University of Szeged

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Department of Pharmacology and Pharmacotherapy

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

Ph.D. Thesis Summary

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Introduction

Previously, adipose tissue was only recognized as an energy storage. Later, a new concept was adopted based on its secretory functions, and it is now widely known as the largest endocrine organ. It is the source of various proteins named adipokines, which are mostly recognized for regulating metabolic processes, but their influence on reproduction has also been documented. Leptin is known for regulating appetite, energy homeostasis and lipid metabolism. More importantly, it signals to the central nervous system about the nutritional status and acts as a link between the adipose tissue and the reproductive system. Besides acting as a regulator of the HPG axis, leptin was also described as a modulator of uterine action. During gestation, the placenta is a site for leptin secretion, therefore maternal serum concentrations are elevated in pregnancy and decline quickly postpartum. Leptin acts by binding to its receptor, LEPR, which is a type I cytokine receptor ubiquitously expressed in the body. LEPR stimulation results in the activation of the JAK2/STAT and MAPK signalling pathways.

Adiponectin is the most abundant adipokine in human plasma. Unlike leptin, its serum levels are inversely correlated with the BMI. Adiponectin has anti-inflammatory and antiatherogen effects and regulates lipid metabolism and glucose uptake. These effects are evolved through two main receptors: AdipoR1 and AdipoR2. The stimulation of AdipoRs results in the activation of AMPK, p38 MAPK and PPARα cascades. In addition, an adaptor protein called APPL1 was reported to mediate AdipoRs signalling. The adiponectin system has also been detected in peripheral reproductive tissues. Yet, previous findings imply that adiponectin signalling might not be critical for the proper functioning of the reproductive system. Maternal adiponectin levels are also elevated in early pregnancy, but a reduction occurs towards term, showing a negative correlation with the maternal BMI. There has been less evidence for adiponectin's impact on uterine activity, but it has been hypothesized that it might be a link between maternal metabolic state and the outcome of pregnancy.

One of the most studied neuropeptides in the last decade was kisspeptin. Kisspeptin fragments are the products of the proteolytical cleavage of a larger inactive preprohormone. All fragments efficiently activate Kiss1r, a $G_{q/11}$ coupled receptor. Elements of the kisspeptin/Kiss1r system were also identified in reproductive tissues. However, kisspeptin is mostly recognized for its central roles: increasing GnRH release, regulating pubertal development and fertility. 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.

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 alteration 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

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.). Non-pregnant fertile female rats in oestrous phase were selected for experiments and also for mating. Sexually mature Sprague-Dawley rats were mated in a special mating cage in the early morning hours. Within 4 hours after possible mating, vaginal smears were taken and checked under a microscope at 1200x magnification. Pregnancy was confirmed by the presence of spermatozoa or visible sperm plug in the vagina. The day of copulation was designated as first day of pregnancy.

In vitro contractility studies

The animals were terminated by CO_2 inhalation. Uteri were removed from non-pregnant rats in oestrous phase or from pregnant rats on different gestational days. Tissues were then prepared for the contractility measurements. During the whole process, carbogen (95% O_2 and 5% CO_2) was bubbled through the buffer and its temperature was maintained at 37°C. After mounting, the initial 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 contractions were then elicited by

25 mM KCl solution. The experiments were also carried out on non-pregnant and pregnant endometrium-denuded myometrium.

Cumulative concentrations of KISS1 94-121 were added to the organ bath system in the concentration range of 10^{-12}–10^{-7} M. Experiments with 5-day pregnant tissues were also performed in the presence of kisspeptin-234 trifluoroacetate, an antagonist of Kiss1r. In case of leptin or adiponectin, contractile responses were observed in the concentration range of 10^{-12}–10^{-8} M. The tension of the muscle rings was measured with a strain gauge transducer and recorded and analysed with the SPEL Advanced ISOSYS Data Acquisition System. 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 EC50 and E_{max} values were calculated.

RT-PCR

After termination, the uterine tissues from non-pregnant and pregnant animals were rapidly removed and placed into RNAlater Solution. Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi. Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-CT-Step One Kit and an ABI StepOne Real-Time cycler. β-actin was used 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.

Western blot analysis

25 µg of sample protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units. Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System. Antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit. The blots were incubated overnight on a shaker with Kiss1r, AdipoR1, AdipoR2, LEPR and 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, 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

Fluorescent immunohistochemistry of Kiss1r

For fluorescent immunohistochemistry, 5 μm-thick cryosections were prepared. In case of Kiss1r, incubation was performed with rabbit anti-Kiss1r primary antibody and anti-rabbit Alexa Fluor 488 secondary antibody. For the triple-labelling of LEPR and AdipoRs, incubation was performed with rabbit anti-AdipoR1, mouse anti-AdipoR2 and chicken anti-Leptin R primary antibodies. Samples were then incubated with anti-rabbit Alexa Fluor 488, anti-mouse CyTM3 and anti-chicken Alexa Fluor 647 secondary antibodies. Negative controls were performed by omitting the primary antibody when no immunoreactivity was observed. Sections were mounted in Fluoroshield TM with DAPI mounting medium, observed and photographed with a fluorescent microscope equipped with a camera.

Statistical analysis

The statistical analysis was carried out with unpaired t-test and ANOVA using Tukey's post hoc test by Prism 5.01.

