Involvement of the calcitonin gene-related peptide system in the modulation of inflamed uterus contractile function in pigs

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This study analyzed severe acute endometritis action on myometrial density and distribution of protein gene product (PGP)9.5- and calcitonin gene-related peptide (CGRP)-like immunoreactive nerve fibers and calcitonin receptor-like receptor (CLR) expression, and on CGRP receptor (CGRPR) participation in uterine contractility in pigs. E. coli suspension (E. coli group) or saline (SAL group) were injected into the uteri, or only laparotomy was performed (CON group). In the E. coli group myometrium, a lack of significant changes in PGP9.5 and CGRP innervation patterns and increased CLR protein level were revealed. In all groups, compared to the pretreatment period, human αCGRP increased amplitude in the myometrium, while reducing it in endometrium/myometrium. In the E. coli group endometrium/myometrium, human αCGRP lowered amplitude vs other groups. Human αCGRP reduced frequency in CON and SAL groups and enhanced it in the E. coli group endometrium/myometrium. The frequency in E. coli group increased vs other groups. CGRPR antagonist, human αCGRP8–37, reversed (CON, SAL groups) and eliminated (E. coli group) the rise in human αCGRP-induced myometrial amplitude. In endometrium/myometrium, human αCGRP8–37 abolished (CON group) and reversed (SAL group) a decrease in frequency, and reduced the rise in frequency (E. coli group) caused by human αCGRP. Collectively, in the myometrium, endometritis did not change PGP9.5 and CGRP innervation patterns and enhanced CLR protein level. CGRPR also mediated in CGRP action on inflamed uterus contractility.

Calcitonin gene-related peptide (CGRP) is a 37 amino-acid sensory neuropeptide belonging to the CGRP family. This family is also composed by calcitonin (CT), calcitonin receptor-stimulating peptide (CRSP), amylin (AMY) and adrenomedullin (AM). To produce biological effects, CGRP binds to the CGRP receptor (CGRPR), which consists of a calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1)1–3. CGRP and CGRPR are widely distributed in the peripheral and central nervous systems and peripheral organs. For example, these factors participate in nociceptive processing in the central nervous system, peripheral sensory processing, vascular regulation, and inflammatory processes in visceral organs4,5.

Under physiological conditions, the presence of CGRP was revealed in the uterus-innervating neurons of the pig paracervical ganglion (PCG)6 and dorsal root ganglia (DRGs)7. CGRP-immunoreactive (IR) nerve fibers lie within the human8, mouse9, rat10,11 and porcine12 endometrium and myometrium, and in the myometrial layer, these fibers supply muscle cells and blood vessels. In healthy uteri of women13, mice14, and rats15–17, CGRP has a relaxing action on smooth muscles of the myometrium. It is also reported that in the myometrium, CGRPR is expressed in women18,19, CLR and RAMP1 in rats20 and CGRP-receptor component protein (CGRP-RCP), a marker of CGRP-receptor expression, is expressed in mice21 and rats22. Moreover, CGRPR antagonists reduced the CGRP-induced decrease in the contractility of the human8 and rat16 myometrium. Regulatory functions of CGRP in implantation, trophoblast proliferation and invasion and fetal organogenesis were also reported2.

Among uterine diseases in domestic animals and women in the postpartum period, endometritis and metritis are of significant importance. These pathologies may often lead to disturbances in reproductive processes, and may cause reduced animal production profitability23,24. Endometritis and metritis are evoked mainly by bacteria, and favoring factors, for example, hard labor and fetal membrane retention, contribute to the occurrence of these diseases25. The origin, development and maintenance of uterine inflammation are due to the dysfunction

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Density and distribution of PGP9.5- and CGRP-like IR nerve fibers. The total numbers of PGP9.5- and CGRP-like IR nerve fibers did not differ significantly in the myometrium of CON, SAL and E. coli groups (PGP9.5: 46.4 ± 2.6, 45.4 ± 2.8, 47.8 ± 4.9; CGRP: 5.5 ± 0.6, 6.1 ± 0.3, 6.9 ± 0.2, respectively). In the CON, SAL and E. coli groups, the numbers of fibers expressing PGP9.5 did not differ significantly around the myometrial muscle cells (43.2 ± 3.1, 43.3 ± 3.1, 45.9 ± 4.8, respectively; Fig. 1A,E,I) or blood vessels (4.8 ± 0.5, 5.1 ± 0.7, 6.3 ± 0.3, respectively; Fig. 1C,G,K). A similar situation in the CON, SAL and E. coli groups concerned CGRP-like IR fibers near the myometrial muscle cells (3.1 ± 0.6, 2.1 ± 1.1, 1.9 ± 1.2, respectively; Fig. 1B,F,J) and blood vessels (0.7 ± 0.12, 0.5 ± 0.5, 0.6 ± 0.3, respectively; Fig. 1D,H,L).

