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The ontogeny of kisspeptin, leptin and adiponectin: their possible role in the uterine quiescence in rats

Ph.D. Thesis

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List of publications

Publication related to Ph.D. thesis

I. **Schaffer, A.;** Ducza, E.; Bódi, N.; Bagyánszki, M.; Szalai, Z.; Mirdamadi, M.; Barna, T.; Szűcs, K. F.; Gáspár, R.: The ontogenies of endometrial and myometrial leptin and adiponectin receptors in pregnant rats: Their putative impact on uterine contractility
LIFE SCIENCES 297 Paper: 120465, 12 p. (2022)
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II. **Schaffer, A.;** Hajagos-Tóth, J.; Ducza, E.; Bódi, N.; Bagyánszki, M.; Szalai, Z.; Gáspár, R.: The ontogeny of kisspeptin receptor in the uterine contractions in rats: Its possible role in the quiescence of non-pregnant and pregnant uteri
EUROPEAN JOURNAL OF PHARMACOLOGY 896 Paper: 173924, 9 p. (2021)
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Presentation related to Ph.D. thesis

I. **Schaffer, A.,** Hajagos-Tóth, J., Ducza, E., Gáspár, R.:
The effects of kisspeptin, leptin and adiponectin on non-pregnant rat uterus in vitro
RECOOP 9th Annual Project Review Meeting, 2018.10.11-14., Bratislava, Slovakia (Poster presentation)

II. **Schaffer, A.,** Hajagos-Tóth, J., Ducza, E., Gáspár, R.:
Ontogeny of the effects of leptin, adiponectin and kisspeptin on pregnant rat uterine contractility in vitro
RECOOP 10th Annual Project Review Meeting, 2019.10.11-12, Wrocław, Poland (Poster presentation)

III. **Schaffer, A.,** Hajagos-Tóth, J., Ducza, E., Gáspár, R.:
A leptin, adiponektin és kisspeptin vemhes uterusz kontraktilitásra gyakorolt hatásának vizsgálata in vitro
XVI. Congressus Pharmaceuticus Hungaricus, 2020.09.10-12., Online conference (Poster presentation)

IV. **Schaffer, A.,** Ducza, E., Bódi, N., Bagyánszki, M., Szalai, Z., Gáspár, R.:
Effects of kisspeptin on non-pregnant and pregnant uterine contractility
RECOOP 15th Bridges in Life Sciences Conference, 2020.10.02., Online conference (Poster presentation)

V. **Schaffer, A.,** Ducza, E., Bódi, N., Bagyánszki, M., Szalai, Z., Gáspár, R.:
Role of leptin and adiponectin in the regulation of pregnant uterine contractility in rats
RECOOP 16th Bridges in Life Sciences Conference, 2021.04.16., Online conference (Poster presentation)

VI. **Schaffer, A.,** Ducza, E., Bódi, N., Bagyánszki, M., Gáspár, R.:
The influencing role of leptin and adiponectin systems in pregnant rat uterine contractility
ISCTICO-HUPHAR-IUPHAR conference, 2021.10.27-30., Pécs, Hungary
(Poster presentation)

Other publication nonrelated to this thesis

I. Gáspár, R.; Hajagos-Tóth, J.; **Schaffer, A.**; Kothencz, A.; Siska-Szabó, L.; Ducza, E.; Csányi, A.; Tábi, T.; Bagaméry, F.; Szökő, É.; Kovács, O.; Barna, T.; Samavati, R.; Mirdamadi, M.; Sztojkov-Ivanov, A.; Szűcs, K. F.; Vari, S. G.: High Fat High Sucrose Diet Modifies Uterine Contractility and Cervical Resistance in Pregnant Rats: The Roles of Sex Hormones, Adipokines and Cytokines
LIFE-BASEL 12: 6 Paper: 794 , 17 p. (2022)
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II. Seres-Bokor, A.; Kemény, K. K.; Taherigorji, H.; **Schaffer, A.**; Kothencz, A.; Gáspár, R.; Ducza, E.: The Effect of Citral on Aquaporin 5 and Trpv4 Expressions and Uterine Contraction in Rat - An Alternative Mechanism
LIFE-BASEL 11: 9 Paper: 897 , 9 p. (2021)
(IF: 3,251, Biochemistry, Genetics and Molecular Biology [miscellaneous], SJR indicator: Q2)

III. Kothencz, A.; Hajagos-Tóth, J.; Szűcs, K. F.; **Schaffer, A.**; Gáspár, R.: α-Tocopherol Potentiates the Cervical Resistance Decreasing Effects of COX Inhibitors in Pregnant Rats: The Putative Role of Cyclooxygenase-2 Inhibition
JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS 368 : 2 pp. 292-298. , 7 p. (2019)
(IF: 3.561, Pharmacology, SJR indicator: Q1)

List of abbreviations

AUC: area under curve

AdipoRs: adiponectin receptor 1 and 2

POMC: pro-opiomelanocortin

KISS1: kisspeptin

Kiss1r: kisspeptin receptor

CGRP: calcitonin gene-related peptide

PNOC: prepronociceptin

N/OFQ: nociceptin

LEPR: leptin receptor

JAK/STAT: Janus kinase/signal transducer and activator of transcription

IRS: insulin receptor substrate

PI3K: phosphatidylinositol 3 kinase

SHP2: SH2-containing protein tyrosine phosphatase 2

AMPK: adenosine monophosphate-activated protein kinase

MAPK: mitogen-activated protein kinase

ACC: acetyl-CoA carboxylase

HPG: hypothalamic-pituitary-gonadal

LH: luteinizing hormone

FSH: follicle-stimulating hormone

GnRH: gonadotropin-releasing hormone

PPARα: peroxisome proliferator-activated receptor alpha

APPL1: adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1

DAG: diacylglycerol

IP3: inositol-1,4,5-trisphosphate

TBS: Tris-buffered saline

DAPI: 4′,6-diamidino-2-phenylindole

Contents

Introduction

Adipokines in general

For decades, adipose tissue was only known as an energy reservoir. Following the discovery of leptin in 1994, a new concept was adopted based on the secretory functions of the adipose tissue. It is now widely considered as the largest endocrine organ, and the source of various proteins named adipokines. These cell signalling factors are secreted directly into the bloodstream and are involved in multiple physiological functions at different levels. They are mostly recognized for their regulatory role in metabolic processes, but their influence on the reproductive system has also been previously documented. [1] It is also known that nutritional status has a major influencing role on reproductive functions in women. Disfunction of reproduction can occur in case of malnutrition or obesity, two conditions that are associated with altered adipokine plasma levels. [2]

Leptin

Leptin is a polypeptide encoded by the *ob* gene and secreted predominantly by the adipocytes of white adipose tissue. Leptin system is reported to be present in the hypothalamus, gastrointestinal tract, skeletal muscle, placenta and mammary gland. [3–8] Main actions of leptin include regulation of appetite, energy homeostasis and lipid metabolism. Leptin controls food intake by acting on POMC neurons that are the main sites of hypothalamic LEPR expression. [9] More importantly, leptin acts as a link between adipose tissue and the reproductive system. It signals to the central nervous system about the body's energy storage and nutritional status, as there is a positive correlation between leptin plasma levels and the amount of body mass. [10] Low concentrations or absence of leptin are a sign of energy deficiency, and as a result, physiological processes are interrupted. [11] Deficiency of leptin or LEPR results in hyperphagia, obesity and diabetes mellitus. Severe obesity is accompanied by elevated plasma leptin levels and leptin resistance, a complex anomaly that is still not fully clarified. [12] Also, gender difference in serum leptin levels is also proved with higher leptin concentrations detected in women. [13]

LEPR is a type I cytokine receptor that has 6 isoforms and is ubiquitously expressed in the body. Binding of leptin results in receptor dimerization, which initiates the activation of the JAK2/STAT and MAPK signalling pathways. Other signal transduction pathways regulated by leptin include IRS/PI3K, SHP2/MAPK and AMPK/ACC. [14]

Leptin is known for its neuroendocrine properties and acts as a major regulator of the HPG axis. LEPRs have been detected both centrally and peripherally through the HPG axis and were proved to be crucial for fertility and the onset of puberty. [15] Since LEPRs are not expressed in GnRH neurons, leptin indirectly stimulates the pituitary GnRH secretion by acting on kisspeptin neurons. [16,17] By increasing the secretion of GnRH, leptin causes elevation in LH and FSH levels. [18] Consequently, dysregulation of the leptin system can cause delayed onset of puberty or impaired ovary functions. [19–21] Leptin was also described before as a modulator of uterine smooth muscle action. It was previously reported that leptin reduces both spontaneous and induced contractions of normal and obese human pregnant myometrium. [22] Leptin also exerted a small inhibitory effect on rat myometrial contractions, but the exact signalling mechanism is still unidentified. [23] Serum concentrations of leptin have been shown to be elevated in rats and humans during gestational period, as compared with non-pregnant plasma levels. The main source of leptin during pregnancy is the placenta, which explains the quick decline of maternal leptin concentrations to the physiological levels postpartum. [24,25]

Adiponectin

Adiponectin is a 30 kDa polypeptide produced primarily by subcutaneous adipose tissue. It is present in the circulation at a considerably high concentration taking up approximately 0,01% of plasma proteins, which makes it the most abundant adipokine in human plasma. It can be found in the serum in monomeric form and tri-, hexa- and multimeric forms with different molecular weights. [26] Adiponectin shows sexual dimorphism as well, meaning that females have 2-3 times higher plasma levels than males. [27] Unlike leptin, its serum concentrations are inversely correlated with the body mass index. Severe obesity and insulin resistance are linked with reduced plasma adiponectin levels, and at the same time, weight loss induces an elevation in plasma concentration. [28–30]

Adiponectin exerts anti-inflammatory and antiatherogenic effects but is mostly known for its active regulatory role in lipid metabolism and glucose uptake. It is also recognized as an insulin-sensitizing adipocytokine. [31] These exerted effects are evolved through two main receptors: AdipoR1 and AdipoR2. However, a third receptor called T-cadherin has also been identified, and it is involved in vascularization processes. [32–34] However, the involvement of T-cadherin in the uterine actions of adiponectin was not an objective of the current study. The distribution of AdipoR1 and AdipoR2 in the peripheral tissues is different, AdipoR1 is expressed ubiquitously, while AdipoR2 can mostly be detected in the liver. However, recent evidence shows that both are downregulated in insulin-resistance associated with obesity. [35] The binding of adiponectin

to these receptors results in the activation of AMPK, p38 MAPK and PPARα signalling cascades. [36] In addition, an adaptor protein called APPL1 was reported to interact with adiponectin receptors and mediate signalling mechanisms. [37] Expressions of AdipoR1 and/or AdipoR2 have been confirmed in peripheral reproductive tissues in different species. The presence of the adiponectin system is proved in human endometrium, human and rat placenta, as well as in pregnant mice and porcine uteri. [38–40] Yet, according to previous studies, deletion of one or both receptors does not result in impaired female fertility, implying that adiponectin signalling might not be critical for the proper functioning of the reproductive system. [34] Presumably, adiponectin influences reproduction through the regulation of metabolic functions. [41]

Previous studies have reported that maternal circulating adiponectin concentration is elevated in the early stages of pregnancy, but a reduction occurs towards term, showing a negative correlation with the increased maternal body fat mass. Generally, obesity is associated with hypoadiponectinaemia and insulin resistance, and these conditions in pregnant women may cause abnormalities during pregnancy and labour. [42]

Contrary to leptin, there has been less evidence for adiponectin's impact on uterine smooth muscle activity. One study revealed that adiponectin reduces contractility of non-pregnant and pregnant mice myometrium, hypothesizing that adiponectin might be a link between maternal metabolic state and the outcome of pregnancy. [43]

Kisspeptin

Several neuropeptides regulate uterine smooth muscle contractility. The most well-known of them is oxytocin, which plays a key role in inducing myometrial contractions at the time of labour. [44] Towards parturition, the uterine oxytocin receptors are markedly upregulated, therefore the responsiveness of the myometrium is intensified. [45,46] In contrast, the sensory neuropeptide CGRP is recognized as a smooth muscle relaxing agent. According to previous studies, CGRP reduces uterine contractions both in humans and rats. [47,48] CGRP caused dose-dependent relaxation of spontaneous and electric field stimulated contractions *in vitro*. However, a loss of its action was observed as term was approached and also after delivery. [49] N/OFQ, a product of the precursor protein PNOC, acts as a natural ligand for the nociceptin receptor. [50,51] Besides its central effects (e.g. pain transmission), N/OFQ also influences the female reproductive axis as a neuroendocrine modulator. [52] Both N/OFQ and its precursor peptide are expressed in the pregnant human myometrium and rat uterus. Results of *in vitro* contractility studies demonstrated that N/OFQ relaxes rat and human myometria. [53,54]

Furthermore, the effects of the neuropeptide nocistatin on the function of rat and human uteri were also reported. Nocistatin presence was confirmed in non-pregnant and pregnant rat uterine tissue, with higher levels of expression on the last gestational day. Previous studies demonstrated that nocistatin inhibits contractions of human and rat myometrium and proved that N/OFQ can enhance this action. The same study also suggested that the relaxant effect of nocistatin is mediated by CGRP in rats. [54,55]

Still, one of the most studied neuropeptides in the last decade was kisspeptin, the product of the KISS1/Kiss1 gene. After the proteolytical cleavage of a larger inactive preprohormone, shorter, biologically active fragments are produced, that are widely referred to as kisspeptins. The most significant cleavage product consists of 54 amino acids, and it was first recognized as a metastasis inhibitor on human malignant melanoma cell lines in 1996. [56,57] Further proteolytic processing generates derivates consisting of 10, 13 or 14 amino acids. All fragments efficiently activate the kisspeptin receptor Kiss1r (initially named GPR54), which is a G-protein coupled receptor. Activation of $G_{q/11}$ leads to PLC activation, which causes the hydrolysis of PIP3 into IP_3 and DAG. Consequently, Ca^{2+} is mobilized from the intracellular stores, while DAG mediates PKC activation, which triggers the phosphorylation of different mitogen-activated protein kinases, like ERK1/2 and p38.

