (green) and channel-rhodopsin-2-IR axons (red) appear. At high power (**b**) the juxtaposition (arrowhead) of a ChR2-expressing axon (red) to the dendritic process of a GnRH neuron (green) is demonstrated. The micrograph corresponds to the boxed area in Fig. c.*

3.3. Effect of optogenetic stimulation of GLP-1/ChR2 axons on the neurons of paraventricular nucleus (PVN): optimizing parameters of the stimulation

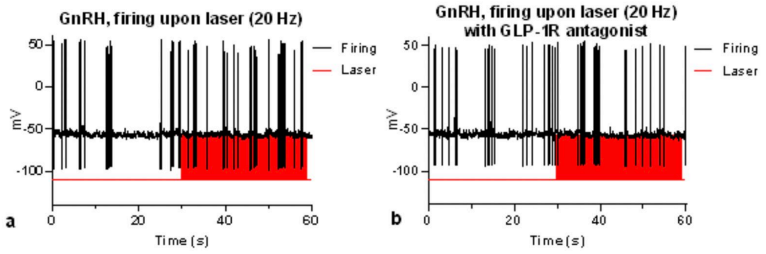
This study examined whether photostimulation of the channelrhodopsin (ChR2) expressing GLP-1 axon terminals soundly modulates neuronal activity in the hypothalamus. The tip of the glass fiber (laser wavelength: 473 nm) was placed on the surface of the acute brain slice preparations prepared from the PVN (coronal cut) or the preoptic area (POA, sagittal cut) of PPG-cre-ChR2-GnRH-GFP triple transgenic adult male mouse. Whole cell patch clamp method was used to measure the effects of the laser stimulation in neurons of the given brain areas (untagged neurons in the PVN and GFP-tagged GnRH neurons in the POA). The tip of the glass fiber was placed at a distance of 200-300 µm from the neurons to be illuminated. We measured PSCs in voltage-clamp at -70 mV holding potential and firing at 0 pA in current-clamp. PSCs were recorded at low frequency (0.2 Hz, 10 ms pulse width, 3 mW laser power), and then average of responses to 60 laser shots was studied. Measurement of the firing was carried out at higher laser shooting frequency (5-40 Hz, 10 ms pulse width, 3 mW laser power), starting with a 30-sec-long registration with no laser (for control purposes).



Responses of PVN neurons for low (0.2 Hz) and high (10 Hz) frequency laser stimulation. ***a.*** *The laser beam activates the axon terminals expressing ChR2/GLP1, resulting in a well-defined PSC response in 50 % of the neurons recorded (5/10 neurons). In the inset, the effect can be observed at a zoomed timescale.* ***b****. The response vanished in the extracellular presence of kynurenic acid, showing that the observed PSC was due to the induced glutamate release.* ***c.*** *The high frequency laser stimulation increased the firing rate (6/10 neurons, from 0.41 ± 0.163 Hz up to its 279 ± 56.6%, p=0.034). This action was abolished by GLP-1 receptor antagonist (112 ± 10.9 %, p=0.298) (****d****). No significant change in the firing rate was detected at higher (20-40 Hz), or lower (5 Hz) laser frequencies. The laser stimulation is marked by red color.*

3.4. Effect of optogenetic stimulation of the GLP-1/ChR2 expressing axons on the function of GnRH neurons in acute brain slices

The high frequency laser stimulation (20 Hz) increased the firing rate of GnRH neurons (6/10 neurons, from 0.47 ± 0.07 Hz to its 175 ± 17.3 %, p=0.0007) (**a**). This effect was abolished by GLP-1 receptor antagonist (111 ± 10.7%, p=0.3236) (**b**). Slightly weaker response was recorded at 10 Hz. Higher (40 Hz), or lower (5Hz) laser frequencies resulted in no significant change in the firing rate.



*Effect of the high frequency (20 Hz) laser stimulation on the firing of GnRH neurons in untreated (**a**) and GLP-1 receptor antagonist treated (b) brain slice fabricated from triple transgenic mouse. The laser stimulation is marked by red color.*

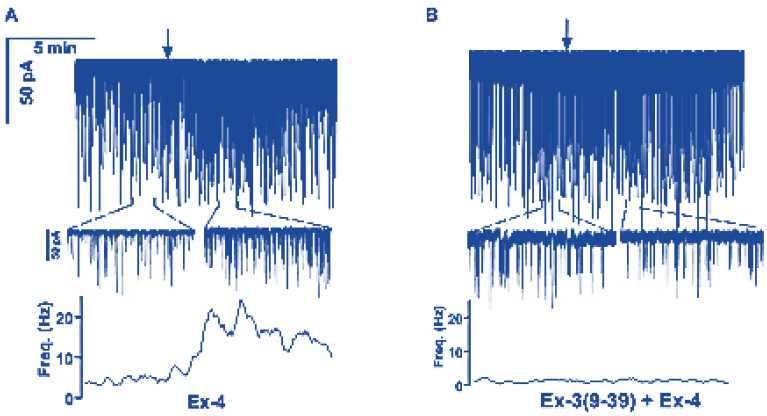
The results indicate that the GLP-1 released from neuronal sources - GLP-/ChR2 fibers – acts on the function of GnRH neurons, by increasing their firing rate. Effect of GLP-1 is specific, because this elevation in the firing rate can be eliminated by GLP-1 receptor antagonist.