Moreover, no significant differences were found between the CON, SAL and E. coli groups in terms of CGRP-like IR fibers normalized against the total population of PGP9.5-like IR fibers (12.8 ± 1.2%, 13.4 ± 0.8%, 14.8 ± 0.9, respectively).

Immunofluorescent staining of the porcine duodenum, as the positive control, showed PGP9.5- and CGRP-like immunoreactivity in the nerve fibers (Supplementary Fig. 1). PGP9.5- and CGRP-like IR fibers were not present after omitting the primary antibodies (Fig. 1M,N, respectively).

Expression of CLR messenger RNA. No significant differences in the myometrial CLR mRNA expression were revealed between the CON, SAL and E. coli groups (Fig. 2).

Expression of CLR protein. Mice and porcine duodenum utilized as positive controls showed bands of approximately 53 kDa, and they were accepted as CLR protein (Supplementary Fig. 2). The band was not found after not using the primary antibody (data not shown). Western blotting of the porcine myometrium indicated protein bands of approximately 53 kDa for CLR (Supplementary Fig. 3).

The CLR protein expression in the myometrium of E. coli group was significantly increased in relation to the CON and SAL groups (Fig. 3).

Distribution of CLR. Immunofluorescent staining of the porcine duodenum, as the positive control, showed CLR-like immunoreactivity (Supplementary Fig. 4). CLR-immunoreactivity was not visible following omitting of the primary antibody (Fig. 4D). CLR-like immunoreactivity was present in the muscle cells and blood vessels (endothelium, muscle layer) of myometrium in the CON (Fig. 4A), SAL (Fig. 4B) and E. coli (Fig. 4C) groups.

Human α-CGRP (hαCGRP) action on the contractility of uterine strips. Comparison of the hαCGRP action in myometrium in the particular groups in relation to the period before its use. The amplitude in myometrium in response to hαCGRP (10^-8 M) was significantly increased in the CON, SAL and E. coli groups (Fig. 5A). HαCGRP at this dose significantly decreased the frequency in the CON and SAL groups (Fig. 5B).

Comparison of the hαCGRP action in myometrium between groups. The frequency in myometrium in the E. coli group was significantly enhanced by hαCGRP (10^-8 M) vs other groups (Fig. 5B). In all groups, the myometrial amplitude did not differ significantly after using hαCGRP (10^-9, 10^-8 M) (Fig. 5A).

Comparison of the hαCGRP action in endometrium/myometrium in the particular groups in relation to the period before its use. The amplitude in endometrium/myometrium of the CON and SAL groups was significantly reduced by hαCGRP (10^-8 M), while in the E. coli group this effect was exerted by hαCGRP at both doses (10^-9,
Figure 1. Representative pictures show protein gene product (PGP)9.5- and calcitonin gene-related peptide (CGRP)-like immunoreactive (IR) nerve fibers in the myometrial layer of gilts from the control (CON), saline (SAL) and *E. coli* (*E. coli*) groups. Note that numbers of PGP9.5- and CGRP-like IR nerve fibers around myometrial muscle cells and arteries were similar in the CON (A–D), SAL (E–H) and *E. coli* (I–L) groups. Negative control (NC) for PGP9.5 (M) and CGRP (N) was obtained by omitting the primary antibodies. MMC myometrial muscle cells, A artery, Arrowhead nerve fiber.

Figure 2. The messenger RNA expression of calcitonin receptor-like receptor (CLR) in the myometrial layer of gilts from the control (CON), saline (SAL) and *E. coli* (*E. coli*) groups, estimated by real-time RT-PCR. Data are expressed as the mean ± SEM (*n* = 5/gilts in each group). mRNA levels are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
10⁻⁸ M) (Fig. 5C). HaCGRP (10⁻⁸ M) significantly decreased the frequency in the tissues of the CON and SAL groups (Fig. 5D). In the E. coli group, hαCGRP (10⁻⁹, 10⁻⁸ M) significantly enhanced values of this parameter.