Expressions of kisspeptin and its receptor have been observed in reproductive tissues of various species. High levels of mRNA and protein expressions of kisspeptin were detected in the syncytiotrophoblast cells of the human placenta. [58] Elements of the kisspeptin/Kiss1r system were also identified in the ovaries and the uterus of both humans and rodents. [59–62] Nonetheless, kisspeptin is mostly recognized for its central roles. Kisspeptin acts as a potent upstream regulator of GnRH release, and therefore induces LH and FSH secretion, and regulates pubertal development and fertility. [63,64] The direct stimulatory effect on gonadotropin secretion is through the activation of Kiss1r co-expressed in most GnRH neurons in the hypothalamus. Studies reported that in the presence of a GnRH antagonist, kisspeptin failed to increase gonadotropin secretion. [65] However, indirect actions of kisspeptin via GABAergic/glutamatergic transmissions are also suspected. [66] Despite being mainly recognized as a neuroendocrine factor, kisspeptin is often referred to as a member of adipokines based on the fact that it is expressed in adipose tissue, and acts as a link between energy homeostasis and reproduction. [67,68] However, further confirmation might be needed whether it should truly be regarded as an adipose-derived factor.

Aims

The ontogenies of LEPR, AdipoRs and Kiss1r have not yet been clarified during pregnancy. In addition, controversial data exists regarding the effects of leptin, adiponectin and kisspeptin on uterine smooth muscle function. The focus of this study was to gain information about the significance of endometrial and myometrial receptors, and how they might change the impact of leptin, adiponectin and kisspeptin on non-pregnant and pregnant uterine contractions.

- Our goal was to investigate the effects of leptin, adiponectin and kisspeptin on non-pregnant and 5-, 15-, 18-, 20- and 22-day pregnant uterine contractility.
- Second aim of this study was to measure LEPR, AdipoRs and Kiss1r expression in non-pregnant uterus and endometrium-denuded myometrium, and to identify the alterations of these expressions during the gestational period in rats.
- Our third aim was to determine whether endometrial receptors modify the uterine actions of leptin, adiponectin and kisspeptin.
- Finally, the fourth aim was to visually demonstrate the changes in uterine distribution of LEPR, AdipoRs and Kiss1r during gestation with fluorescent immunohistochemistry.

Materials and methods

Housing and handling

The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (registration number: IV./3071/2016.). The animals were kept in rooms with controlled temperature (22 ± 3°C), relative humidity (30%–70%), and light (12-hour light/dark cycle), with tap water and standard rodent pellet (Animalab Hungary Ltd, Vác, Hungary) available *ad libitum*.

Mating of the animals, selection of non-pregnant females

Non-pregnant fertile female rats in oestrous phase were selected for experiments and also for mating. The oestrous cycle was detected by vaginal impedance measurement with Oestrus Cycle Monitor (Fine Science Tools, Foster City, CA), and rats with impedance values between 7-9 kΩ were chosen.

Sexually mature Sprague-Dawley rats (Animalab Hungary Ltd, Vác, Hungary) were mated in a special mating cage in the early morning hours. Female (180-220 g) and male (220-260 g) rats were separated with a time-controlled metal door, which was opened with an electronic switch before dawn (5.00 a.m.). Within 4 hours after possible mating, vaginal smears were taken from the female rats and checked under a microscope at 1200x magnification. Pregnancy was confirmed by the presence of spermatozoa in the sample or visible sperm plug in the vagina. The day of copulation was designated as first day of pregnancy.

In vitro contractility studies

Uterus preparation

On the day of the experiment, the animals were terminated by CO_2 inhalation. After abdominal incision, uteri were removed from non-pregnant rats in oestrous phase or from pregnant rats on gestational days 5, 15, 18, 20 or 22. Uterine tissues were then prepared for the *in vitro* contractility measurements. Briefly, two rings were sliced from the middle part of each horn (4 rings from 1 rat), including implantation sites in case of pregnancy, and then immediately placed in a Petri dish containing de Jongh solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4, and 6 mM glucose [pH 7.40]). Carbogen (95% O_2 and 5% CO_2) was bubbled through the buffer and its temperature was maintained at 37°C.

The muscle rings previously cleaned of fat and connective tissue were individually mounted on tissue holders and were placed in the isolated organ bath chambers under the same conditions described before. After mounting, the initial resting tension was set at about 1.5 g, and the muscle strips were equilibrated for at least 60 minutes, with a buffer change every 15 minutes. Rhythmic uterine contractions were then elicited by adding freshly made, 25 mM KCl solution to the chambers. When the response became stable (after approximately 5-7 minutes), rhythmic contractions were recorded for 5 minutes.

Removal of the endometrium

The experiments were also carried out on non-pregnant and pregnant endometrium-denuded myometrium. For the removal of the endometrial layer, uterine tissues were turned inside out, and the endometrium was removed by gentle scraping with the blunt side of a surgical blade.

Kisspeptin, leptin and adiponectin studies

Cumulative concentrations of the KISS1 94-121 fragment (Sigma-Aldrich Kft., Budapest, Hungary) were added to each chamber of the organ bath (10 ml) in the concentration range of 10^{-12}–10^{-7} M, and 5-minute periods were recorded. In case of leptin (PeproTech EC, Ltd., London, United Kingdom) or adiponectin (Sigma-Aldrich Kft., Budapest, Hungary), contractile responses were observed in the concentration range of 10^{-12}–10^{-8} M for 5-minute intervals. The stock solutions were prepared with distilled water and were stored at -20°C. The working dilutions were freshly made before the start of the experiment. The tension of the uterine or myometrial rings was measured with a strain gauge transducer (SG-02; MDE GmbH., Walldorf, Germany) and recorded and analysed with the SPEL Advanced ISOSYS Data Acquisition System (MDE GmbH, Walldorf, Germany). The AUCs of 5-minute periods were evaluated, and the effects of kisspeptin, leptin or adiponectin were expressed as a percentage of the KCl-evoked contractions. The concentration-response curves were plotted and the concentration for 50% of the maximum effect (EC_{50}) and the maximum contraction-inhibiting value (E_{max}) were calculated. Experiments with 5-day pregnant tissues were also performed in the presence of kisspeptin-234 trifluoroacetate (Sigma-Aldrich Kft., Budapest, Hungary), an antagonist of Kiss1r.

RT-PCR and Western blot studies

Tissue isolation

After the termination of the rats, the uterine tissues from non-pregnant and pregnant animals (n=6) were rapidly removed and placed into RNAlater Solution (Sigma-Aldrich, Budapest). The tissues were frozen in liquid nitrogen and stored at -75°C until the extraction of total RNA.

Total RNA preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi. [69] After precipitation with isopropanol, the RNA was washed with 75% ethanol and then re-suspended in diethyl pyrocarbonate-treated water. RNA purity was controlled at an optical density of 260/280 nm with BioSpec Nano (Shimadzu, Kyoto, Japan); all samples exhibited an absorbance ratio in the range of 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis.

Real-time quantitative reverse-transcriptase PCR

Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-CT-Step One Kit (Life Technologies, Budapest, Hungary) and an ABI StepOne Real-Time cycler. Reverse-transcriptase PCR amplifications were performed as follows: at 48°C for 15 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. The following primers were used: assay ID Rn00576940_m1 for Kiss1r water channel, assay ID Rn01483784_m1 for AdipoR1, Rn01463173_m1 for AdipoR2, Rn01433205_m1 for LEPR and Rn00667869_m1 for β-actin as endogenous control. All samples were run in triplicates. The fluorescence intensities of the probes were plotted against PCR cycle number. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as the threshold cycle (C_T).

Western blot analysis

The uterine tissues were homogenized using a Micro-Dismembrator (Sartorius AG, Germany) and centrifuged at 5,000×g for 15 min at 4 °C in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Budapest, Hungary) with protease and phosphatase inhibitor cocktail. Total protein amounts from the supernatant were determined with spectrophotometry (BioSpec-nano, Shimadzu, Japan). 25 µg of sample protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Life Technologies, Hungary). Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer

System (Life Technologies). Antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Thermo Fisher Scientific, Budapest, Hungary). The blots were incubated overnight, at 4°C on a shaker with Kiss1r, AdipoR1, AdipoR2, LEPR or β-actin polyclonal primary antibody in the blocking buffer. The incubation of the secondary antibody solution was carried out based on the protocol of the WesternBreeze® Chromogenic Immunodetection Kit. Images were taken with the EDAS290 imaging system (Kodak Ltd., Hungary), the optical densities of immunoreactive bands were determined with Kodak 1D Images analysis software. Optical densities were expressed as arbitrary units after the subtraction of the local area background.

Immunohistochemistry

Tissue collection

After the termination of the rats, the uterine samples taken for immunohistochemical studies were embedded in cryomatrix medium (Shandon Cryomatrix, Thermo Scientific), snap-frozen by submerging in liquid nitrogen and then stored at -20°C.

Fluorescent immunohistochemistry of Kiss1r

The dissected tissue samples of the uterus were processed for fluorescent microscopy. For fluorescent immunohistochemistry, 5 µm-thick cryosections were prepared from different days of gestation. After washing in TBS with 0.025% Triton X-100 and blocking in TBS containing 1% bovine serum albumin (Sigma-Aldrich, Budapest, Hungary) and 10% normal goat serum (Sigma-Aldrich, Budapest, Hungary) (2 h, room temperature), the samples were incubated overnight with anti-Kiss1r (rabbit; Alomone Labs, final dilution 1:50) primary antibody at 4 °C. After washing in TBS, samples were incubated with anti-rabbit Alexa Fluor 488 (Life Technologies Corporation, Molecular Probes, Inc., Eugene, OR; final dilution 1:200) secondary antibody for 1 h at room temperature. Negative controls were performed by omitting the primary antibody when no immunoreactivity was observed. Sections were mounted in Fluoroshield TM with DAPI mounting medium (Sigma-Aldrich, Budapest, Hungary), observed and photographed with a Zeiss Imager Z.2 fluorescent microscope equipped with an Axiocam 506 mono camera.

Triple labelling of LEPR and AdipoRs

The dissected tissue samples of uterus were processed for fluorescent microscopy. For triple-labelling fluorescent immunohistochemistry, 5 µm-thick cryosections were prepared from different days of gestation. After washing in TBS with 0.025% Triton X-100 and blocking in

TBS containing 1% bovine serum albumin (Sigma-Aldrich, Budapest, Hungary) and 10% normal goat serum (Sigma-Aldrich, Budapest, Hungary) (2 hours, room temperature), the samples were incubated overnight with anti-AdipoR1 (rabbit; Novus Biologicals, final dilution 1:100), anti-AdipoR2 (mouse; Santa Cruz Biotechnology, final dilution 1:100) and anti-Leptin R (chicken; Novus Biologicals, final dilution 1:200) primary antibodies at 4 °C. After washing in TBS, samples were incubated with anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, final dilution 1:200), anti-mouse CyTM3 (Jackson ImmunoResearch Laboratories, final dilution 1:200) and anti-chicken Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, final dilution 1:200) secondary antibodies for 1 hour at room temperature. Negative controls were performed by omitting the primary antibodies when no immunoreactivity was observed. Sections were mounted in FluoroshieldTM with DAPI mounting medium (Sigma-Aldrich, Budapest, Hungary) (Fig. 1.), observed and photographed with a Zeiss Imager Z.2 fluorescent microscope equipped with an Axiocam 506 mono camera. Simultaneous immunostainings of all investigated receptors were done, but the results of triple-labelling are not shown. Instead, leptin and adiponectin receptors are shown individually for better visibility and interpretability.