Results

In vitro contractility measurements

Leptin inhibited non-pregnant uterine contractions, but the removal of the endometrium significantly weakened its action. In pregnant uteri, 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. In contrast, leptin was able to maintain its effect in the endometrium-denuded uterus through the gestational period. 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 18, and significantly increased on day 20 and day 22 (Fig. 1., Table 1.).

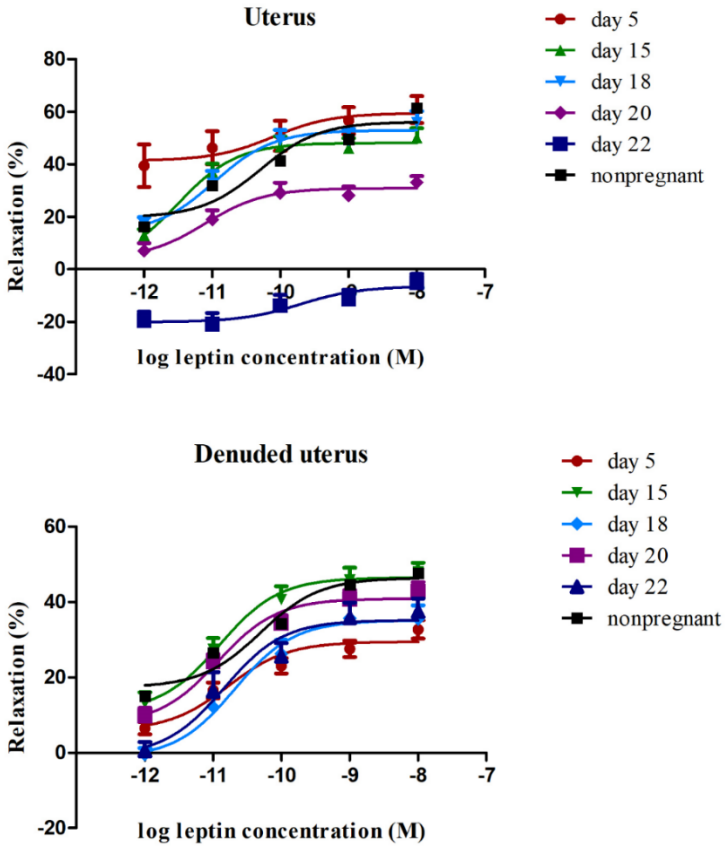

Figure 1.: Dose-response curves demonstrating the effects of leptin on KCl-stimulated contractions of non-pregnant and pregnant intact and endometrium-denuded uteri. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M., n=6 for each group.

	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 (10^{-12}-10^{-8}M) 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 reduced the contractility of non-pregnant and pregnant rat uterus (Fig. 2.). The endometrium removal significantly decreased the relaxing effect in non-pregnant cases. In pregnancy, an increase in the E_{max} was detected from 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. In early pregnant denuded uteri, a strong inhibitory effect was observed, but a significant decrease was seen on day 20, and adiponectin was not able to elicit a markable relaxation on the last day. According to these, the E_{max} values of denuded uteri were significantly higher on days 5, 15 and 22 as compared with the whole uteri (Table 2.).

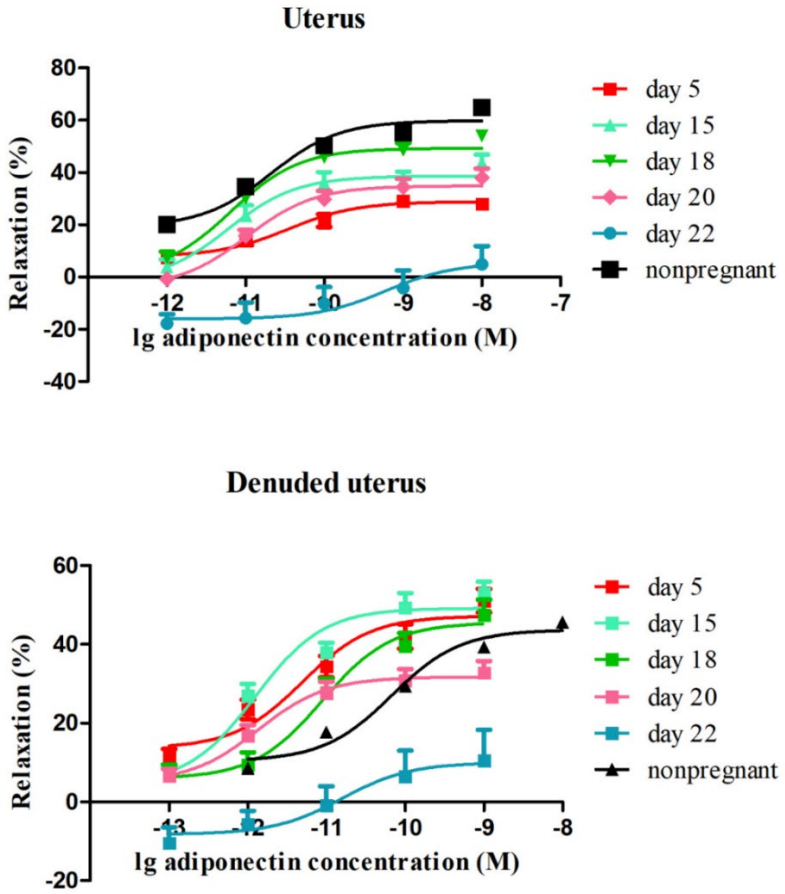

Figure 2.: Dose-response curves demonstrating the effects of adiponectin on KCl-stimulated contractions of non-pregnant and pregnant rat uterus or endometrium-denuded uterus on different days of gestation. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M., 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 (10^{-12}-10^{-8}M) 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