Comparison of the hαCGRP action in endometrium/myometrium between groups. After using hαCGRP (10⁻⁹, 10⁻⁸ M), the amplitude in endometrium/myometrium of the E. coli group significantly lowered vs the CON and SAL groups (Fig. 5C). In turn, the frequency in the E. coli group in response to hαCGRP (10⁻⁹, 10⁻⁸ M) was significantly higher than in other groups (Fig. 5D).

Human α-CGRP8–37 (haCGRP8–37, CGRPR antagonist) and haCGRP action on the contractility of uterine strips. Comparison of the haCGRP8–37 and haCGRP action in myometrium in the particular groups in relation to the period before their use. After the application of haCGRP8–37 (10⁻⁷ M) with haCGRP (10⁻⁹, 10⁻⁸ M), the amplitude in myometrium of the CON group was significantly dropped (Fig. 6A). Similar results were evoked by haCGRP8–37 and haCGRP in the myometrium of the SAL group (haCGRP: 10⁻⁸ M) and E. coli (haCGRP: 10⁻⁸ M) groups. HaCGRP8–37 and haCGRP (10⁻⁸ M) significantly decreased the frequency in myometrium of the SAL, while the haCGRP8–37 and haCGRP (10⁻⁸ M) significantly increased it in the E. coli group (Fig. 6B).

Comparison of the haCGRP8–37 and haCGRP action in myometrium between groups. The myometrial amplitude (Fig. 6A) and frequency (Fig. 6B) in the E. coli group after the application of haCGRP8–37 and haCGRP (10⁻⁸ M) were significantly enhanced vs other groups.

Figure 3. The protein expression of calcitonin receptor-like receptor (CLR) in the myometrial layer of gilts from the control (CON), saline (SAL) and E. coli (E. coli) groups, estimated by Western blot analysis. Data are expressed as the mean ± SEM (n = 5/gilts in each group). Protein levels are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Blot with representative bands for each group is presented in Supplementary Fig. 3. ***P < 0.001 compared between groups.

Figure 4. Representative pictures show calcitonin receptor-like receptor (CLR)-like immunoreactivity in the myometrial layer of gilts from the control (CON), saline (SAL) and E. coli (E. coli) groups. CLR-like immunoreactivity is visible in muscle cells and arteries (endothelium, muscle layer) of the myometrium in the CON (A), SAL (B) and E. coli (C) groups. Negative control (NC) for CLR (D) was obtained by omitting the primary antibody. MMC myometrial muscle cells, A artery.
Comparison of the hαCGRP8–37 and hαCGRP action in endometrium/myometrium in the particular groups in relation to the period before their use.

HαCGRP8–37 (10−7 M) and hαCGRP (10−9, 10−8 M) significantly reduced the amplitude in endometrium/myometrium of the CON and SAL groups while it significantly increased this parameter in the *E. coli* group (Fig. 6C). In the SAL and *E. coli* groups, hαCGRP8–37 together with hαCGRP (10−9, 10−8 M) significantly increased the frequency in endometrium/myometrium (Fig. 6D). HαCGRP8–37 and hαCGRP (10−9 M) significantly increased the frequency in the SAL group vs CON group.

Comparison of the hαCGRP8–37 and hαCGRP action in endometrium/myometrium between groups. HαCGRP8–37 (10−7 M) with hαCGRP (10−9, 10−8 M) caused a significant rise in the amplitude in the endometrium/myometrium of the *E. coli* vs other groups (Fig. 6C). After using hαCGRP8–37 and hαCGRP (10−9, 10−8 M), the frequency in the endometrium/myometrium of the *E. coli* group was significantly higher than in the CON group (Fig. 6D). HαCGRP8–37 and hαCGRP (10−9 M) significantly increased the frequency in the SAL group vs CON group.