4. Effects of GLP-1 receptor agonist and antagonist compounds on the firing of GnRH neurons

At first, we determined the dose-response curves of the GLP-1 receptor agonist, Exendin-4 (Ex-4) and antagonist, Exendin-3 (9-39) (Ex-3) compounds. Ex-4 was effective at 1-5 µM. We used it at 1 µM concentration in loose and whole cell patch experiments on acute hypothalamic slice preparations. Ex-4 increased the mean firing rate to 434 ± 69.9% of the control. The effect was abolished by the use of the specific GLP-1R antagonist, Ex-3.

4.1 *Effect of Exendin-4 on the GABAergic mPSCs of GnRH neurons*

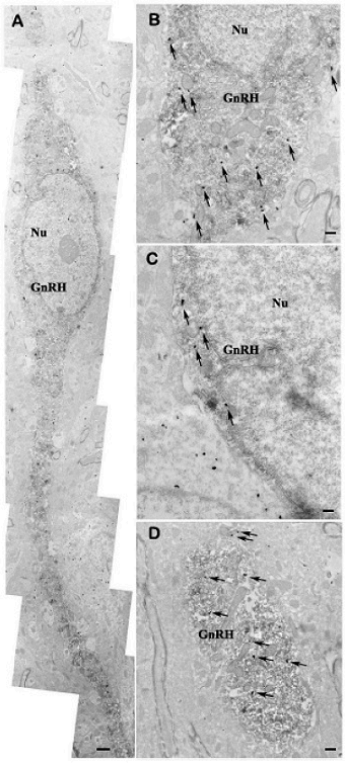
Ex-4 powerfully increased (240.7±30,42%) the frequency of mPSCs of the control which was also blocked by Ex-3 pretreatment.



Effects of GLP-1 receptor specific agonist and agonist compounds on frequency of mPSCs in GnRH neurons. A. Exendin-4 increased the frequency of mPSCs. B. Ex-3 blocked the effect of the agonist.

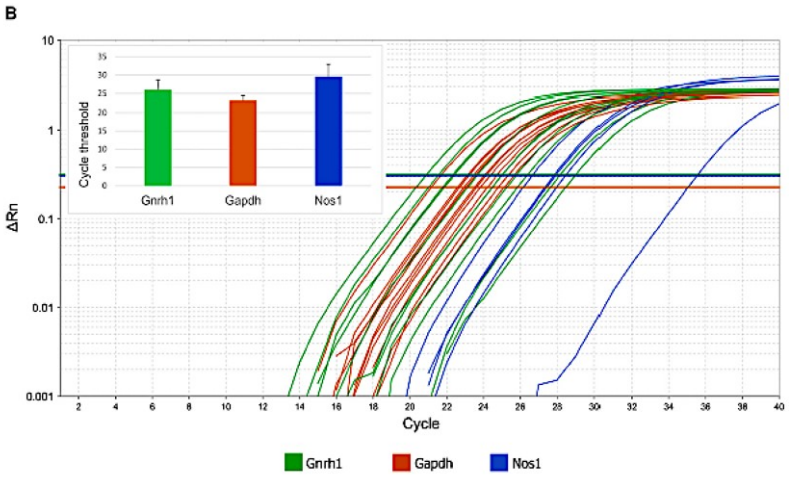
4.2. Role of nitric oxide signaling mechanisms in the regulatory process

Our original results have shown the first time that GnRH neurons express neuronal nitic oxide synthase (nNOS) and are capable of synthesizing nitric oxide. The gas neurotransmitter diffuses out from GnRH neurons and targets GABAergic presynaptic boutons.



*Ultrastructural detection of nNOS immunoreactivity in mouse GnRH neurons. **A.** Fusiform GnRH neuron shown at low power. In micrographs made at higher power (**B-D**), in the DAB-labeled cytoplasm of GnRH neurons silver intensified colloidal gold particles (arrows) identify the occurrence of nNOS enzyme. Nu: nucleus.*

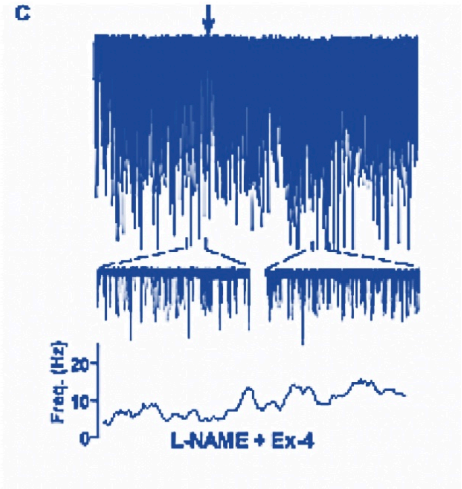
Quantitative real-time PCR studies have also confirmed the expression of nNOS mRNA in cytoplasmic samples of GnRH neurons.