Statistical analysis

The statistical analysis was carried out with unpaired t-test and ANOVA using Tukey's post hoc test by Prism 5.01 (GraphPad Software, San Diego, CA).

Results

In vitro contractility measurements

Leptin

Leptin reduced the KCl-stimulated contractions both in non-pregnant and pregnant uteri (Fig. 1.).

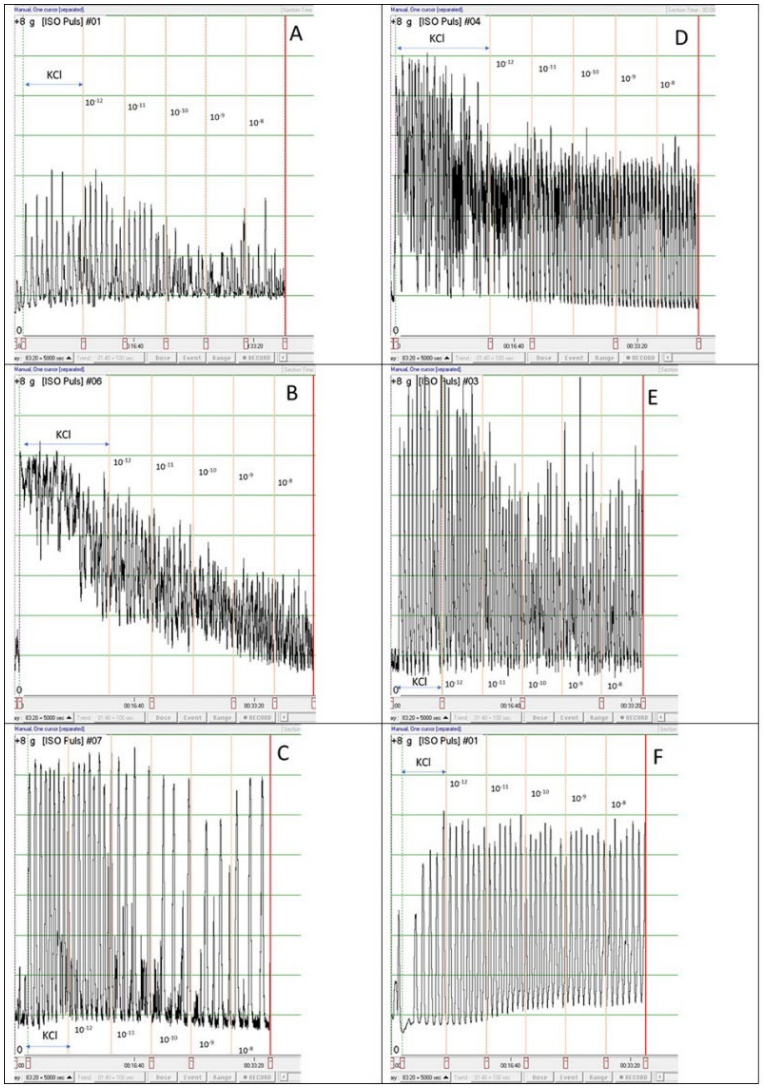

Figure 1.: Representative raw traces of KCl-stimulated uterine contractions and the effects of cumulative doses of leptin on non-pregnant (A) and pregnant (B-F) uteri.

Leptin inhibited non-pregnant uterine contractions in a concentration-dependent manner, but the removal of the endometrium significantly weakened its action.

In pregnant uteri, cumulative concentrations of leptin caused a strong relaxation of the intact uterus in the early phase of gestation, but this effect dramatically decreased towards the end of

pregnancy. Particularly, the relaxant effect of leptin ceased on the last gestational day. In contrast, leptin was able to maintain its uterorelaxant effect in the endometrium-denuded uterus through the gestational period. An increase was observed in the relaxing effect from day 5 to day 15, but from there the calculated maximal relaxing effect did not change significantly until the end of pregnancy. Comparison of the E_{max} values of intact and denuded uteri shows that the leptin-induced relaxations of denuded uteri were significantly decreased on day 5 and day 18, and significantly increased on day 20 and day 22 (Fig. 2., Table 1.).

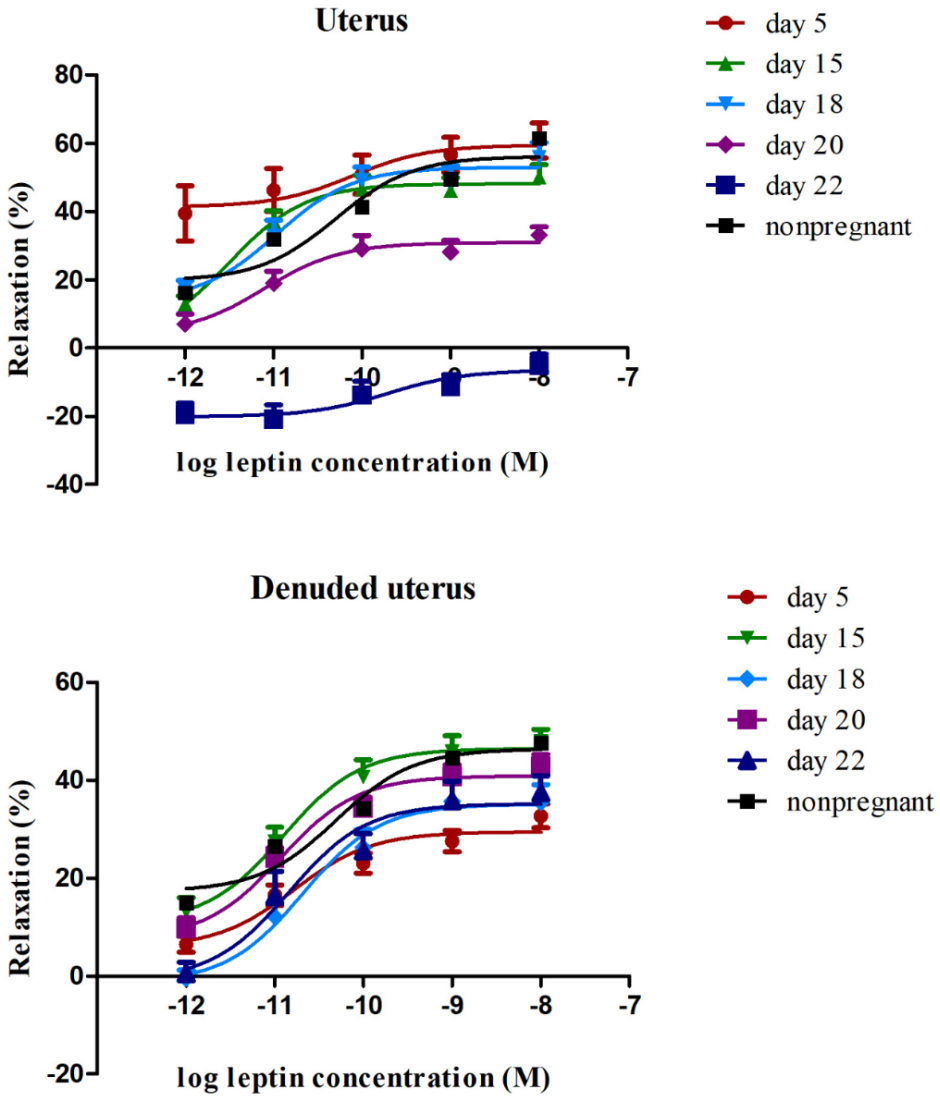

Figure 2.: Dose-response curves demonstrating the effects of leptin on KCl-stimulated contractions of non-pregnant and pregnant intact and endometrium-denuded uteri. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M., n=6 for each group.

	EC50 (M ± S.E.M.)		Emax (% ± S.E.M)		Comparison of intact and denuded uteri	
	Uterus	**Denuded uterus**	**Uterus**	**Denuded uterus**	**EC50**	**Emax**
non-pregnant	$4.9x10^{-10} \pm 1.7x10^{-10}$	$1.1x10^{-10} \pm 2.1x10^{-11}$	61.2 ± 2.9	51.4 ± 1.9	#	#
day 5	$7.7x10^{-10} \pm 5.3x10^{-11}$	$3.3x10^{-11} \pm 6.9x10^{-12}$	63.0 ± 5.1	30.9 ± 2.0	ns	###
day 15	$2.9x10^{-11} \pm 1.6x10^{-12}$	$2.5x10^{-11} \pm 6.5x10^{-12}$	57.1 ± 3.3	46.0 ± 2.9***	ns	ns
day 18	$6.0x10^{-11} \pm 1.8x10^{-12}$	$2.4x10^{-11} \pm 5.0x10^{-12}$	56.9 ± 4.5	39.7 ± 2.6	ns	###
day 20	$3.0x10^{-10} \pm 1.9x10^{-11}$	$2.1x10^{-11} \pm 3.9x10^{-12}$	32.2 ± 2.2***	40.3 ± 2.3	ns	#
day 22	$1.1x10^{-9} \pm 5.5x10^{-10}$	$1.0x10^{-10} \pm 4.7x10^{-11}$	-4.5 ± 3.4***	37.7 ± 3.5	ns	###

Table 1.: EC50 values and the mean maximal relaxing effect of leptin on rat uteri and denuded myometria. *: compared to the previous gestational day; #: compared to the intact uteri; #p < 0.05, ###p<0.001, ***p<0.001, ns: not significant

Adiponectin

Adiponectin modified the KCl-induced contraction responses both in non-pregnant and pregnant uteri (Fig. 3.).

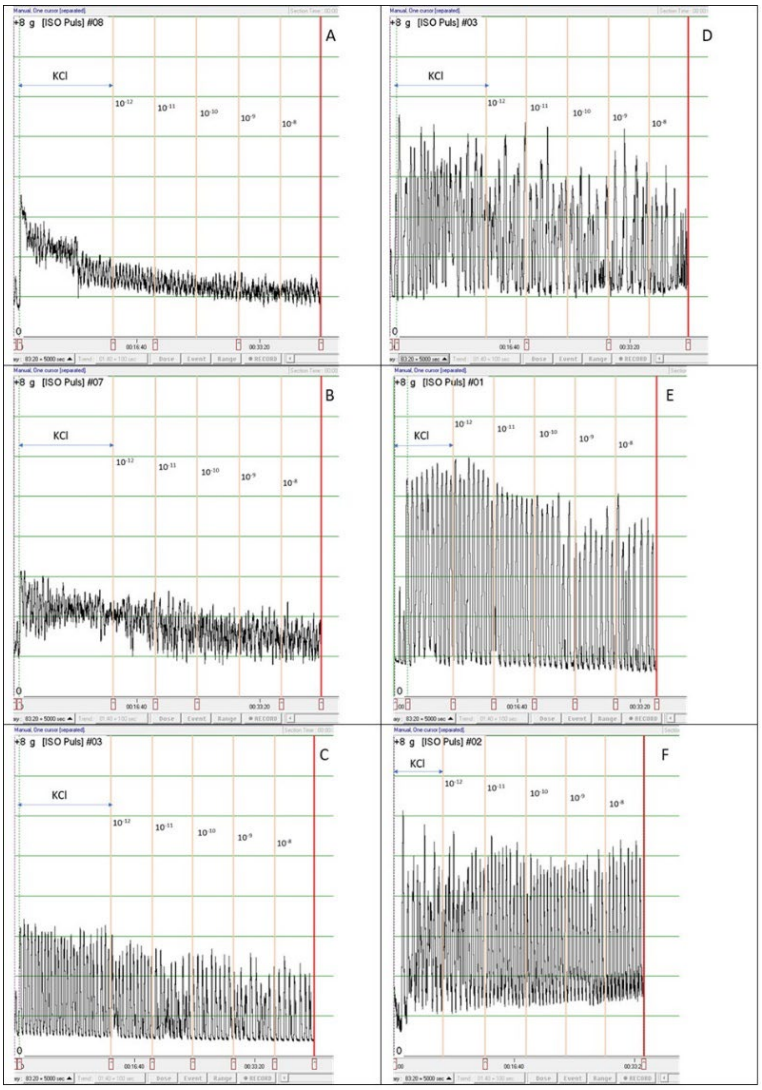

Figure 3.: Representative raw traces of KCl-stimulated uterine contractions and the effects of cumulative doses of adiponectin on non-pregnant (A) and pregnant (B-F) uteri.