KISS1 94-121 reduced the contractions of non-pregnant uteri and denuded myometrium (Fig. 3a and 3b). 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.). KISS1 94-121 exerted a relaxant effect in pregnant uterus and myometrium as well. The maximal relaxation of KISS1 94-121 gradually declined towards term, but the effect was still detectable on the last day of pregnancy. The strongest relaxation was measured in case of 5-day pregnant uterus. In case of denuded myometrium, we did not find a gradually decreasing tendency in the effect. A significant reduction was seen on the 18^{th} and 22^{nd} days, 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.

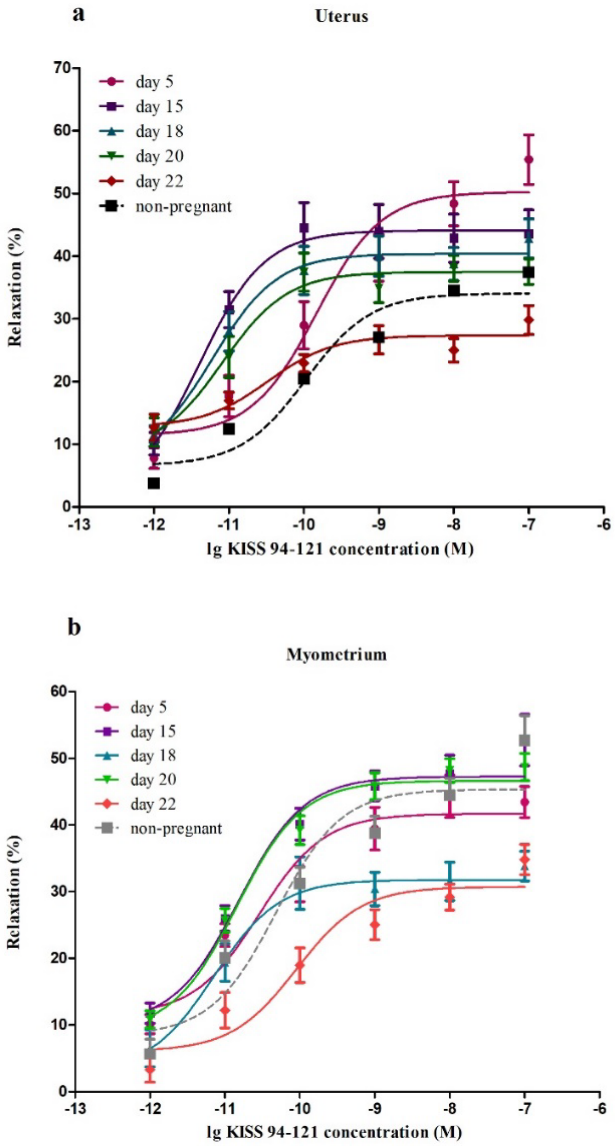

Figure 3.: Dose-response curves demonstrating the effects of KISS1 94-121 in the range of 10^{-12} to 10^{-7} M on KCl-evoked contractions of the non-pregnant and pregnant rat uterus (**a**) or endometrium-denuded myometrium (**b**). The change in contraction was calculated via the area under the curve 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 of gestation	$1.5x10^{-10} \pm 1.4x10^{-10}$ ns	$2.8x10^{-11} \pm 9.8x10^{-12}$ ns	b
day 15 of gestation	$4.0x10^{-12} \pm 1.2x10^{-11}$ ns	$1.5x10^{-11} \pm 1.1x10^{-11}$ ns	ns
day 18 of gestation	$6.0x10^{-12} \pm 5.4x10^{-12}$ ns	$7.1x10^{-12} \pm 8.9x10^{-11}$ ns	ns
day 20 of gestation	$8.2x10^{-12} \pm 4.2x10^{-12}$ ns	$1.4x10^{-11} \pm 5.6x10^{-12}$ ns	ns
day 22 of gestation	$3.3x10^{-11} \pm 8.4x10^{-9}$ 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 of gestation	50.3 ± 2.3 b	41.7 ± 1.7 ns	ns
day 15 of gestation	44.1 ± 1.8 ns	47.3 ± 1.4 ns	ns
day 18 of gestation	40.4 ± 1.6 ns	31.8 ± 1.8 c	ns
day 20 of gestation	37.5 ± 1.4 ns	46.6 ± 1.1 c	c
day 22 of gestation	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 3a and 3b. 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