Quantitative detection of GnRH1, Gapdh and Nos1 mRNA-s in GnRH neuron samples by PCR.

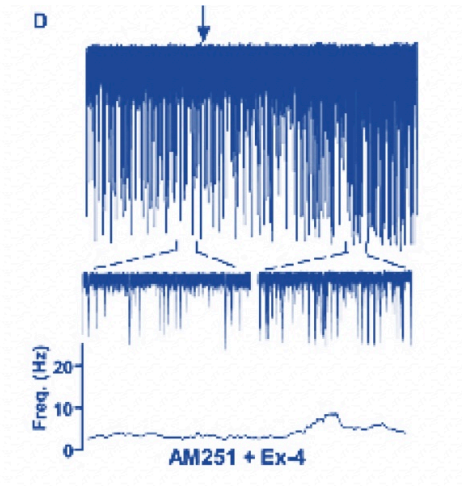
The inhibition of NO production (L-NAME, NPLA) significantly, but not totally decreased the observed effects of Ex-4 administration. The use of the NO scavenger, CPTIO gave similar results.

4.3. Role of 2-arachidonilglicerol (2-AG) retrograde signaling mechanism in the process



C. L-NAME pretreatment blocks only partially the effect of Ex-4 on the increase of frequency of mPSCs in GnRH neurons.

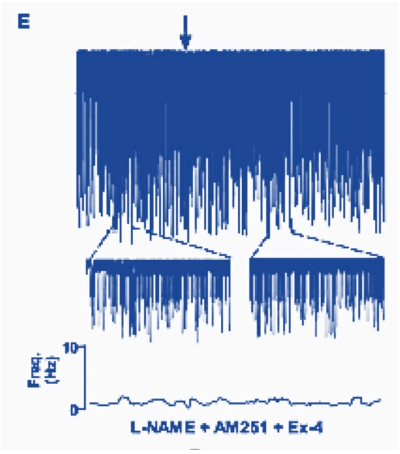
Our previous studies have shed light on the participation of retrograde endocannabinoid signaling in the control of GnRH neurons. 2-AG was shown to act on CB1 receptors expressed in afferent boutons of GABAergic neurons impinging on GnRH cells. Binding of 2-AG to CB1 decreases the probability of GABA release which is manifested in a decreasing frequency of mPSCs and subsequent inhibition of GnRH neurons. The current results indicate that 2-AG signaling also contributes to the effects of GLP-1 receptor activation because antagonizing the CB1 receptor by AM251 partially decreases the Ex-4-evoked change in the frequency of mPSCs in GnRH neurons.



D. Effect of AM251 pretreatment on the GnRH cell response evoked by Ex-4. Note the partial decrease.

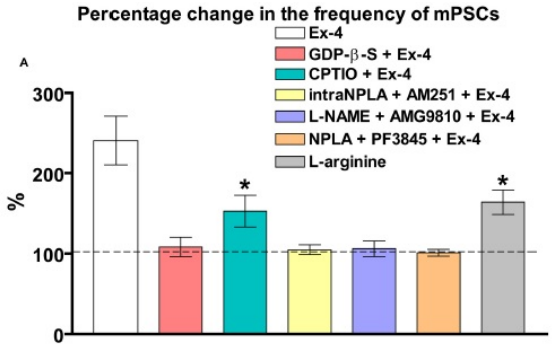
The detailed pharmacological analysis of the 2-AG signaling proved that activation of GLP-1 receptor decreases the 2-AG production via the anandamide (AEA)-transient receptor potential vanilloid 1 (TRPV1)-diacylglycerol lipase (DGL) pathway. The evoked change in this signaling mechanism disinhibits the endocannabinoid break of GABAergic afferents resulting the excitation of GnRH neurons.

The simultaneous pharmacological blockade of both endocannabinoid and NO retrograde signaling mechanisms totally abolished the effect of Ex-4 on the frequency of mPSCs in GnRH neurons.

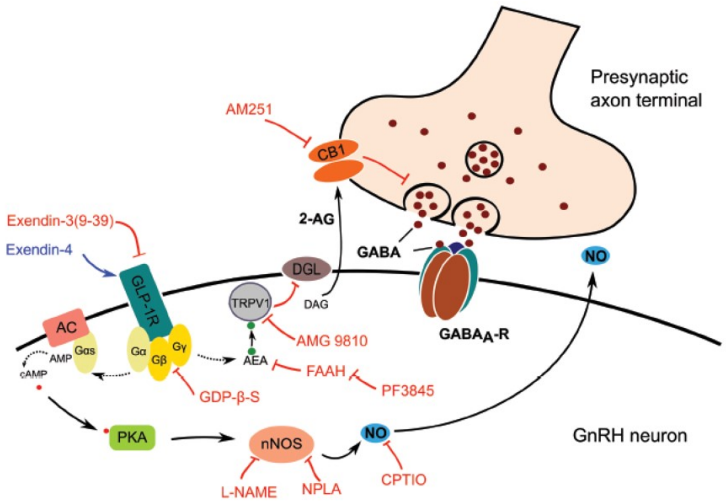


E. The combined pretreatment of the slice with AM251 and L-NAME totally prevents the effect of Ex-4 on frequency of mPSCs.