Adiponectin dose-dependently reduced the contractility of non-pregnant rat uterus. However, the consequence of endometrium removal was a significant decrease in the relaxing effect. Cumulative concentrations of adiponectin also inhibited pregnant uterus contractions. An increase in the calculated maximal relaxation was detected from pregnancy day 5 to day 18, followed by a decrease in the effect on the 20-day pregnant uterus. The relaxant action of adiponectin practically ceased on the last day of pregnancy.

In early pregnant denuded uteri (days 5, 15 and 18), a strong inhibitory effect was observed, but a significant decrease was seen on gestational day 20. Adiponectin was not able to elicit a markable relaxation on the last day of pregnancy in the case of denuded uteri. The E^{max} values of denuded uteri were significantly higher on days 5, 15 and 22 as compared with the whole uteri (Fig. 4., Table 2.).

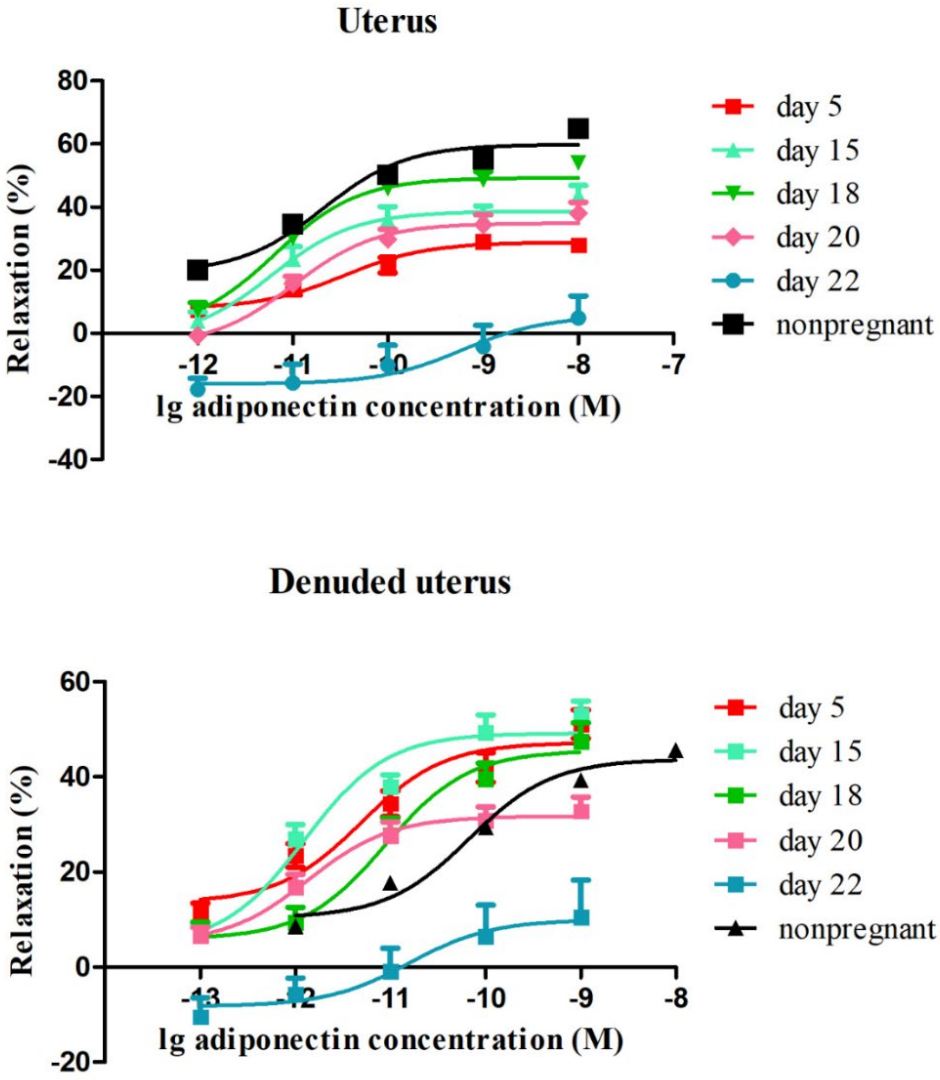

Figure 4.: Dose-response curves demonstrating the effects of adiponectin on KCl-stimulated contractions of non-pregnant and pregnant rat uterus or endometrium-denuded uterus on different days of gestation. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M., n=6 for each group.

	EC50 (M ± S.E.M.)		Emax (% ± S.E.M)		Comparison of intact and denuded uterus	
	Uterus	**Denuded uterus**	**Uterus**	**Denuded uterus**	**EC50**	**Emax**
non-pregnant	$4.0x10^{-11} \pm 8.6x10^{-12}$	$2.3x10^{-10} \pm 9.9x10^{-11}$	62.7 ± 2.6	46.4 ± 2.8	ns	###
day 5	$5.2x10^{-11} \pm 1.6x10^{-12}$	$4.7x10^{-12} \pm 7.8x10^{-13}$	30.2 ± 2.5	48.8 ± 2.8	##	###
day 15	$2.1x10^{-10} \pm 1.1x10^{-11}$	$2.3x10^{-12} \pm 6.0x10^{-13}$	37.6 ± 3.0	49.7 ± 2.9	#	##
day 18	$2.3x10^{-11} \pm 7.9x10^{-12}$	$1.6x10^{-11} \pm 5.7x10^{-12}$	50.8 ± 2.7***	45.9 ± 3.7	ns	ns
day 20	$1.1x10^{-11} \pm 1.5x10^{-12}$	$2.6x10^{-12} \pm 5.9x10^{-13}$	36.2 ± 2.8**	32.9 ± 2.5**	###	ns
day 22	$8.4x10^{-10} \pm 6.8x10^{-11}$	$2.8x10^{-11} \pm 7.3x10^{-12}$	6.5 ± 6.8***	17.0 ± 6.4**	ns	#

Table 2.: EC50 values and the mean maximal relaxing effect of adiponectin on rat uteri and denuded myometria.

*: compared to the previous gestational day; #: compared to the intact uteri; #P < 0.05, ##p<0.01, ###p<0.001, **p<0.01, ***p<0.001, ns: not significant

Kisspeptin

In the presence of KISS1 94-121, rhytmic contractions elicited by KCl-solution were reduced both in non-pregnant and pregnant uteri (Fig. 5.).

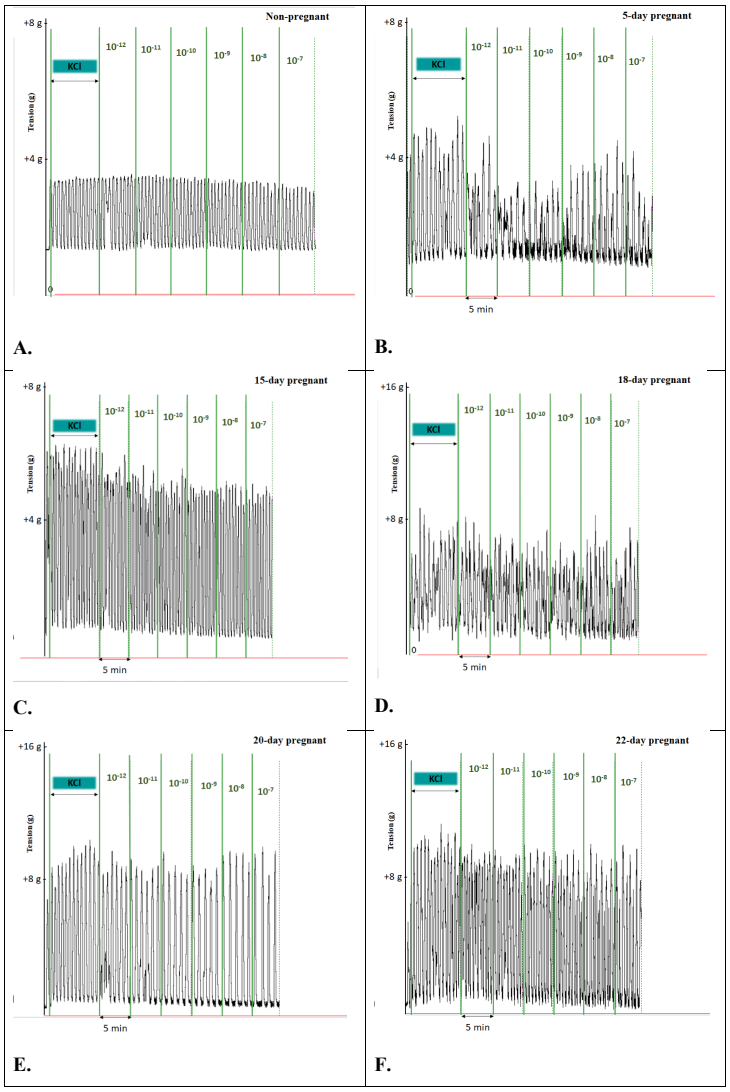

Figure 5.: Representative raw traces of KCl-stimulated uterine contractions and the effects of KISS1 94-121 on non-pregnant **(A)** and pregnant **(B-F)** uteri.

The KISS1 94-121 fragment reduced the KCl-induced contractions of non-pregnant uteri and denuded myometrium in the range of 10^{-12} to 10^{-7} M in a dose-dependent manner (Fig. 6a and 6b). In the endometrium-denuded myometrium, the calculated maximal relaxation was significantly higher and the EC50 value was shifted to the left as compared with the intact uterus (Table 3.).

Results of contractility studies performed on pregnant uterus and myometrium also showed that KISS1 94-121 exerts a relaxant effect in a concentration-dependent manner. The calculated

maximal relaxation of KISS1 94-121 gradually declined towards term, but the effect was still detectable on the last day of pregnancy (Fig. 6a, Table 3.). The highest mean maximal relaxation was measured in case of 5-day pregnant uterus. No significant change was detected between the EC_{50} values of KISS 94-124 during the gestational period.

Regarding the relaxing effect of KISS1 94-121 on denuded myometrial muscle strips, we did not find a gradually decreasing tendency (Fig. 6b). A significant reduction in the mean maximal relaxation value was seen on the 18^{th} and 22^{nd} days of gestation, while a temporary increase was found on day 20. The comparison of uterine and myometrial relaxations showed that KISS1 94-121 was more effective at the end of pregnancy when the endometrium was removed, although the difference between the E_{max} values was only significant on day 20 of gestation. Adding the highest concentration dose of KISS1 94-121 (10^{-8} M) to the system did not increase the maximal relaxing effect.

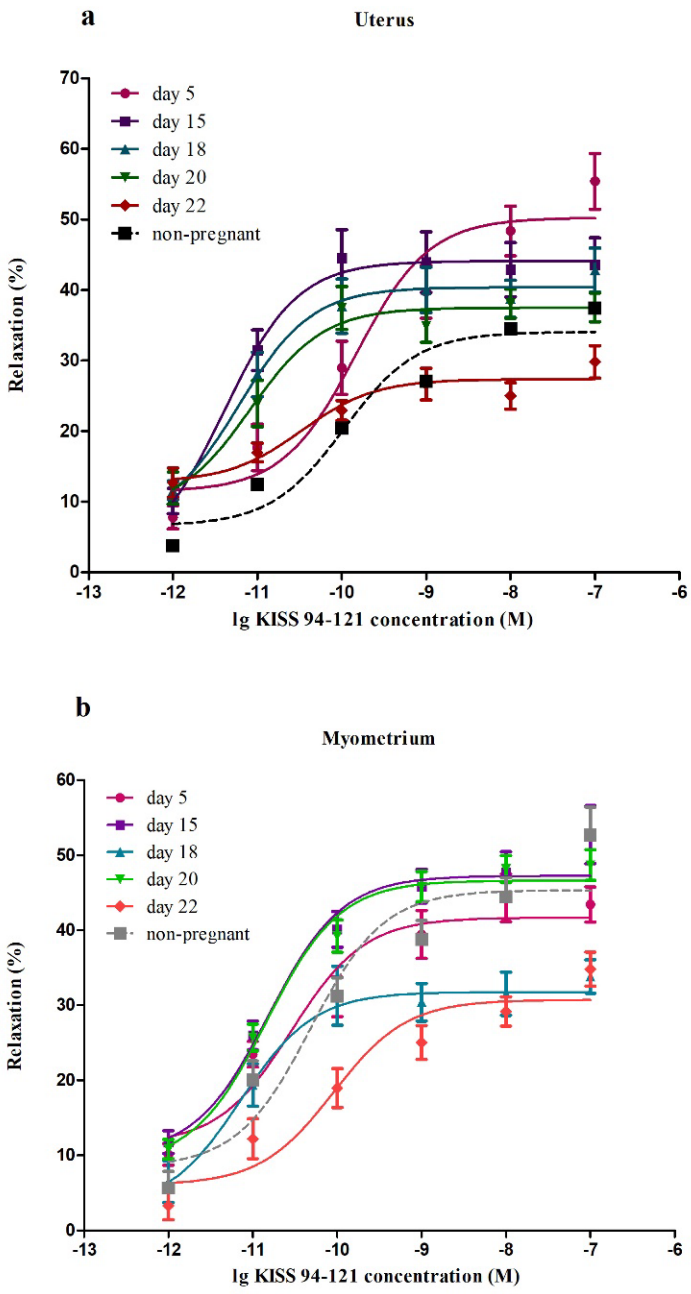

Figure 6.: Dose-response curves demonstrating the effects of KISS1 94-121 in the range of 10^{-12} to 10^{-7} M on KCl-evoked contractions of the non-pregnant and pregnant rat uterus (**a**) or endometrium-denuded myometrium (**b**). The change in contraction was calculated via the AUC and expressed in % ± S.E.M.; n=6 for each group.