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

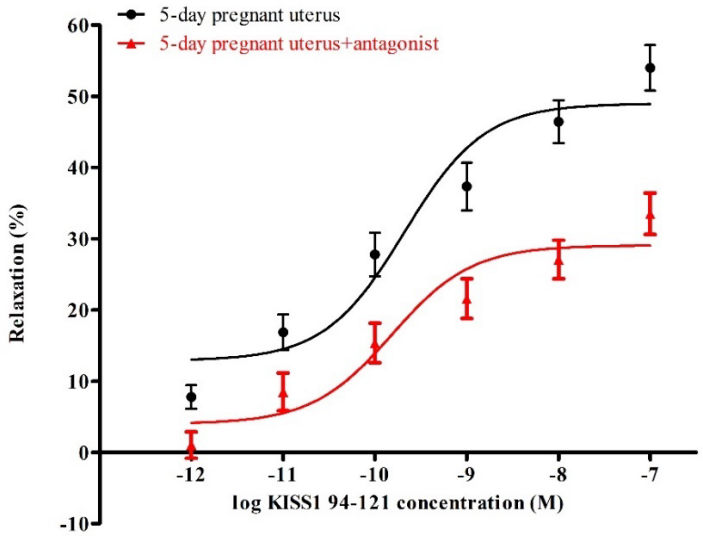

Figure 4.: Dose-response curves demonstrating the effects of KISS1 94-121 (10^{-12} to 10^{-7} M) in the presence of kisspeptin-234 trifluoroacetate (Kiss1r antagonist) on KCl-evoked contractions on 5-day pregnant rat uterus. The change in contraction was calculated via the area under the curve and expressed 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 demonstrated in Figure 4. 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

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

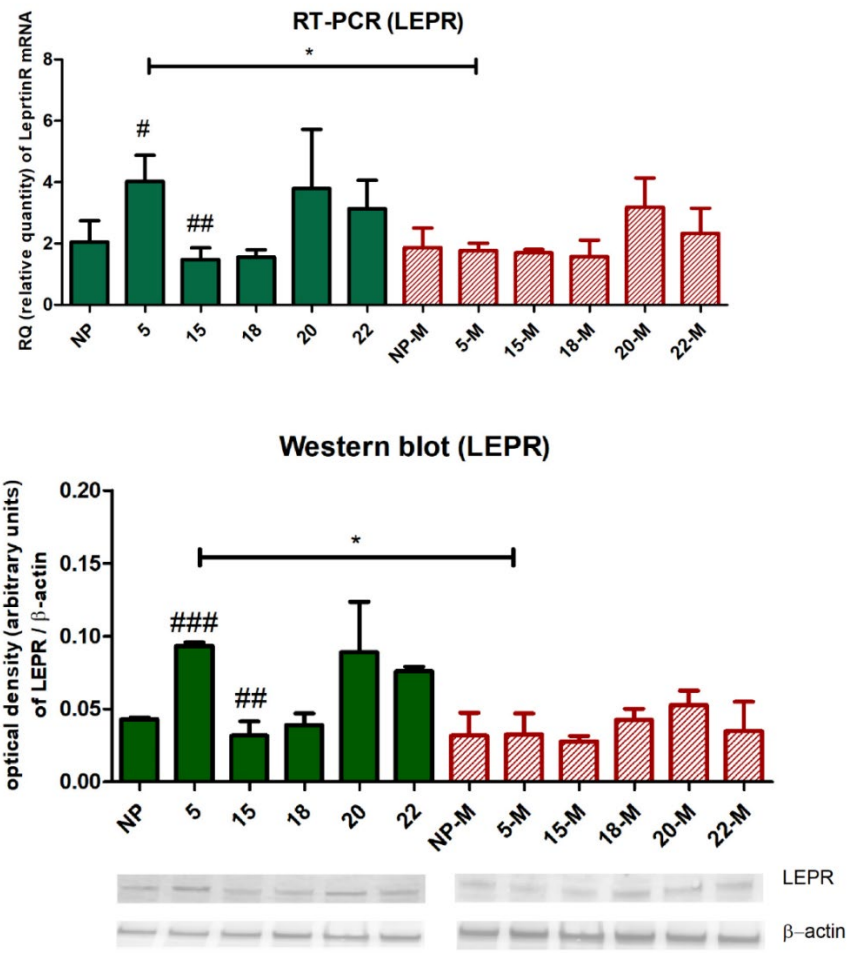

Figure 5.: RT-PCR and Western blot analysis of the expression of leptin receptor (LEPR, 156 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; *p<0.05, #p< 0.05, ##p<0.01, ###p<0.001

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 were moderated, and from day 18 the endometrial denuding did not significantly alter the mRNA and protein levels (Fig. 6.).

AdipoR2 mRNA and protein expressions peaked on day 15 but were significantly lower in the end of pregnancy. The endometrial removal dramatically reduced receptor mRNA expressions on days 15 and 20, and the protein expressions through the whole pregnancy period. Presence of AdipoR2 was basically minimal in late pregnant myometrium (Fig. 7.).