Summary of the effects of different compounds - linked to GLP-1 receptor signaling pathway in GnRH neurons - on the percentage change in the frequency of mPSCs.



5. Schematic summary of the cellular components of GLP-1 receptor-associated signal transduction pathway in GnRH neurons, as explored in the project



Specific activation of GLP-1 receptor increases the nitric oxide generation and decreases the 2-AG synthesis in GnRH neurons that have impact on GABA release from presynaptic afferents and result in activation of the GnRH neuron.

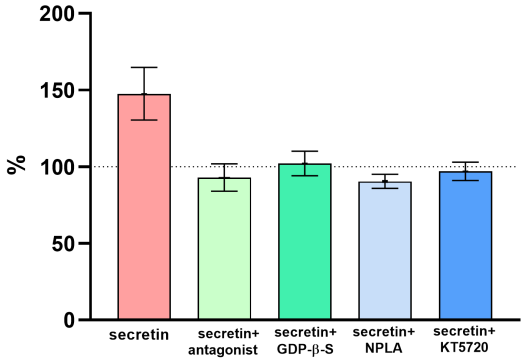
II. *Exploration of the regulatory role of secretin on GnRH neurons in vitro*

1. Secretin directly regulates GnRH neurons

We have discovered that secretin, a member of the secretin-glucagon-vasoactive intestinal peptide (VIP) hormone family, regulates directly - via secretin receptor - GnRH neurons and the hypothalamo-pituitary gonadal axis (HPG) axis in male mice. Administration of secretin (100 nM) increased the frequency of both the spontaneous postsynaptic (118.0±2.64%) and the miniature postsynaptic 147.6±19.19% currents. Secretin administration has also changed the resting membrane potential (12.74±4.539mV) and the firing of GnRH cells (after 1 min to 144.3 ± 10.8% and after 3 min up to 138.2 ± 11.24%). Secretin receptor antagonist (3 μM), or G-protein blocker (GDP-β-S, 2 mM) treatment abolished the secretin-evoked biological response.

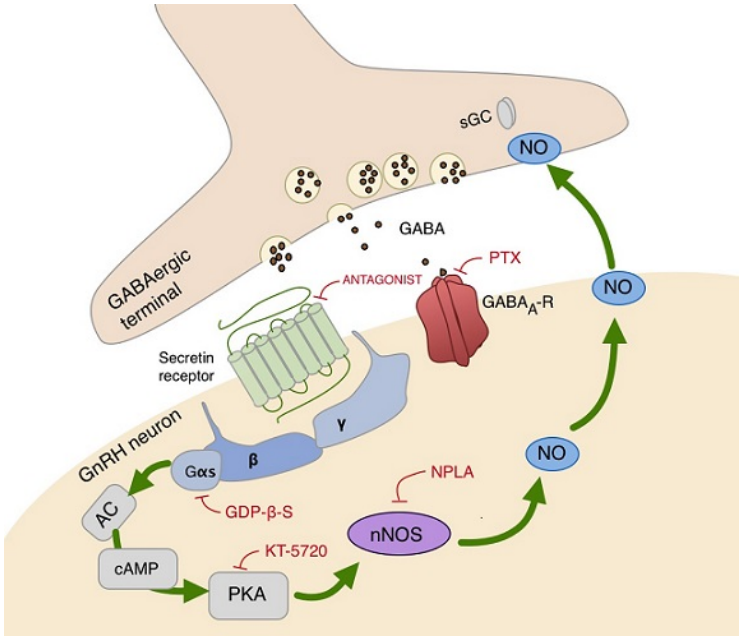
2. Elucidation of the secretin receptor-linked signal transduction pathway

We have also studied the downstream signal transduction mechanisms that are associated with the secretin receptor activation. Intracellular blockade of protein kinase A (PKA) (KT5720, 2 μM) or nNOS (NPLA, 1 μM) prevented the effects of secretin exerted on GnRH neurons. Our current results also elucidated the role of the cAMP/PKA/nNOS signaling pathway in the execution of the secretin receptor-mediated activation. The activation of this intracellular signaling pathway results in the increase of nitric oxide (NO) production that in turn, facilitates the operation of GnRH neurons via the excitatory $GABA_A$ receptor mediated neurotransmission.



*Effects of intracellularly applied nNOS (NPLA) (**A, C**) and PKA (KT5720) (**B, C**) blockers on the secretin-evoked mPSCs in GnRH neurons*

3. Schematic summary of the secretin receptor-coupled signal transduction pathway in GnRH neurons



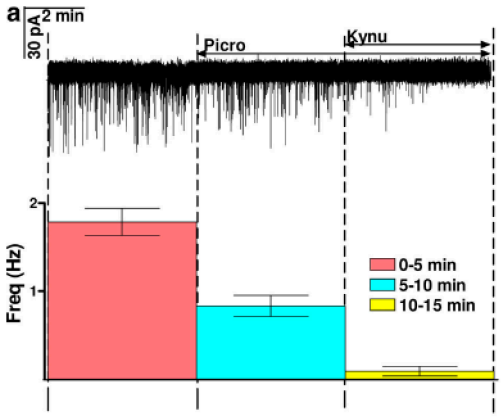
The activation of the secretin receptor increases the NO production via the cAMP/PKA/nNOS pathway that acts on the NO receptors (sGC) of presynaptic GABAergic afferents. The increasing GABA release exerts facilitatory influence upon GnRH neurons via $GABA_A$ receptors.