EC_{50} (M ± S.E.M.)			
	Uterus	**Myometrium**	**Uterus compared with myometrium**
non-pregnant	$1.1x10^{-10} \pm 4.5x10^{-10}$	$4.8x10^{-11} \pm 3.2x10^{-11}$	a
day 5	$1.5x10^{-10} \pm 1.4x10^{-10}$ ns	$2.8x10^{-11} \pm 9.8x10^{-12}$ ns	b
day 15	$4.0x10^{-12} \pm 1.2x10^{-11}$ ns	$1.5x10^{-11} \pm 1.1x10^{-11}$ ns	ns
day 18	$6.0x10^{-12} \pm 5.4x10^{-12}$ ns	$7.1x10^{-12} \pm 8.9x10^{-11}$ ns	ns
day 20	$8.2x10^{-12} \pm 4.2x10^{-12}$ ns	$1.4x10^{-11} \pm 5.6x10^{-12}$ ns	ns
day 22	$3.3x10^{-11} \pm 8.4x10^{-9}$ ns	$9.0x10^{-11} \pm 2.2x10^{-10}$ ns	ns
E_{max} (% ± S.E.M.)			
	Uterus	**Myometrium**	**Uterus compared with myometrium**
non-pregnant	34.1 ± 2.0	45.3 ± 1.8	a
day 5	50.3 ± 2.3 ^{b}	41.7 ± 1.7 ^{ns}	ns
day 15	44.1 ± 1.8 ^{ns}	47.3 ± 1.4 ^{ns}	ns
day 18	40.4 ± 1.6 ^{ns}	31.8 ± 1.8 ^{c}	ns
day 20	37.5 ± 1.4 ^{ns}	46.6 ± 1.1 ^{c}	c
day 22	27.4 ± 1.2 ^{a}	30.7 ± 1.5 ^{c}	ns

Table 3.: The calculated EC50 and E_{max} values of the dose-response curves demonstrated in Figure 6a and 6b. E_{max} and EC_{50} values of intact uterus and endometrium-denuded myometrium were compared to each other. Each investigated gestational day was compared to the previous gestational day, and day 5 was compared to the non-pregnant values. ns: not significant, $aP < 0.05$, $bP<0.01$, $cP<0.001$

Kisspeptin studies in the presence of a Kiss1r antagonist

Contractility studies were also conducted in the presence of kisspeptin-234 trifluoroacetate (Kiss1r antagonist) on 5-day pregnant uteri. Kisspeptin-234 trifluoroacetate inhibited the effect of KISS1 94-121, since the calculated E_{max} value was reduced by 40%. (Fig. 7., Table 4.).

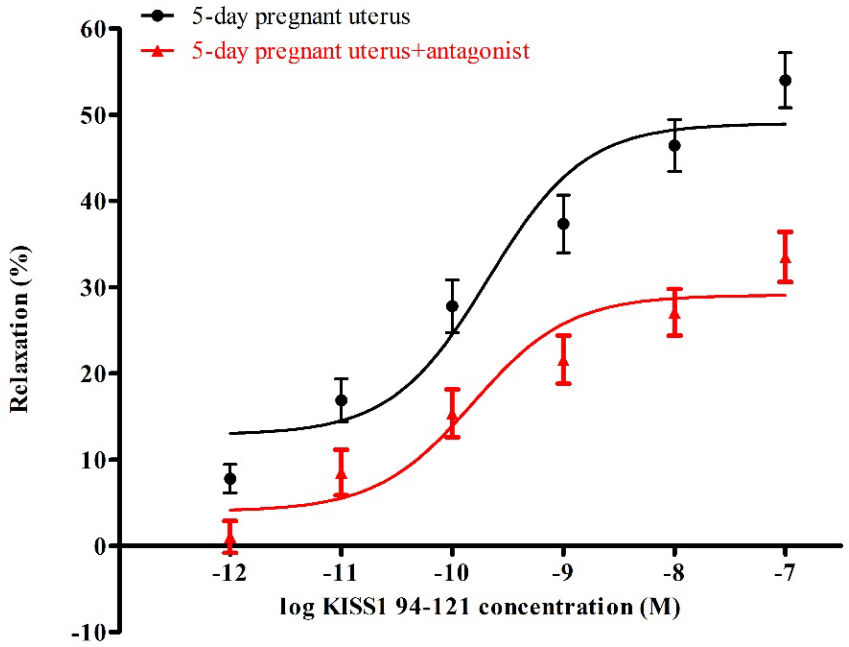

Figure 7.: Dose-response curves demonstrating the effects of KISS1 94-121 (10^{-12} to 10^{-7} M) in the presence of kisspeptin-234 trifluoroacetate (Kiss1r antagonist) on KCl-evoked contractions on 5-day pregnant rat uterus. The change in contraction was calculated via the AUC and expressed in % ± S.E.M.; n=6 for each group.

	EC_{50} (M ± S.E.M.)	**E_{max} (% ± S.E.M.)**
5-day pregnant uterus	$2.1x10^{-10} \pm 3.3x10^{-10}$	49.0 ± 3.1
5-day pregnant uterus+antagonist	$1.5x10^{-10} \pm 1.0x10^{-10}$ ns	29.1 ± 3.1[c]

Table 4.: The calculated EC_{50} and E_{max} values of the dose-response curves. The E_{max} and EC_{50} of KISS1 94-121 were compared to its action in the presence of the antagonist; ns: not significant, cP < 0.001

RT-PCR and Western blot studies

Leptin

In general, LEPR presence is significant both in non-pregnant and pregnant rat uteri. The highest expression was measured on pregnancy day 5, and in the late phase of gestation (day 20 and 22). On days 15 and 18 the expressions were reduced. The reduction in the amount of LEPR mRNA and protein after endometrium removal was significant only on day 5 (Fig. 8.).

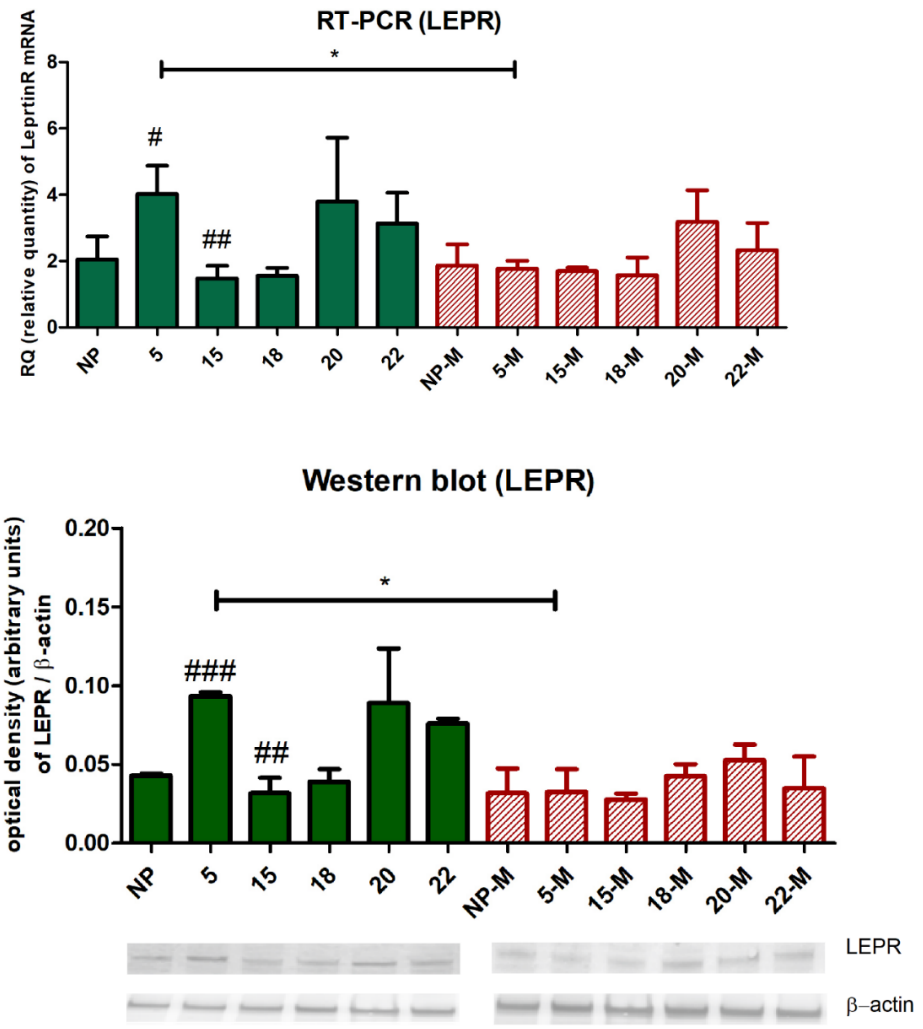

Figure 8.: RT-PCR and Western blot analysis of the expression of leptin receptor (LEPR, 156 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; *p<0.05, #p< 0.05, ##p<0.01, ###p<0.001

AdipoR1 and AdipoR2

The highest expression of AdipoR1 was detected in non-pregnant and 5-day pregnant uteri. In non-pregnant samples, the removal of the endometrium significantly reduced the mRNA and protein expressions. From day 15, the expressions of the receptors were moderated, and from day 18 the endometrial denuding did not significantly alter the mRNA and protein levels (Fig. 9.). AdipoR2 mRNA and protein expressions peaked on gestational day 15 but were significantly lower in the end of pregnancy. The endometrial removal dramatically reduced receptor mRNA expressions on gestational days 15 and 20, and the protein expressions through the whole pregnancy period. Presence of AdipoR2 was basically minimal in late pregnant myometrium (Fig. 10.).

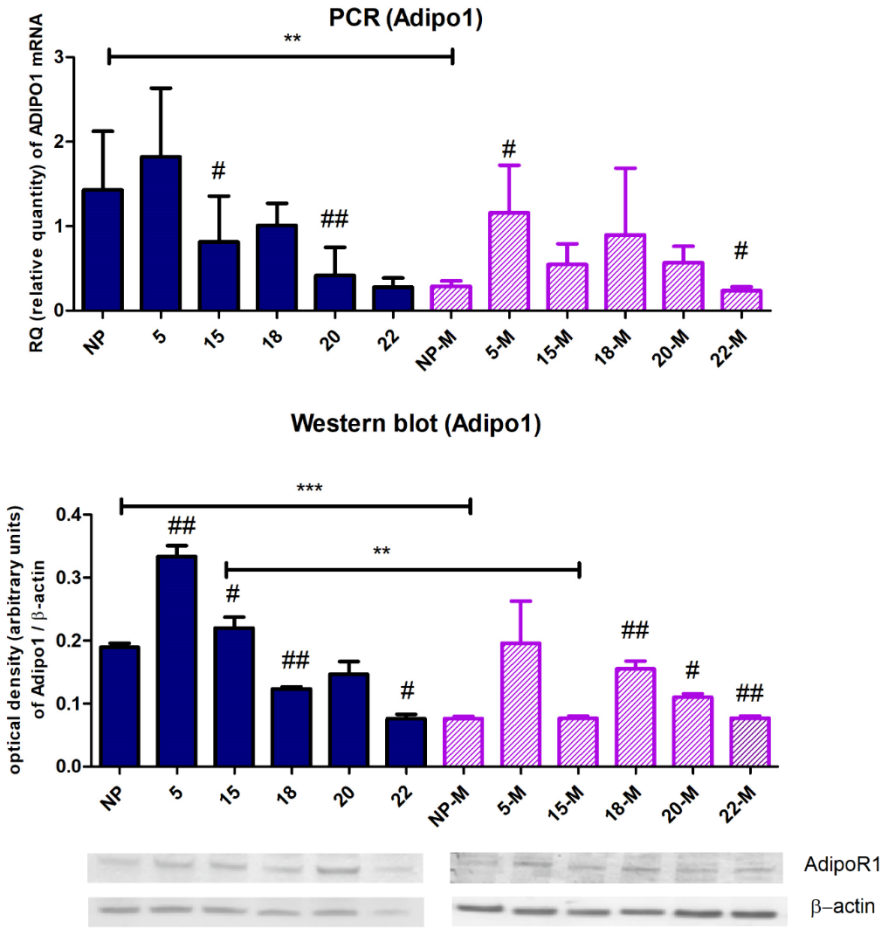

Figure 9.: RT-PCR and Western blot analysis of the expression of AdipoR1 (~52 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; **p<0.01, ***p<0.001, #p < 0.05, ##p < 0.01