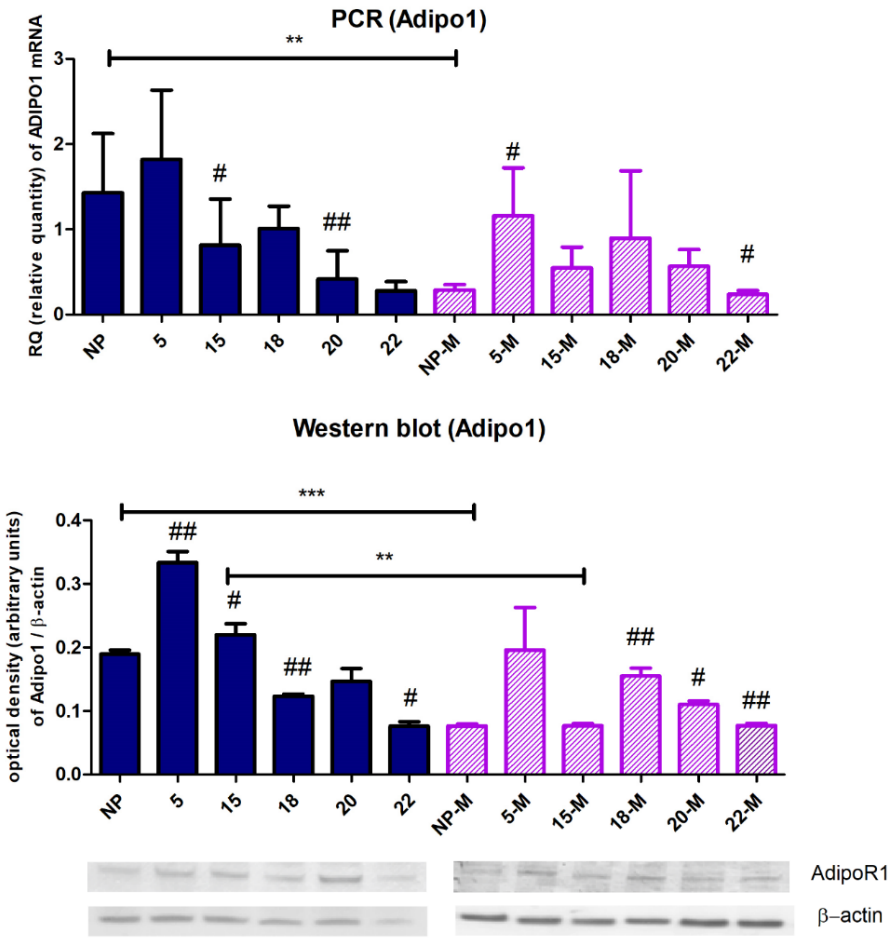

Figure 6.: RT-PCR and Western blot analysis of the expression of AdipoR1 (AdipoR1, ~52 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; **p<0.01, ***p<0.001, #p < 0.05, ##p < 0.01

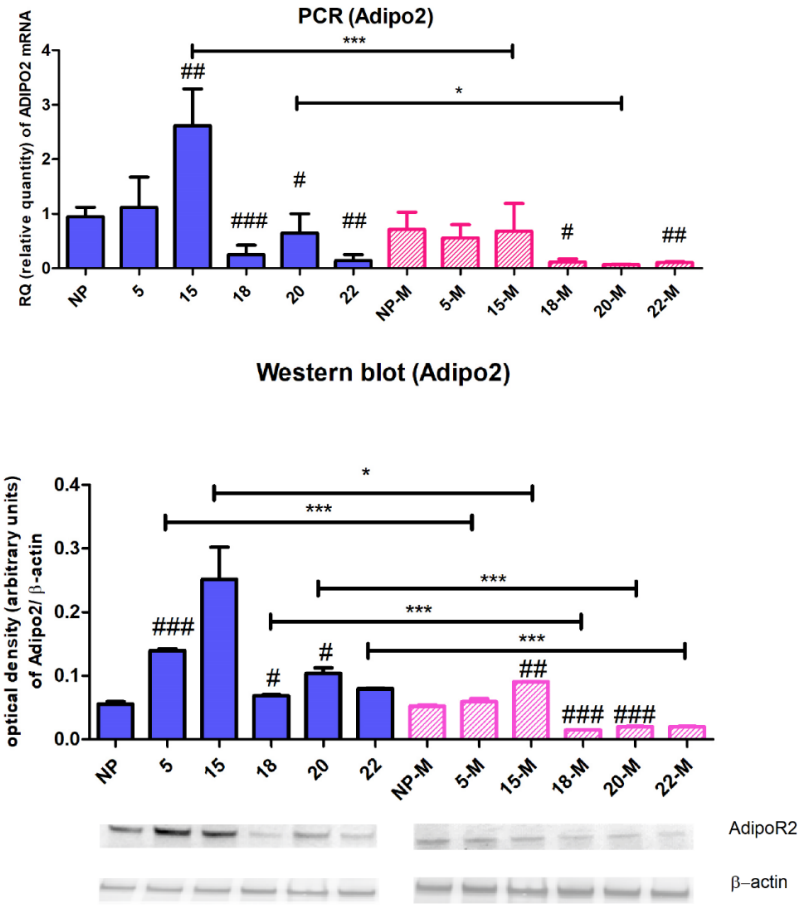

Figure 7.: RT-PCR and Western blot analysis of the expression of AdipoR2 (AdipoR2, ~52 kDa) and β-actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: non-pregnant intact uterus, NP-M: non-pregnant denuded myometrium, #: 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

Uterine and myometrial mRNA expressions of Kiss1r were determined in non-pregnant and pregnant tissues (Fig. 8.). The highest mRNA levels were found in the non-pregnant and the 5-day pregnant uteri, and the lowest expression was detected on day 15. The removal of the endometrium caused a significant decrease in 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. 9.), but high expression was observed on the 5th day 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.