III. Effects of high and low levels of (E2) on the physiology of GnRH neurons and the medial preoptic area

1. Direct effects of positive E2 feedback on GnRH neurons: proestrus phase of the gonadal cycle

1.1. Significance of glutamate and GABA neurotransmission in the regulatory process

During proestrus the ovary increases its E2 production and release which influence the GnRH neurons and their different neuronal afferent systems via the positive feedback regulatory mechanism. The final outcome is manifested in the increased release of GnRH, the GnRH surge which is a prerequisite of the subsequent ovulation. Our current electrophysiological data proved that the preovulatory GnRH neurons **1.** possess glutamate and GABA neurotransmitter inputs and **2**. estradiol enhances the frequency of spontaneous and miniature postsynaptic currents. The data also suggest the participation of an E2-regulated retrograde messenger system in the physiological process.



In proestrus, the frequency of mPSCs of GnRH neurons is decreased partially by picrotoxin treatment. The residual currents are eliminated by the blockade of ionotropic glutamate receptors with kynurenic acid.

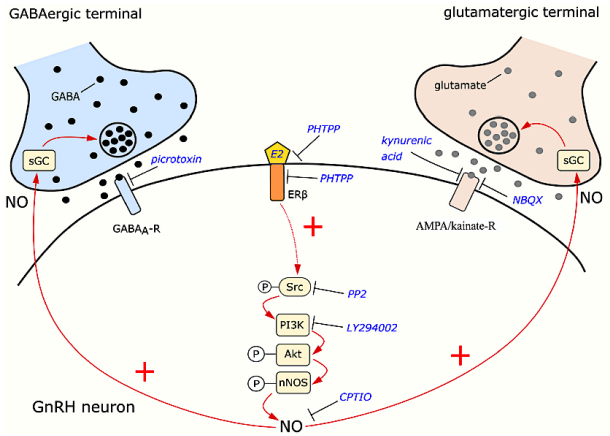
1.2. Detection of nitric oxide receptor in GABA- and glutamatergic neuronal afferents of GnRH neurons

By the use of fluorescent triple label immunocytochemistry and confocal laser microscopy, we provided evidence of the expression of NO receptor, the soluble guanylate cyclase, in GABA- and glutamatergic regulatory boutons innervating GnRH neurons.

1.3 Involvement of estrogen receptor beta and neuronal nitric oxide synthase (nNOS) in the regulatory process.

Earlier our group discovered the expression of estrogen receptor beta (ERβ) in rodent and human GnRH neurons and proved the existence of NO retrograde signaling from this cell population. The current results indicate that the contribution of both the functional ERβ and the neuronal nitric oxide synthase (nNOS) are mandatory in the successful completion of the positive E2 feedback regulation in GnRH neurons. Accordingly, antagonizing ERβ (PHTPP, 1 μM) or arresting the NO production in GnRH neurons powerfully counteract the effect of estradiol on the increment of frequency of mPSCs. We identified the ERβ/Src/PI3K/Akt/nNOS signal transduction pathway taking part and controlling the E2-modulated NO production.

1.4. Schematic summary of molecular mechanisms executing the positive E2 feedback regulation in GnRH neurons.



In proestrus, E2 induces NO production via the ERβ-Src-PI3K-Akt-nNOS regulatory pathway. The retrograde neurotransmitter binds to its receptor (sGC) in glutamate- and GABAergic afferents of GnRH neurons that results in an increased release of the neurotransmitters. The facilitation of GnRH neurons is accomplished via AMPA/kainate and GABA$_A$ receptors.

2. Impact of proestrus on expression of genes encoding neurotransmitter receptors in GnRH neurons

We provided the first evidence for the marked footprint of proestrus on the expression of neurotransmitter receptor genes of GnRH neurons. The evoked change of the transcriptome was rather widespread substantiating the fact that the induction of the preovulatory surge depends on the simultaneous cooperation of many neurotransmitter systems. The table beneath summarizes the major findings:

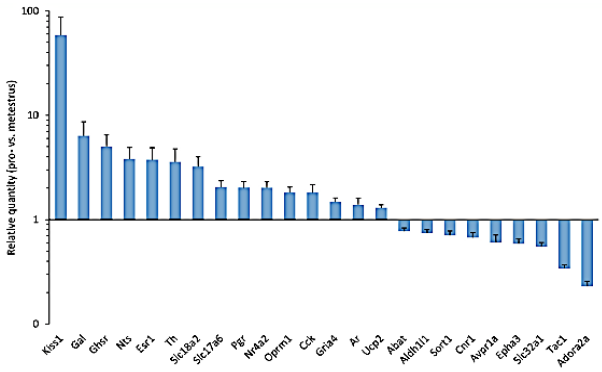
Upregulated genes in proestrus					
Affymetrix ID	**AE**	**Symbol**	**Description**	**FC**	**adj *p*-value (FDR)**
Cholinergic signaling					
1420744_PM_at	123	Chrnb2	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	2,65	2,17E-02
GABAergic signaling					
1455021_PM_at	269,3	Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	2,8	5,95E-04
1421263_PM_at	145,2	Gabra3	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3	1,9	1,77E-02
1428205_PM_x_a	30,9	Gabrb2	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2	1,97	1,36E-02
1435021_PM_at	35,3	Gabrb3	gamma-aminobutyric acid (GABA) A receptor, subunit beta 3	3,05	1,53E-02
1418177_PM_at	76,6	Gabrg2	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	2,2	2,96E-02
1416937_PM_at	481,4	Gabarap	gamma-aminobutyric acid receptor associated protein	1,82	4,36E-03
G-proteins and downstream effectors					
1427510_PM_at	241,6	Gnai1	guanine nucleotide binding protein (G protein), alpha inhibiting 1	2,59	1,49E-03
1435652_PM_a_a	98,2	Gnai2	guanine nucleotide binding protein (G protein), alpha inhibiting 2	1,88	4,71E-02
1450186_PM_s_a	1687	Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	1,73	8,27E-04
1455296_PM_at	58	Adcy5	adenylate cyclase 5	2,7	1,84E-02
1450519_PM_a_a	109,1	Prkaca	protein kinase, cAMP dependent, catalytic, alpha	2,06	5,66E-04
1420611_PM_at	496,7	Prkacb	protein kinase, cAMP dependent, catalytic, beta	1,86	1,22E-03
1427562_PM_a_a	87,5	Prkca	protein kinase C, alpha	1,78	7,51E-03
Glutamatergic signaling					
1421970_PM_a_a	99,7	Gria2	glutamate receptor, ionotropic, AMPA2 (alpha 2)	3,13	1,35E-03
1435239_PM_at	30,6	Gria1	glutamate receptor, ionotropic, AMPA1 (alpha 1)	2,36	2,84E-02
1437968_PM_at	65	Grin1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	2,24	2,73E-02
1438866_PM_at	39,9	Grin3a	glutamate receptor ionotropic, NMDA3A	1,8	2,11E-02
Solute carrier family					
1418610_PM_at	173	Slc17a6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), membe	1,77	1,46E-02
1423549_PM_at	74	Slc1a4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2,24	1,46E-02
1436137_PM_at	189	Slc6a17	solute carrier family 6 (neurotransmitter transporter), member 17	3,55	1,30E-03
Others					
1422314_PM_at	169,2	Clcn6	chloride channel 6	2,27	2,81E-04
Downregulated genes in proestrus					
Adenosinergic signaling					
1427519_PM_at	21,7	Adora2a	adenosine A2a receptor	0,5	7,47E-04
1450214_PM_at	13,7	Adora2b	adenosine A2b receptor	0,59	7,64E-03
Adrenergic signaling					
1422183_PM_a_a	24	Adra1b	adrenergic receptor, alpha 1b	0,49	5,18E-04
1423022_PM_at	25	Adra2a	adrenergic receptor, alpha 2a	0,37	5,07E-04
1422335_PM_at	50	Adra2c	adrenergic receptor, alpha 2c	0,56	1,22E-03
Cholinergic signaling					
1450575_PM_at	15	Chrm4	cholinergic receptor, muscarinic 4	0,58	1,20E-02
1420560_PM_at	30,3	Chrne	cholinergic receptor, nicotinic, epsilon polypeptide	0,52	4,98E-03
Dopaminergic signaling					
1422278_PM_at	16	Drd3	dopamine receptor D3	0,6	4,99E-02
1422829_PM_at	36,7	Drd4	dopamine receptor D4	0,45	8,28E-05
GABAergic signaling					
1457763_PM_at	16,3	Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0,58	4,98E-03
G-proteins and effectors					
1421959_PM_s_a	34,1	Adcy3	adenylate cyclase 3	0,5	9,04E-03
1452481_PM_at	32,8	Plcb2	phospholipase C, beta 2	0,42	3,99E-04
Glutamatergic signaling					
1438827_PM_at	118,3	Gls	glutaminase	0,56	4,53E-03
1425700_PM_at	57,6	Grm1	glutamate receptor, metabotropic 1	0,53	5,78E-03
1421393_PM_at	15,1	Grin2d	glutamate receptor, ionotropic, NMDA2D (epsilon 4)	0,37	1,49E-04
Serotonergic signaling					
1422288_PM_at	37,3	Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	0,58	1,14E-02
Purinergic signaling					
1422218_PM_at	24	P2rx7	purinergic receptor P2X, ligand-gated ion channel, 7	0,59	2,20E-03
Others					
1422277_PM_at	20,6	Glra1	glycine receptor, alpha 1 subunit	0,49	2,76E-03

3. Proestrus differentially regulates ion channel and calcium homeostasis-regulating genes in GnRH neurons.

Comparing the transcriptome of GnRH neurons obtained from proestrus and metestrus animals revealed that proestrus markedly modifies the expression of different voltage-gated sodium, potassium and calcium gene subunits. The results are in harmony with the increased activity and burst type firing of GnRH neurons seen in proestrus. Certain responses in case of potassium channel expression envisage the preparation of GnRH neurons for a decreasing cellular activity characterizing the post-surge period. 37 ion channel and 8 calcium homeostasis regulating genes showed differential expression that are introduced and discussed in the corresponding publication.