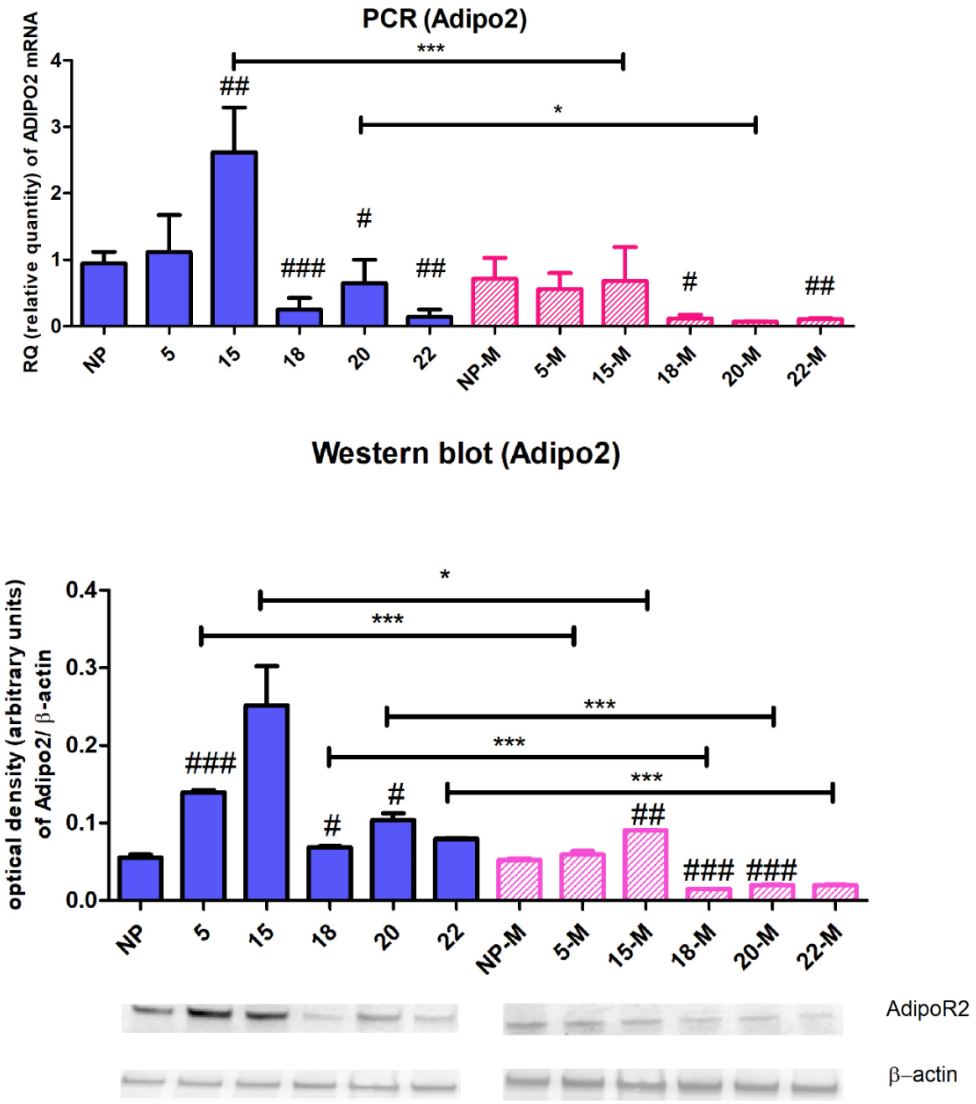

Figure 10.: RT-PCR and Western blot analysis of the expression of AdipoR2 (~52 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; *p<0.05, ***p<0.001, #p < 0.05, ##p<0.01, ###p<0.001

Kisspeptin

Uterine (Fig. 11. full columns) and myometrial (Fig. 11. empty columns) mRNA expressions of Kiss1r were determined in non-pregnant and pregnant tissues. The highest levels of Kiss1r mRNA were found in the non-pregnant and the 5-day pregnant uteri. The lowest Kiss1r mRNA levels were measured on day 15. From there, a slight elevation was seen, but further alterations were not detected. The removal of the endometrium caused a significant decrease in the amount of receptor mRNA only in the case of non-pregnant myometria.

The highest Kiss1r protein levels were measured in the non-pregnant uteri and denuded myometria (Fig. 12.), but high optical density was observed on the 5th day of pregnancy as well. Major reduction was seen in the Kiss1r protein levels from day 5 to 15, but no significant decrease was detected towards the end of pregnancy. The removal of the endometrium caused a proportional decrease in Kiss1r levels, but these changes were not significant.

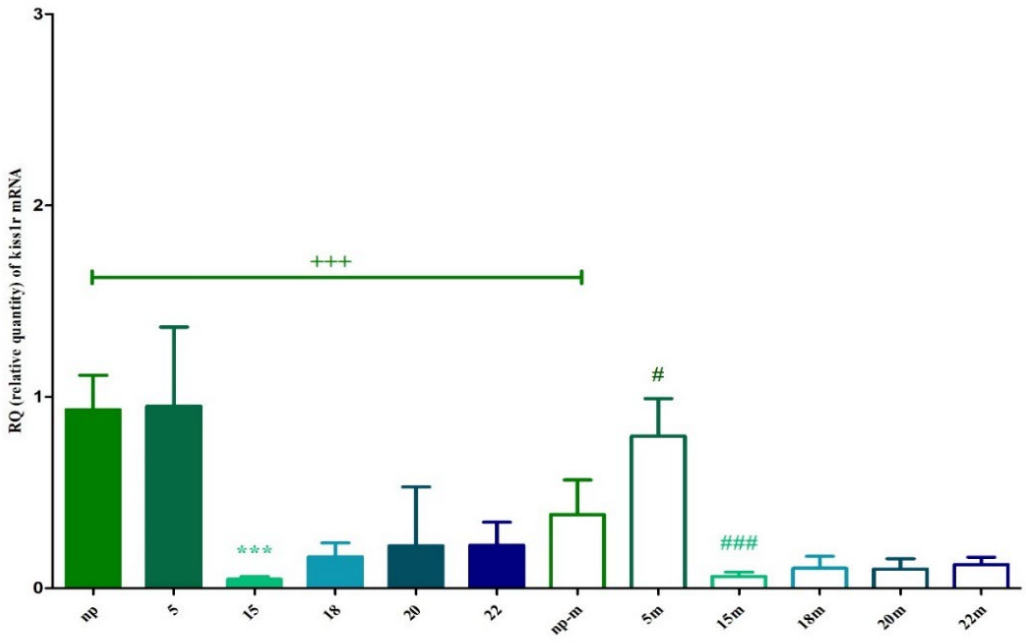

Figure 11.: Changes in the uterine (full columns) and myometrial (empty columns) mRNA expressions of Kiss1r throughout gestation and in non-pregnant samples. Kiss1r expression in the uterus was compared to the myometrial expression on each gestational day as well as in non-pregnant samples (+). Also, day 5 was compared to the non-pregnant values, and each investigated day of pregnancy was compared to the previous gestational day (* and #). np: non-pregnant, np_m: non-pregnant (denuded) myometrium, +P < 0.05, ###P<0.001, ***P<0.001, n=4 for each group

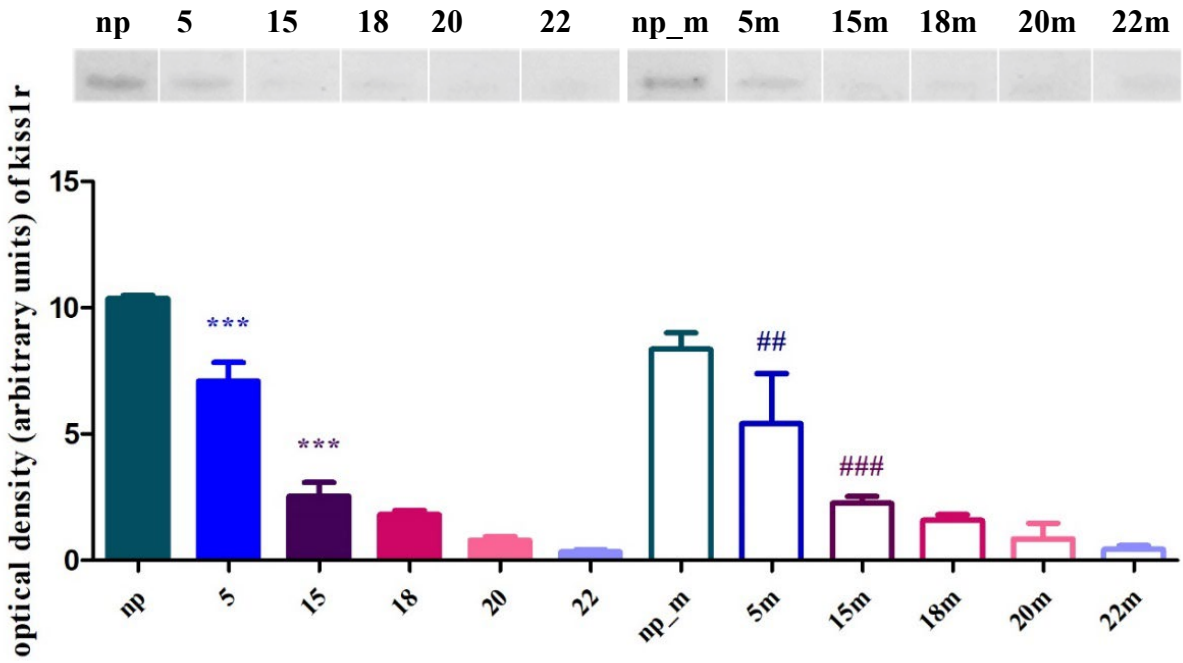

Figure 12.: The uterine (full columns) and denuded myometrial (empty columns) protein expression of Kiss1r on different days of gestation and in non-pregnant samples along with representative blots. Kiss1r expressions show a declining trend throughout pregnancy as compared to the previous day in uteri (*) or denuded myometria (#). The results in the uterus were also compared to the myometrial expressions on each gestational day as well as in non-pregnant samples. np: non-pregnant uterus, np_m: non-pregnant (denuded) myometrium, ***P<0.001, ##P< 0.01, ###P<0.001, n=4-7 for each group

Fluorescent immunohistochemistry

LEPR

The intensity of LEPR staining was strong both in non-pregnant and pregnant samples. LEPRs were widely distributed in non-pregnant uteri, but the expression was more significant in the circular and longitudinal layers of the myometrium. In the pregnant samples, the presence of LEPR was mostly restricted to the myometrial layer, except on day 5, when higher intensities were detected in the endometrium (Fig. 13.).