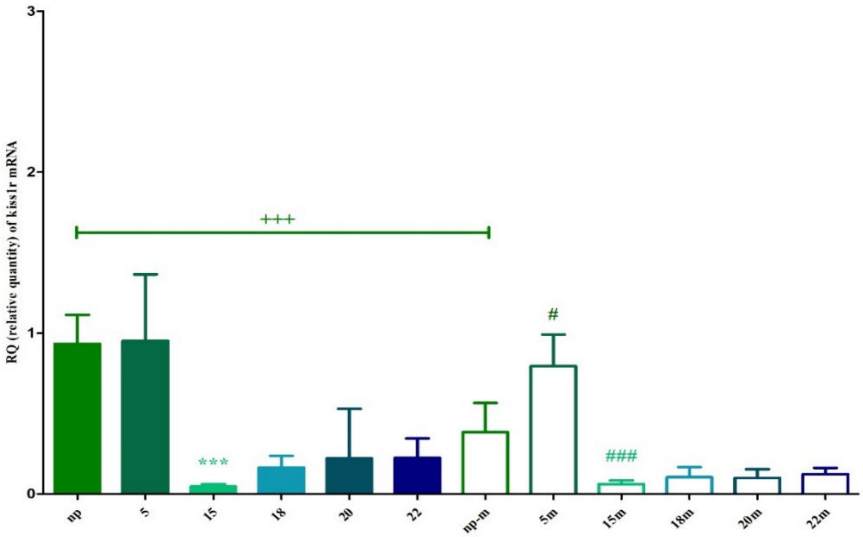

Figure 8.: Changes in the uterine (full columns) and myometrial (empty columns) mRNA expressions of Kiss1r throughout gestation and in non-pregnant samples. Kiss1r expression in the uterus was compared to the myometrial expression on each gestational day as well as in non-pregnant samples (+). Also, 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

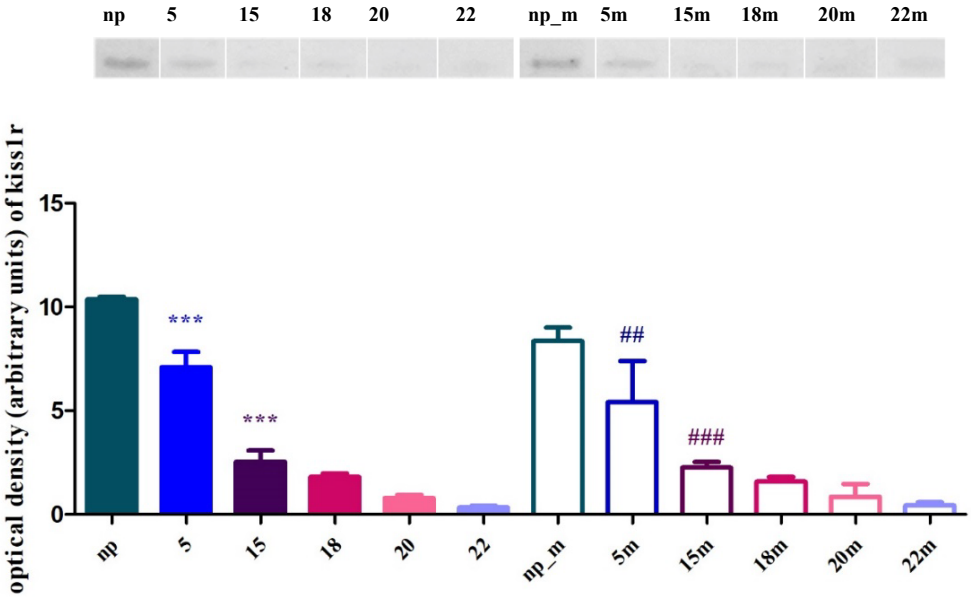

Figure 9.: The uterine (full columns) and denuded myometrial (empty columns) protein expression of Kiss1r on different days of gestation and in non-pregnant samples along with representative blots. Kiss1r expressions show a declining trend throughout pregnancy as compared to the previous day in uteri (*) 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

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. 10.).

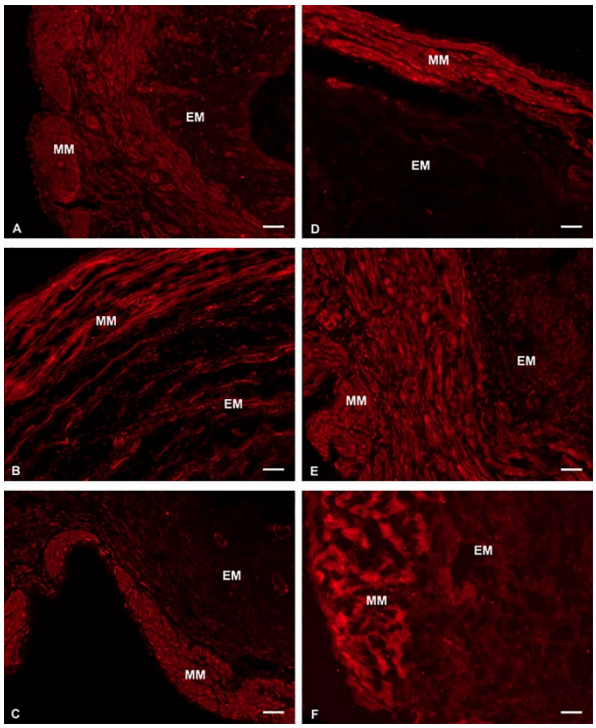

Figure 10.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after LEPR immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μm

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. 11.). A peak in AdipoR2 expression on 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 (Fig. 12.).