Figure 5. Influence of human α-calcitonin gene-related peptide (hαCGRP) on the contractile amplitude (A,C) and frequency (B,D) in the myometrium (MYO; (A,B)) and endometrium/myometrium (ENDO/MYO; (C,D)) strips of gilts from the control (CON; grey bars), saline (SAL; hatched bars) and *E. coli* (*E. coli*; black bars) groups. Data are expressed as the mean ± SEM (n = 5/gilts in each group). The actions of individual hαCGRP doses are depicted as percentages of the baseline (pre-treatment period) contractile amplitude and frequency, taken as 100% (horizontal lines). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the basal value in each group; AP < 0.05, AAP < 0.01, AAA P < 0.001 compared between the CON and *E. coli* groups for the same treatment; BBP < 0.01, BBBP < 0.001 compared between the SAL and *E. coli* groups for the same treatment.
Discussion

Here we present the endometritis influence on the myometrial density and distribution of PGP9.5- and CGRP-like IR nerve fibers and CLR expression as well as the role of CGRP and CGRPR in the contractile function of the porcine inflamed uterus. Results of macroscopic and histopathologic examination of uteri used in the current study were reported earlier. Macroscopically, no inflammatory changes were observed in the endometrium of the CON and SAL groups. In turn, the \textit{E. coli} injections led to the accumulation of inflammatory exudate in the horns, reddening and swelling of the endometrium. Histopathological examination of uterine sections stained with the hematoxylin–eosin method, according to the criteria described previously did not reveal any changes indicating an inflammatory process in the CON and SAL groups. In the \textit{E. coli} group, a severe acute endometritis has been diagnosed based on the presence of the following changes: edema, hyperemia, damage to the luminal and glandular epithelium and statistically higher number of neutrophils than in the healthy uterus.

Figure 6. Influence of human α-calcitonin gene-related peptide (hαCGRP) on the contractile amplitude (A,C) and frequency (B,D) in the myometrium (MYO; (A,B)) and endometrium/myometrium (ENDO/MYO; (C,D)) stripes of gilts from the control (CON; grey bars), saline (SAL; hatched bars) and \textit{E. coli} (\textit{E. coli}; black bars) groups after the application of human α-calcitonin gene-related peptide receptor (hαCGRP8–37, CGRPR antagonist) (a dose of $10^{-7}$ M). Data are expressed as the mean ± SEM (n = 5/gilts in each group). The actions of hαCGRP8–37 and individual hαCGRP doses are depicted as percentages of the baseline (pretreatment period) contractile amplitude and frequency, taken as 100% (horizontal lines). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the basal value in each group; \textsuperscript{A}P < 0.05, \textsuperscript{AA}P < 0.001 compared between the CON and \textit{E. coli} groups for the same treatment; \textsuperscript{BB}P < 0.001 compared between the SAL and \textit{E. coli} groups for the same treatment; \textsuperscript{C}P < 0.05 compared between the CON and SAL groups for the same treatment.
The current study found that endometriosis did not significantly change the myometrial total population of nerve fibers, as revealed by PGP9.5-like immunoreactivity as well as the numbers of CGRP-like IR fibers (both total number and number in relation to the total population of PGP9.5-like IR fibers). The lack of changes in the myometrial population of CGRP-like IR nerve fibers in response to endometriosis is consistent with the unchanged number of uterine perikarya expressing CGRP in the DRGs of pigs suffering from this pathology. Reports also show that inflammation increased the population of PGP9.5- and CGRP-IR nerve fibers in murine vagina and CGRP-like IR fibers in pig descending colon. It is known that peptides of the CGRP family share a similar secondary molecular structure, and the majority of their functions overlap. Thus, it is possible that the antibody used in the present study to stain CGRP fibers also binds AM, AMY, and CRSP. This supposition is based on reports showing the immunoreactivity for AM in perivascular nerve fibers in rat mesenteric artery, AMY in perikarya in cat trigeminal ganglion, and CRSP in pig central nervous system.

As mentioned earlier, CGRP is a potent dilator seen in cerebral, coronary, and kidney vascular beds. This function is essentially inhibited by the CGRP antagonist. CGRP plays an important role in the course of inflammatory reaction because its release results in edema formation, increased blood flow, and recruitment of inflammatory cells to the local area. Moreover, the relaxing actions of CGRP in the human and rat uterine arteries are mediated through CGPR. In light of the above data, the role of CGPR in the CGRP action on the blood vessels of the pig myometrium under physiological and inflammatory conditions is possible.