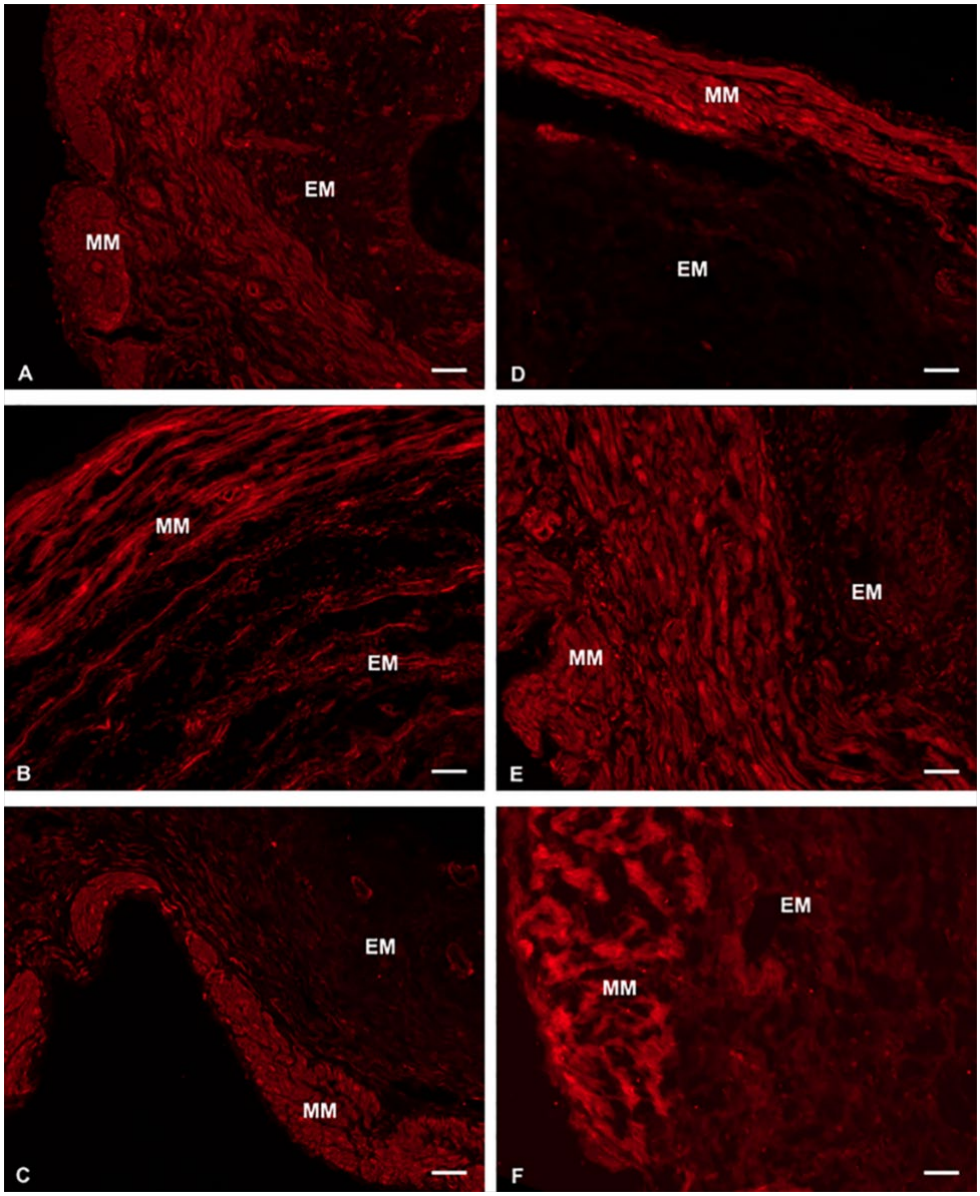

Figure 13.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after LEPR immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 µm

AdipoRs

The muscular and the endometrial layers of non-pregnant and early pregnant samples exhibited a strong immunostaining for AdipoR1. Towards the end of gestation, immunoreactivities became less intense and mainly localized in the muscular layers (Fig. 14.).

A peak in AdipoR2 expression on gestational day 15 was confirmed by strong immunolabelling. The lowest staining intensities were seen in non-pregnant, 5-day and 18-day pregnant tissues. Also, the endometrial presence of AdipoR2 was stronger in the mid and late phases of pregnancy (day 15 and 20) (Fig. 15.).

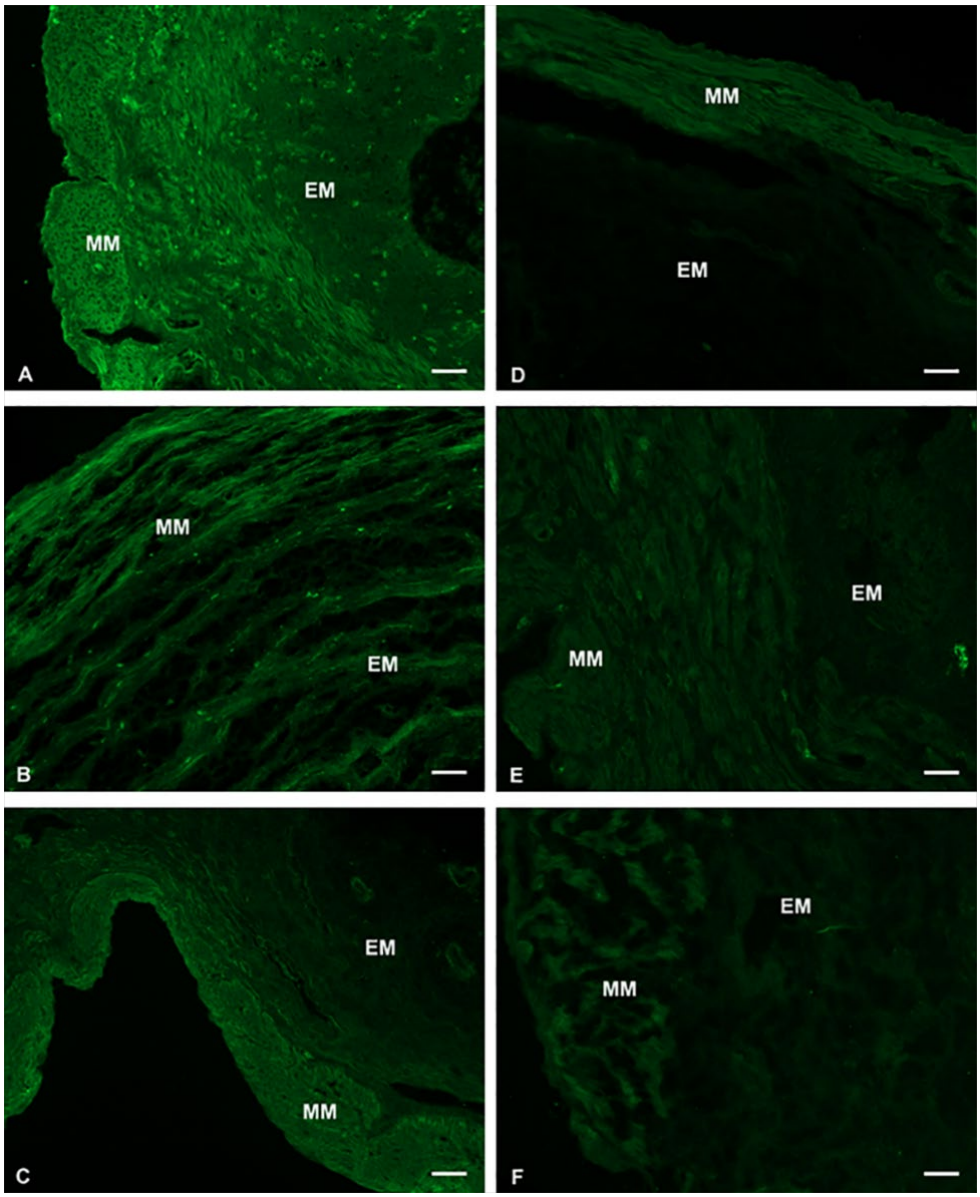

Figure 14.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after AdipoR1 immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μm

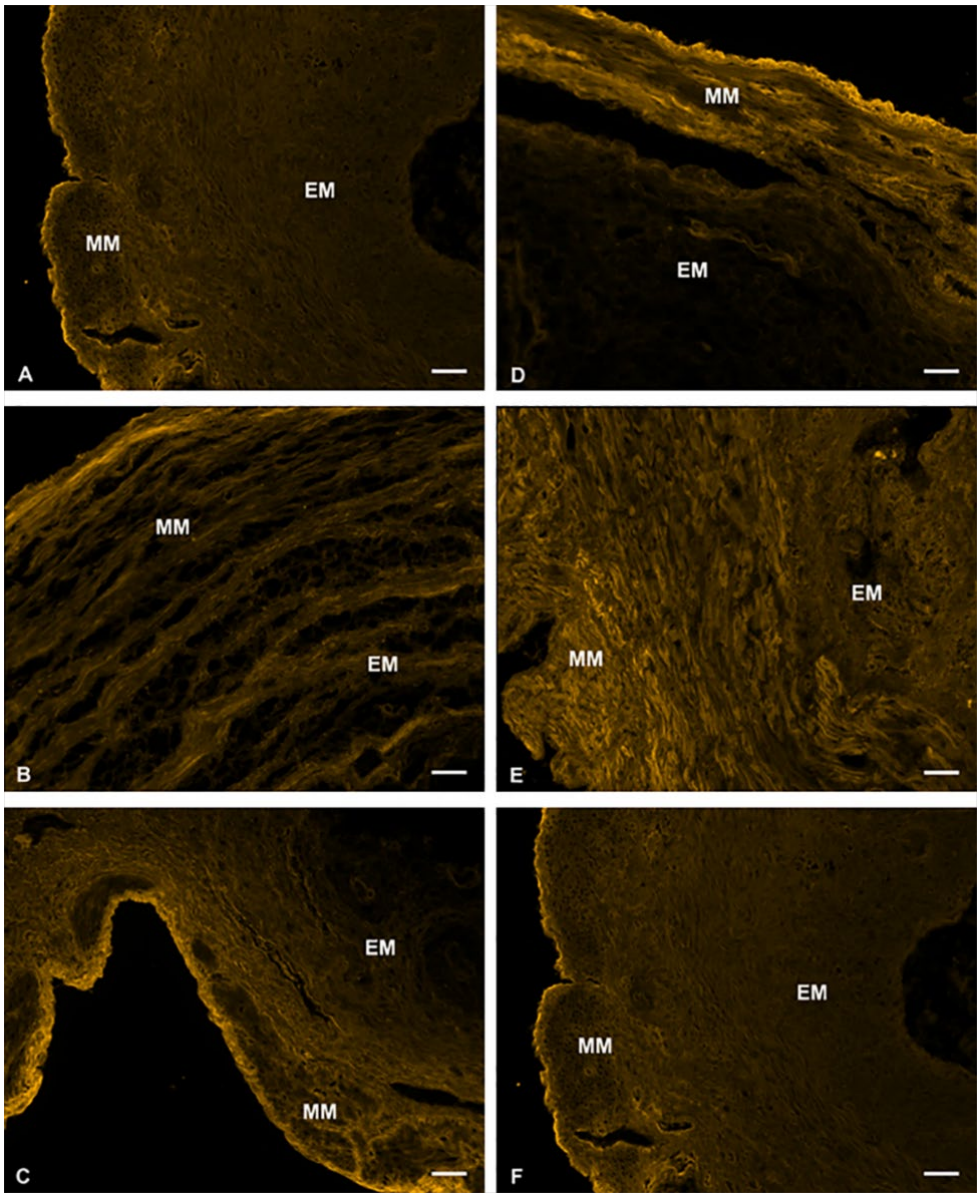

Figure 15.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after AdipoR2 immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μm

Kiss1r

Kiss1r presence in non-pregnant and pregnant uteri was further confirmed by immunohistochemistry (Fig. 16.). The most intensive staining of the receptors was observed in the muscular layer, while the endometrial presence of Kiss1r was found less significant. Kiss1r was also highly distributed in vascular smooth muscle cells. Receptor presence in the non-pregnant and the 5-day pregnant uteri was intense, but the expression was gradually descending towards the last day of pregnancy, and this reduction was especially spectacular in the endometrial layer. From day 18, the endometrial activities became negligible, and receptors were primarily localized in the muscle layers of the uterus. The immunoreactivity of 22-day pregnant uteri was marginal compared to the non-pregnant or 5-day pregnant samples.