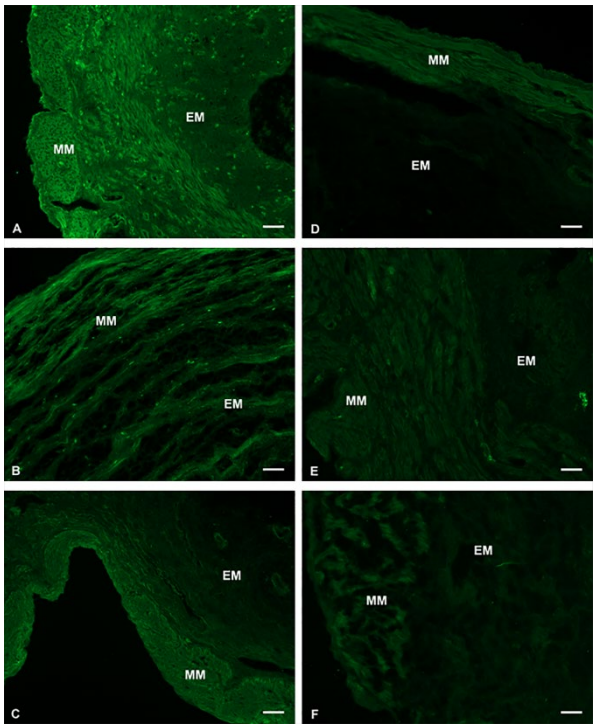

Figure 11.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after AdipoR1 immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 µm

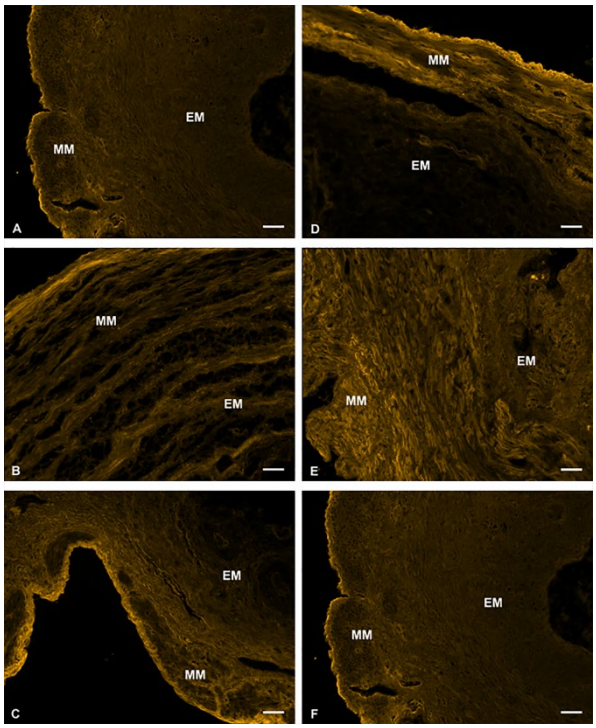

Figure 12.: Representative fluorescent micrographs of cryosections from non-pregnant and pregnant rat uterus after AdipoR2 immunohistochemistry. A: non-pregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 µm

The most intensive staining of Kiss1r was observed in the muscular layer, while the endometrial presence was found less significant (Fig. 13.). Kiss1r is 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.

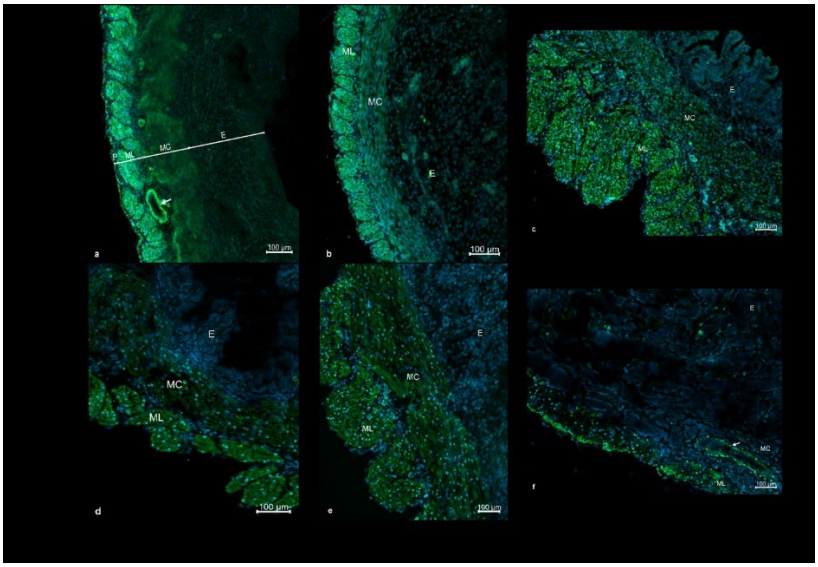

Figure 13.: Representative fluorescent micrographs of cryosections from the uterus of non-pregnant rat **(A)**, and on gestational days 5 **(B)**, 15 **(C)**, 18 **(D)**, 20 **(E)** and 22 **(F)** after Kiss1r immunohistochemistry (green). DAPI was used to label the cell nuclei (blue). P: perimetrium, ML: longitudinal layer of myometrium, MC: circular layer of myometrium, E: endometrium, arrow: large blood vessel in the stratum vasculare.