The current study was also devoted to defining the participation of CGRP and its receptor in the contractile function of the inflammatory-changed uterus. As mentioned before, the disturbances in uterine contractility are a significant cause of the origin, development, and maintenance of an inflammatory state. The use of ACh in the study confirmed the viability and utility of uterine tissues for research. ACh enhanced the amplitude and frequency in healthy uteri (CON and SAL groups). In organs with inflammation (E. coli group) in response to ACh, the frequency was increased, while the amplitude was decreased, which is in line with earlier reports. The current report, for the first time, shows the contractility of healthy pig uteri under the influence of CGRP and the role of CGPRP. Moreover, the current results concern the role of the CGRP system in the contractile function of inflamed uteri. However, the values of contractility parameters and the myometrial expression of CLR mRNA and protein did not differ significantly between the CON and SAL groups. As it was mentioned previously, CGRP, under physiological conditions, decreased the myometrial contractility in humans and rodents. In turn, the effect of CGRP in the gastrointestinal tract was excitatory or inhibitory. Literature data also show the functional role of CGPRP in the motility control of the human and rat myometrium and the rat colon.
In the myometrium of the *E. coli* group, in relation to the period before haCGRP application, haCGRP enhanced the amplitude and did not significantly change the frequency. In turn, in the endometrium/myometrium, a decrease in amplitude and a rise in frequency after using this neuropeptide were found. The direction and level of significance of changes after using haCGRP8–37 and haCGRP indicated that CGRPR mediates CGRP action on the contractility of an inflamed uterus, similarly to the healthy uterus. It is important to add that changes in the contractility of inflamed uteri (also healthy) in response to haCGRP may have also resulted from the action of this peptide via AMY1R (formed by CTR and RAMP1). Moreover, the effect of AM and AMY by CGRPR on the contractile activity of porcine uteri is possible. Based on the higher affinity of CGRPR8–37 for CGRPR than AMY1R, it is supposed that the changes in uterine contractility noted in the current study after the use of haCGRP8–37 result mainly from the blockage of CGRPR. Moreover, one cannot exclude that changes of the uterine contractility observed through all experimental groups might be affected by sensibilization and/or desensibilization of the CGRPR. In response to haCGRP, the amplitude in the endometrium/myometrium of the *E. coli* group lowered and the frequency in both kinds of strips was enhanced vs the CON and SAL groups. These changes in contractility of the inflamed uterus coincided with an augmentation in myometrial CLR protein expression. The lowered amplitude in the endometrium/myometrium and the increased frequency in strips of the *E. coli* group in response to haCGRP in relation to a healthy uterus could also be due to the indirect influence of this neuropeptide. Existing data demonstrate that CGRPR modify the ACh, SP, VIP, and GAL-stimulated uterine contractions in rodents. It should be stressed that ACh and GAL markedly change the contractility of porcine inflamed uteri. On the other hand, it is known that the action of CGRP on pig uterine contractility may be dependent on nitric oxide (NO) and PGE2, which was indicated earlier. The authors' previous studies reported a significant rise in NO and PGE2 production in the pig uterus with inflammation. These tasks require further research.

Further studies are also necessary to explain the varied CGRP influence on the amplitude in both kinds of strips of the CON, SAL and *E. coli* groups and on the frequency in the latter group. We can only assume that this situation is due to the different innervation of the endometrium and myometrium, and relationships between CGRPR and other neurotransmitters in relation to uterine contractility (e.g. SP, GAL and ACh), as mentioned above. Moreover, varied contractility after using CGRP may be dependent on the different content in the endometrium and myometrium of substances modulating this neuropeptide effect. For example, a difference in NO and PGE2 amounts between the particular layers in both healthy and inflamed pig uteri was demonstrated.

Earlier it was reported that NA, ACh, NPY, VIP, GAL and PGE2 have lowering action on the amplitude in pig inflamed uteri. Thus, it is possible, that CGRP by decreasing the value of this parameter (present study) is another substance which contributes to the accumulation of inflammatory exudate inside the uterine lumen. In parallel we have now demonstrated that CGRPR increased the frequency in the inflamed uterus, similarly to ACh, PGE2 and LTG. Moreover, the participation of CGRPR in the influence of CGRP on uterine inflammation, shown in the current study, may constitute the basis for the development of drugs (agonists, antagonists) to increase the contractility of uteri with inflammation. This may contribute to an improvement in the effectiveness of treatment and prevent postpartum diseases of the reproductive tract and, thus, to better fertility and economic results on farms.