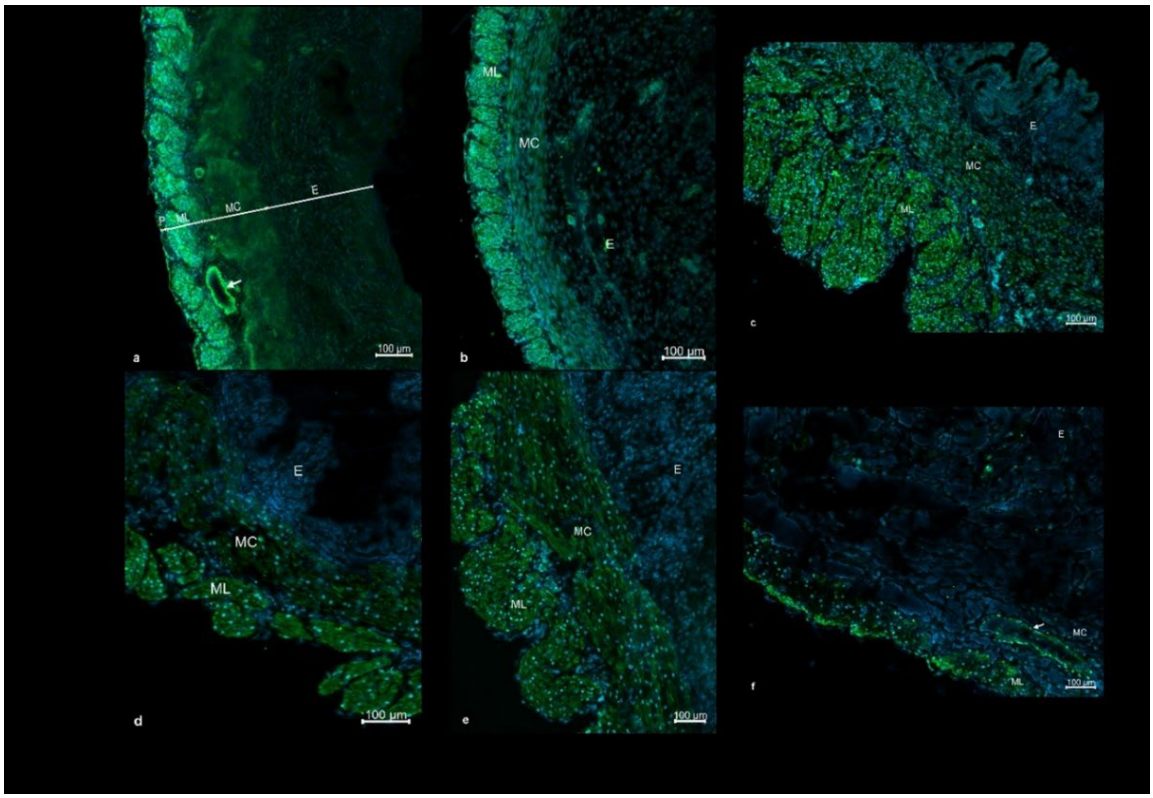

Figure 16.: Representative fluorescent micrographs of cryosections from the uterus of non-pregnant rat **(A)**, and on gestational days 5 **(B)**, 15 **(C)**, 18 **(D)**, 20 **(E)** and 22 **(F)** after Kiss1r immunohistochemistry (green). DAPI was used to label the cell nuclei (blue). P: perimetrium, ML: longitudinal layer of myometrium, MC: circular layer of myometrium, E: endometrium, arrow: large blood vessel in the stratum vasculare.

Discussion

The purpose of the current study was to contribute to the rapidly expanding knowledge about the roles of adipokines in reproduction. Previous research has indicated that various adipokines are able to affect smooth muscle functions. [70] Relevantly, studies have found that leptin reduces contractility of both human and rat pregnant uteri. [22,71] However, we have limited knowledge about the impact of adiponectin on uterine smooth muscle function. Additionally, there is no data about how the effect of these adipokines changes during the pregnancy period. As part of this study, we confirmed that leptin, adiponectin and kisspeptin significantly affects uterine contractility during the whole pregnancy period and also in non-pregnant cases. In addition, this study demonstrates the changes in myometrial and endometrial receptor expressions and how the effects of these adipokines are altered during the gestational period.

First, we demonstrated the presence of LEPR in uterine tissues. Moreover, our findings from Western blot analysis and immunohistochemical studies emphasise that leptin receptors are mainly expressed in the myometrial layer of non-pregnant uteri. Although the reduction in LEPR expression induced by endometrium removal was not significant, we detected a decreased relaxation in the presence of leptin in non-pregnant endometrium-denuded uterus. Our results suggest that the small amount of endometrial LEPR may modify uterine contractility in non-pregnant tissues. We also determined the endometrial and myometrial expression of LEPR on different days of gestation. In early pregnancy (day 5), the uterine presence of LEPR is significant with a markedly high endometrial distribution. According to our results, LEPR expression is decreased in the mid-term of pregnancy, and most of the receptors seem to be distributed in the myometrium at this time. From there, the amount of both endometrial and myometrial leptin receptors tends to rise towards the end of pregnancy. We found that the uterorelaxant effect induced by leptin in the intact uterus gradually decreased during the gestational period, but when the endometrial layer was previously removed, leptin was able to maintain its relaxant effect throughout pregnancy. Our results also show that the lack of endometrial factors reduces the relaxing effect of leptin only on gestational day 18. Although the immunohistochemical studies were not quantitative, the alterations in endometrial and myometrial LEPR distributions correlate with both the RT-PCR and Western blot results. Our findings suggest that in non-pregnant cases and in early- and mid-pregnancy, both endometrial and myometrial LEPRs mediate relaxant effects. On the last days of gestation (days 20 and 22), it seems that the myometrial LEPRs are responsible for the responses. However, the modification in the relaxing effect of leptin is inconsistent with the changes seen in LEPR

expression during the gestational period. This suggests that a possible alteration of signal mechanisms or in the sensitivity of LEPRs might be responsible for the diminishing inhibitory effect towards term. Since the primary signalling cascade for LEPRs is the JAK/STAT pathway, it is possible that the modified phosphorylation/dephosphorylation of different proteins contributes to this alteration. [72] Additionally, leptin can modify and cross-talk with several signal pathways, such as inducing vasoconstriction in vascular tissues by increasing intracellular Ca^{2+} levels, or causing endothelium-dependent relaxation by inducing nNOS expression. [73,74] We propose that any change in these mechanisms during gestation might also modify the final response in the uterine smooth muscle.

Regarding AdipoRs, earlier findings confirmed the presence of both AdipoR1 and AdipoR2 in pregnant uterus, but the local uterine effects of adiponectin are more obscure. [75] In this study we demonstrated that adiponectin was able to reduce uterine contractility, but the interpretation of this effect is complicated because this action is evolved via two main receptors that activate mainly the AMPK and PPARα pathways. [76] Our findings revealed that there are significant differences in the amount and distribution of the two AdipoRs in the uterus. The expression of AdipoR1 is significant in the non-pregnant endometrium, while AdipoR2 is mainly present in the muscular layer of non-pregnant uteri. Based on the results of contractility measurements, adiponectin relaxes both the intact and the denuded non-pregnant uteri, but the absence of endometrial layer decreased this effect. These findings suggest that endometrial AdipoR1 enhances the adiponectin-induced relaxation in non-pregnant uteri. Our results also show that adiponectin elicits relaxation in both the intact and the denuded uteri in early- and mid-term, but on the last day of gestation, adiponectin was not able to maintain its uterorelaxant effect in either case. Correspondingly, the expressions of both AdipoRs are reduced towards the end of gestation, and these modifications were further confirmed by immunohistochemistry. As stated before, endometrial AdipoRs contribute to the relaxation of non-pregnant uteri, but the opposite was seen during most of the gestational period. On gestational days 5 and 15, the maximal relaxation caused by adiponectin was greater in the intact uterus, presumably because of the lack of endometrial factors. We hypothesize that in early pregnancy, endometrial AdipoRs are likely to mediate contraction in uterine smooth muscle. Interestingly, at the end of gestation, no significant differences were detected in the mean maximal relaxation values, suggesting that endometrial AdipoRs lost their remarkable impact on uterine response. On the last gestational day, however, the removal of the endometrium again enhanced relaxation, but this increase in the mean maximal relaxation is less than that in the case of leptin. The modification seen in the relaxing effect of adiponectin was in positive correlation with the alteration of AdipoRs

expression. These findings indicate that the sensitivities and the signal mechanisms of AdipoR1 and AdipoR2 are not significantly changed during the gestational period. However, it would be an interesting challenge for future studies to differentiate the roles of endometrial and myometrial AdipoR1 and AdipoR2 in the regulation of uterine contractility.

As mentioned in the literature review, the kisspeptin/Kiss1r system was previously identified in uterine tissue [59–62], but until now, no clear data has been available about the possible role of kisspeptin in uterine contractility. In our *in vitro* studies, the KISS1 94-121 fragment consisting of 28 amino acid was used to elicit and investigate kisspeptin action. While this fragment binds with strong affinity to Kiss1r [77], it also has better stability and water solubility than the other kisspeptin fragments [78], which were essential qualities for our organ bath contractility measurements. In view of our results, KISS1 94-121 inhibits the contractions both in non-pregnant and pregnant uteri in a dose-dependent manner. To prove that these effects were evolved via Kiss1r, KISS1 94-121 action was also investigated in the presence of kisspeptin-234 trifluoroacetate, a specific Kiss1r antagonist. 5-day pregnant uterine samples were chosen for this measurement because the most potent relaxing effect of KISS1 94-121 was observed on that gestational day. Our findings prove that the relaxing effect is indeed mediated through Kiss1r, since the inhibitory action of KISS1 94-121 was diminished by the specific inhibitor. We found that the mean maximal relaxation of KISS1 94-121 was the highest in case of non-pregnant and early pregnant (day 5) uteri, while towards the end of gestation a gradually decreased effect was seen, reaching the weakest inhibition on the last, 22nd day of gestation. In correlation with the *in vitro* results, a reduction was found in the amount of Kiss1r mRNA and protein towards the end of the gestational period. The same phenomenon was further confirmed by the immunohistochemical studies, which revealed weaker staining intensities in all layers of the uterus as the end of the gestational period approached. The highest immunostaining intensity was seen in the non-pregnant uteri, whereas the lowest Kiss1r expression was found on the last gestational day, when receptor presence was practically detected only in the myometrial layer. In general, we demonstrated that the receptors were predominantly located in the longitudinal and circular smooth muscle layers, and low expression was found in the endometrium. This was further proved by the fact that the removal of the endometrium did not significantly alter the Kiss1r protein expression in non-pregnant uteri or on any of the investigated gestational days. From these findings we can conclude that the main site of Kiss1r expression is the myometrium, and that Kiss1r participates directly in the modulation of uterine smooth muscle contractions as a relaxant agent. This is corroborated by the molecular biology studies demonstrating that the expressions of Kiss1r were the highest in non-pregnant and 5-day

pregnant samples. The activity of the non-pregnant uterus is usually low in rats [79], while the 5th day of gestation is considered as the time of embryo implantation in rats [80], which requires relative quiescence of the uterus. As the demand for enhanced contractility increases as pregnancy progresses, kisspeptin gradually loses its relaxant effect. These results indicate kisspeptin's role in the maintenance of uterine quiescence. Interestingly, KISS1 94-121 elicited a more potent uterorelaxant effect in the non-pregnant and 20-day pregnant endometrium-denuded myometrium as compared to the intact uterus. In addition, a significant reduction was found in the mRNA level of Kiss1r after denuding the non-pregnant uteri. Furthermore, the endometrium removal ceased the decreasing tendency in the relaxing effect of KISS1 94-121. All of these suggest the importance of the small amount of endometrial Kiss1r in the contractile response of the pregnant uterus. These findings might also indicate that a currently unknown mechanism, possible networking with other receptors or endometrial secretory responses could be involved in the Kiss1r signal transduction modulating uterine contractility. It has been reported before that the endometrial layer has a fundamental role in the control of uterine contractility by locally producing different agents that have an impact on smooth muscle function [81]. Also, a networking has already been demonstrated with kisspeptin neurons in the central nervous system [82] and the reproductive system [83], but still no data is available for the uterine smooth muscle in this respect.

Finally, regarding the results of immunohistochemistry, it should be clarified that the methods used in this study were not suitable for quantitative measurements. However, considering the fact that rat uterus has a low connective tissue content and its amount is further reduced due to hormonal changes towards the end of pregnancy [84], it can be stated that the amount of connective tissue is marginal as compared with that of smooth muscle. Generally, we can state that immunostaining revealed the adipokine receptor expressions in the smooth muscle of the non-pregnant and pregnant rat uteri. This is also supported by the high-resolution images where the stained receptors are clearly located in the circular and longitudinal muscular layers.

Conclusion

In general, leptin, adiponectin and kisspeptin systems are present both in non-pregnant and pregnant uterus playing a role in the regulation of contractility. We can conclude that endometrial LEPR, AdipoRs and Kiss1r have regulatory roles in the actions of these adipokines both in non-pregnant and pregnant conditions. Our results support the idea that these adipokines contribute to the maintenance of uterine quiescence in early- and mid-pregnancy, but they also indicate that their involvement is less significant towards labour. The modified responses of denuded myometria suggest that the endometrial adipokine receptors might have some networking and cross-talking with other mechanisms. The clarification of these putative mechanisms would be an interesting topic for future studies.

To the best of our knowledge, this is the first evidence for the ontogeny of LEPR, AdipoR1, AdipoR2 and Kiss1r expression and distribution, and for the modification of these adipokine actions on uterine contractility throughout gestation. Our results contribute to the better understanding of the uterine effects exerted by these adipokines during pregnancy and might also predict the importance of these adipokines in uterine contractility under altered adipokine level conditions. Finally, we propose that maintaining a balance among the plasma levels of these adipokines during pregnancy might be crucial for healthy pregnancy outcomes.

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