4. Effects of proestrus on the transcriptome of the medial preoptic area (mPOA).

The mPOA plays a vital role in the regulation of GnRH neurons under the positive E2 feedback. Its neurons express alpha and beta subtypes of estrogen receptor whose antagonization abolishes the GnRH surge. Furthermore, the region also contains kisspeptin-synthesizing neurons that are also responsible for the mediation of the positive E2 feedback and proper execution of the GnRH surge. The transcriptome analysis of the region in proestrus showed that the expression of functionally fundamental genes encoding neurotransmitter, neuropeptide and gonadal steroid receptors changed.



Demonstration of proestrus-regulated genes in the medial preoptic area of the mouse brain as revealed by q-RT-PCR.

5. Role of estrogen receptor beta and 2-arachidonilglicerol (2-AG) in the direct regulation of GnRH neurons by the negative estradiol feedback

Estradiol applied at low concentration (10 pM) markedly decreased the frequency of spontaneous PSCs (49.62±7.6%) in GnRH neurons tested at metestrus. The specific ERβ agonist, DPN (10 pM) increased the frequency of mPSCs which effect was abolished by the administration of an ERβ selective antagonist (PHTTP, 1 μM.) The results corroborate the fundamental role of estrogen receptor beta in the mediation of the negative E2 feedback effect Furthermore, in the orchestration of inhibition the retrograde 2-AG signaling also has a major contribution. The pharmacological blockade of the presynaptic CB1 receptor (AM251, 1 μM) hindered the manifestation of the negative feedback.

IV. Studies on extrahypothalamic tissues and organs regulated dominantly by estrogen signaling

1. Effects of ovariectomy on the transcriptome of the hippocampal formation of the middle-aged rat.

The study was undertaken to clarify the effects of long-term, surgical ovariectomy (OVX) on the hippocampal transcriptome in middle-aged rats. At age of 13 months, intact, control and long-term ovariectomized groups were formed. All animals were sacrificed 5 weeks after the time of gonadectomy, the hippocampal formations were removed and processed for transcriptome analysis. Microarray and quantitative real-time PCR studies identified 252 and 61 genes, respectively, whose expression was significantly altered by long-term ovariectomy. KEGG pathway analysis revealed the impact on neuroactive ligand-receptor interaction, retrograde endocannabinoid signaling, estrogen signaling, apoptosis and proteoglycan synthesis among others.

Network and interaction analyses of predicted proteins encoded by long-term ovariectomy-regulated genes revealed the upregulation of growth/ troph/transcription factor signaling assembly (Mdk, Fgf1, Igf2, Ngf, Ngfr, Ntf3, Ntrk1, *Otx2,* Hif1a, Esr1, Nr4a3), peptides/receptor cluster (Cartp, Kl, Ttr, Gnrhr), neurotransmitter signaling group (Grm1, Gria4, Gls, Slc18a2), a potassium channel (Kcj6) and proteins serving immune functions (C3, A2m, Ccl2, Itgam, Cfh, Fcgr3a, Il1b, RT1-EC2, Tlr3 and Tlr4). The downregulated protein clusters included neuropeptides and their receptors (Adcyap1, Cbln2, Cck, Cckbr, Crhr1 and 2, Oprd1, Nts, Penk, Sstr1, Vip), members of neurotransmitter signaling family (5Htr2c, Chrna3, Chrm4, Grm8, Hrh3, Slc17a6) and various potassium channels (Kcnk9, Kcnj9, Kcnma1, Kcnc2). Several solute carriers (9) also underwent downregulation. In the category of growth factor/transcription regulation, powerful candidates appeared (Arnt2, Crabp1, Hsp90ab1, Igfbp4, Rxra, Trhb). Protein cluster serving immune response/defense was represented by Apitd1, Bcl2, Clql3, Ilr3a, Sod1, Sncb in the downregulated group. The findings indicate that surgical gonadectomy carried out at middle-age in female rats robustly changes gene expression and signaling mechanisms of the hippocampal formation that alter neurogenesis, synaptic plasticity and immune modulation and may lead to cognitive dysfunctions.