**Conclusions**

Severe acute endometritis did not change the total population of nerve fibers, including the CGRP-like immunoreactive fibers and increased the CLR protein expression in pig myometrium. In the inflamed uterus, CGRP by CGRPR increases the contractile amplitude in the myometrium and reduces this parameter in the endometrium/myometrium, and increases the frequency in strips of the CON, SAL and *E. coli* groups and on the frequency in the latter group. We can only assume that this situation is due to the different innervation of the endometrium and myometrium, and relationships between CGRPR and other neurotransmitters in relation to uterine contractility (e.g. SP, GAL and ACh), as mentioned above. Moreover, varied contractility after using CGRP may be dependent on the different content in the endometrium and myometrium of substances modulating this neuropeptide effect. For example, a difference in NO and PGE2 amounts between the particular layers in both healthy and inflamed pig uteri was demonstrated.

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**Materials and methods**

**Animals.** Fifteen gilts (female pig after puberty before farrowing, Large White × Landrace, age 7–8 months, body weight/BW/90–120 kg) from the “Wronie” breeding farm (Wronie, Poland) were used in the experiment. There were no reproductive disturbances in these animals (vaginal discharges did not occur and the second estrous cycle was regular). Behavioral estrus was determined by a tester boar. Transport of gilts from a farm to the animal house (University of Warmia and Mazury, Olsztyn, Poland) took place three days before the start of the research. The animals were kept in individual pens (an area: approx. 5 m2) under 14.5 ± 1.5 h of natural daylight and 9.5 ± 1.5 h of night, and 18 ± 2 °C of temperature. They were fed commercial diets and had access to water ad libitum. The study procedures were conducted according to the relevant Polish and EU regulations in the field of Animal Protection and Welfare (Leg. Decree 26/2014 implementing EU directive 2010/63/EU), and were approved by the Local Ethics Committee (Consent no. 65/2015).

**Study procedures.** The gilts were allocated (randomly), on day 3 of the second estrous cycle (day 0 of the research), into three groups: Escherichia coli (*E. coli*), saline (SAL), control (CON) (five animals in a particular group).

The research procedures have been reported in detail. The premedication was evoked using atropine (0.05 mg/kg BW; Atropinum sulf. WZF, Warszawskie Zakłady Farmaceutyczne Polfa S.A., Poland), azaperone (2 mg/kg BW; Stresnil, Janssen Pharmaceutica, Beerse, Belgium) and ketamine hydrochloride (10 mg/kg BW; Ketamina, Biowet, Pulawy, Poland). Ketamine hydrochloride (supplementary doses: 1 mg/kg BW every 5 min) was
also used for induction and maintenance of general anesthesia. After median laparotomy into each uterine horn in the E. coli group 50 ml of E. coli suspension (strain O25K23/a:H1; Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland), containing 10⁹ colony-forming units/ml were injected. In the SAL group, 50 ml of saline solution was injected. In the gilts from the CON group, only median laparotomy was made. The animals were left untreated in the time from surgery to euthanasia. The euthanasia was performed on day 8 of the experiment (the expected day 11 of the estrous cycle) using an overdose of sodium pentobarbital and the uteri were harvested. For real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) and Western blot analyses, fragments of the horn were collected from three parts: paraoviducal, middle, and paracervical. Using a scalpel blade and a dissecting microscope, endometrial and myometrial layers were separated. The fragments of myometrium about the thickness of the entire layer were snap-frozen in liquid nitrogen and stored at –80°C for real-time RT-PCR and Western blot analysis. For the immunofluorescent method, the fragments of horn from three parts were divided into smaller pieces and placed in a 4% paraformaldehyde solution (pH 7.4) for 24 h. After fixation, the pieces were rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and cryoprotected in an 18% buffered solution of sucrose (pH 7.4) until sectioning. To measure the uterine contractility, fragments of the horn from its middle part were placed on ice and transported to the laboratory (within 5 min following collection).

RNA extraction, and real-time RT-PCR. Total RNA was isolated from myometrial tissues. They were homogenized in a TRI Reagent solution (Invitrogen, Thermo Fisher Scientific, USA) using a FastPrep 24 homogenizer (MP Biomedicals, LLC, USA). For phase separation, a BCP reagent (Molecular Research Center Inc., USA) was used, and the RNA was then purified by using an RNasy Mini Kit (QIAGEN, USA) in accordance with the manufacturer’s instructions. RNA was stored until further use at –80°C in RNase-free water with the addition of RNase Inhibitor (Applied Biosystems, Thermo Fisher Scientific, USA). The quantity and quality of extracted RNA were determined by the use of the NanoDrop 1000 (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA with an RNA Integrity number ranging from 7.0 to 9.6 was used in real-time RT-PCR.