Discussion

As part of this study, we confirmed that leptin, adiponectin and kisspeptin significantly affects uterine contractility. We also demonstrate the changes in myometrial and endometrial receptor expressions and how the effects of these adipokines are altered during the gestational period.
LEPR presence was proved in the uterus previously, but our results emphasise that they are mainly expressed in the myometrium in non-pregnant cases. Although the reduction in the expression after endometrium removal was not significant, we detected a decreased relaxation in non-pregnant cases. Our results suggest that the endometrial LEPRs may modify uterine contractility in non-pregnant tissues. In early pregnancy, the endometrial distribution of LEPR is markedly high. LEPR expression is decreased in mid-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 LEPRs tends to rise towards term. The relaxant effect induced by leptin in the intact uterus gradually decreased during the gestation, but when the endometrial layer was previously removed, leptin was able to maintain its relaxant effect throughout pregnancy. The alterations in endometrial and myometrial LEPRs distributions revealed by immunolabelling correlated 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. However, the modifications in the effect were 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 effect.

The presence of AdipoRs in the uterus has also been confirmed by previous studies. However, our results revealed that there are significant differences in the amount and distribution of AdipoRs in the uterus. The expression of AdipoR1 is significant in the non-pregnant endometrium, while AdipoR2 is mainly present in the myometrium. Adiponectin relaxed both the intact and the denuded non-pregnant uteri, but the absence of endometrium decreased the effect, suggesting that endometrial AdipoR1 enhances the adiponectin-induced relaxation. During gestation, adiponectin elicited relaxation in both the intact and the denuded uteri, but it was not able to maintain its effect until the end of pregnancy. Correspondingly, the expressions and immunostaining intensities of AdipoRs are reduced towards term. 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 E_{max} values, suggesting that endometrial AdipoRs lost their impact on uterine response.

The kisspeptin/Kiss1r system was also previously identified in uterine tissue, but until now, no clear data has been available about the role of kisspeptin in uterine contractility. We demonstrated that KISS1 94-121 inhibits the contractions both in non-pregnant and pregnant uteri. 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. Our findings confirm that the effect is indeed mediated through Kiss1r, since the inhibitory action of KISS1 94-121 was diminished by the specific inhibitor in 5-day pregnant samples. We found that the relaxant effect of KISS1 94-121 was the strongest in non-pregnant and early pregnant uteri, while a decreased effect was seen towards the end of gestation, indicating a role for kisspeptin in the maintenance of uterine quiescence. We also demonstrated that the Kiss1r expressions were the highest in non-pregnant and 5-day pregnant samples. The activity of the

non-pregnant uterus is usually low in rats, while the 5th day of gestation is considered as the time of embryo implantation, which requires relative quiescence of the uterus. The reduction seen in the Kiss1r expression towards term was further confirmed by immunolabelling, which revealed weaker intensities in all layers of the uterus as term approached. In general, we demonstrated that the receptors were predominantly located in the muscle layers. This was further proved by the fact that the removal of the endometrium did not significantly alter the Kiss1r protein expression in any cases. Interestingly, KISS1 94-121 elicited a more potent relaxant effect in the non-pregnant and 20-day pregnant denuded myometrium as compared to the intact uterus. The endometrium removal also ceased the decreasing tendency in the relaxing effect. All of these suggest the importance of endometrial Kiss1r in the contractile response of the pregnant uterus. It has been reported before that the endometrium has a role in the control of uterine contractility by producing agents that have an impact on smooth muscle function. Also, a networking has already been demonstrated with kisspeptin neurons in the central nervous system and the reproductive axis. These findings might indicate that currently unknown mechanisms or endometrial secretory responses could be involved in the Kiss1r signal transduction.

Conclusion

Leptin, adiponectin and kisspeptin systems are present both in non-pregnant and pregnant uterus and they modify uterine contractility. We conclude that endometrial LEPR, AdipoRs and Kiss1r have regulatory roles in the actions of these adipokines. Our results support the idea that 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. To the best of our knowledge, this is the first evidence for the ontogeny of LEPR, AdipoRs and Kiss1r expression and distribution, and for the modification of these adipokine actions on uterine contractility throughout gestation.

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)
(**IF: 6,78**, Pharmacology, Toxicology and Pharmaceutics [miscellaneous] SJR indikátor: D1)

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)
(**IF: 5,195**, Pharmacology SJR indikátor: Q1)

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
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(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)
(**IF: 3,251**, Biochemistry, Genetics and Molecular Biology [miscellaneous] SJR indikátor: Q2)

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 indikátor: 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 indikátor: Q1)