2. Chronic amyloid β oligomer infusion evokes sustained inflammation and microglial changes in the rat hippocampus via NLRP3

Microglia are instrumental for recognition and elimination of amyloid $\beta_{1\text{-}42}$ oligomers (AβO), but the long-term consequences of AβO-induced inflammatory changes in the brain are unclear. We explored microglial responses and transcriptome-level inflammatory signatures in the rat hippocampus after chronic AβO challenge. Middle-aged Long Evans rats received intracerebroventricular infusion of AβO or vehicle for 4 weeks, followed by treatment with artificial CSF or MCC950, an NLRP3 inhibitor, for the subsequent 4 weeks. AβO infusion evoked a sustained inflammatory response including activation of NF-κB, triggered microglia activation and increased the expression of pattern recognition and phagocytic receptors. $A\beta_{1\text{-}42}$ plaques were not detectable likely due to microglial elimination of infused oligomers. In addition, we found upregulation of neuronal inhibitory ligands and their cognate microglial receptors, whilst downregulation of *Esr1* and *Scn1a*, encoding estrogen receptor alpha and voltage-gated sodium-channel Na(v)1.1, respectively, was observed. These changes were associated with impaired hippocampus-dependent spatial memory and resembled early

neurological changes seen in Alzheimer's disease. To investigate the role of inflammatory actions in memory deterioration, we performed MCC950 infusion, which specifically blocks the NLRP3 inflammasome. MCC950 attenuated AβO-evoked microglia reactivity, restored expression of neuronal inhibitory ligands, reversed downregulation of ERα, and abolished memory impairments. Furthermore, MCC950 abrogated AβO-invoked reduction of serum IL-10. These findings provide evidence that in response to AβO infusion microglia change their phenotype, but the resulting inflammatory changes are sustained for at least one month after the end of AβO challenge. Lasting NLRP3-driven inflammatory alterations and altered hippocampal gene expression contribute to spatial memory decline.

3. Glycinergic input to the mouse basal forebrain cholinergic neurons

The basal forebrain (BF) receives afferents from brainstem ascending pathways, which has been implicated first by Moruzzi and Magoun (1949) to induce forebrain activation and cortical arousal/waking behavior; however, it is very little known about how brainstem inhibitory inputs affect cholinergic functions. In the current study, glycine, a major inhibitory neurotransmitter of brainstem neurons, and gliotransmitter of local glial cells, was tested for potential interaction with BF cholinergic (BFC) neurons in male mice. In the BF, glycine receptor alpha subunit-immunoreactive (IR) sites were localized in choline acetyltransferase (ChAT)-IR neurons. The effect of glycine on BFC neurons was demonstrated by bicuculline-resistant, strychnine-sensitive spontaneous IPSCs recorded in whole-cell conditions. Potential neuronal as well as glial sources of glycine were indicated in the extracellular space of cholinergic neurons by glycine transporter type 1 (GLYT1)- and GLYT2-IR processes found in apposition to ChAT-IR cells. Ultrastructural analyses identified synapses of GLYT2-positive axon terminals on ChAT-IR neurons, as well as GLYT1-positive astroglial processes, which were localized in the vicinity of synapses of ChAT-IR neurons. The brainstem raphe magnus was determined to be a major source of glycinergic axons traced retrogradely from the BF. Our results indicate a direct effect of glycine on BFC neurons. Furthermore, the presence of high levels of plasma membrane glycine transporters in the vicinity of cholinergic neurons suggests a tight control of extracellular glycine in the BF.

4. Long-term selective estrogen receptor-beta agonist treatment modulates gene expression in bone and bone marrow of ovariectomized rats

Gonadal hormones including 17β-estradiol exert important protective functions in skeletal homeostasis. However, numerous details of ovarian hormone deficiency in the common bone marrow microenvironment have not yet been revealed and little information is available on the tissue-specific acts either, especially those via estrogen receptor beta (ERβ). The aim of the present study was therefore to examine the bone-related gene expression changes after ovariectomy (OVX) and long-term ERβ agonist diarylpropionitrile (DPN) administration. We found that OVX produced strong and widespread changes of gene expression in both femoral bone and bone marrow. In the bone out of 22 genes, 20 genes were up- and 2 were downregulated after OVX. It is noteworthy that DPN restored mRNA expression of 10 OVX-induced changes (Aldh2, Col1a1, Daam1, Fgf12, Igf1, Il6r, Nfkb1, Notch1, Notch2 and Psen1) suggesting a modulatory role of ERβ in bone physiology. In bone marrow, out of 37 categorized genes, transcription of 25 genes were up- and 12 were downregulated indicating that the marrow is highly responsive to gonadal hormones. DPN modestly affected transcription, only expression of two genes (Nfatc1 and Tgfb1) was restored by DPN action. The PI3K/Akt

signaling pathway was the most affected gene cluster following the interventions in bone and bone marrow, as demonstrated by canonical variate analysis (CVA). We suggested that our results contribute to a deeper understanding of alterations in gene ex- pression of bone and bone marrow niche elicited by ERβ and selective ERβ analogs.

Scientometric data of the published results of the project

Number of papers	11
Cumulative impact value	43.284
All citations	83
Independent citations	67

V. Publication list

Farkas I, Vastagh C, Farkas E, Bálint F, Skrapits K, Hrabovszky E, Fekete C, Liposits Z.
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