Real-time RT-PCR was carried out by the use of TaqMan tests (Table 1) and a one-step PCR Master mix (Applied Biosystems). Each reaction (10 μl) contained: 15 ng of total RNA in a volume of 3 μl, 5 μl 2× TaqMan RT-PCR Mix, 0.25 μl 40× TaqMan RT Enzyme Mix, 0.5 μl 20× TaqMan Gene Expression Assays and 1.25 μl RNase-free water (Applied Biosystems). The real-time RT-PCR reaction was performed in duplicates in 384-well plates using the following conditions: reverse transcription for 15 min at 48°C, initial denaturation for 10 min at 95°C, followed by 45 cycles of 15 s of denaturation at 95°C and then 1 min of annealing at 60°C, in an ABI Prism 7900HT system (Applied Biosystems). The negative control was prepared by replacing the RNA template with RNase-free water. Data obtained were analyzed by the use of the Miner method. The NormFinder algorithm was utilized to choose the most stable housekeeping gene among: β-actin (ACTB), hypoxanthine–guanine phosphoribosyl transferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The best stability value was determined for the combination of ACTB and GAPDH genes (0.171). The expression levels for each target gene were normalized relative to the geometric mean of ACTB and GAPDH gene expression.

Western blot analysis. The myometrial tissues were homogenized on ice with a cold buffer (composition: 50 mmol/l Tris–HCl, pH 8.0; 150 mmol/l NaCl; 1% Triton X-100, 10 mg/ml aprotinin, 52 mmol/l leupeptin, 1 mmol/l pepstatin A, 1 mmol/l EDTA, 1 mol/l PMSF) and centrifuged (17,500× g, at 4°C, for 10 min). The supernatants were centrifuged (2500 g, at 4°C, for 1 h). The separated supernatants were then freeze-frozen in liquid nitrogen and stored at –80°C. The Bradford method was used to estimate the protein content. Protein extracts (20 μg) were dissolved in a sodium dodecyl sulfate (SDS) gel-loading buffer (composition: 50 mmol/l Tris–HCl, pH 6.8; 4% SDS, 20% glycerol and 2% β-mercaptoethanol), heated (at 95°C, for 4 min) and separated by 10% SDS–polyacrylamide gel electrophoresis. The separated proteins were then electro-blotted onto nitrocellulose membrane (0.22 μm) in transfer buffer (composition: 20 mmol/l Tris–HCl buffer, pH 8.2; 150 mmol/l glycine, 20% methanol, 0.05% SDS). To block the non-specific bindings, membranes were incubated with 5% fat-free dry milk in a TBS-T buffer (at 21°C, for 1.5 h). Next, they were incubated (at 4°C, for 18 h) with primary antibody rabbit CRLR/CGRPR1 polyclonal antibody (dilution: 1:500, cat. no. bs-1860R), from Bioss Antibodies Inc. After rinsing in TBS-T buffer, the membranes were incubated (at 21°C, for 1 h) with biotinylated goat anti-rabbit IgG (dilution: 1:3000, cat. no. PK-6101, Vectastain Elite ABC-HP Kit, Vector Labs, Burlingame, CA, USA). To visualize antibody binding, incubation (for 3–4 min) with a mixture of 3,3′-diaminobenzidine tetrachloride (cat. no. D5637, Sigma Aldrich, St. Louis, MO, USA) and H₂O₂ in Tris-buffered saline (pH 7.2) was performed. To demonstrate the specificity of the primary antibody utilized, it was excluded from the analysis (the negative control). Mice and porcine duodenal proteins were used as the positive control. Images were gained and quantified by a Quan-
The pieces of horns of uteri were cut using a cryostat (Reichert–Jung, Nußloch, Germany). Sections (thickness 10 μm) were stained using the single-immunofluorescent method to estimate immunoreactivity to CLR, and the double-labeling immunofluorescence method to determine PGF9.5- and CGRP-like IR nerve fibers. In short, uterine sections after drying (at 21 °C, for 30 min) and rinsing (0.1 M PBS, pH 7.4, three times, each for 15 min) were incubated (at 21 °C, for 18 h) in a humid chamber, with a primary antibody against CLR/SGPR1 (dilution: 1:200), the same as for Western blotting. On the next day, the sections were washed (as given above) and incubated with biotinylated anti-rabbit IgG (dilution: 1:1000, cat. no. AP132B, Chemicon International, Temecula, CA, USA) (at 21 °C, for 1 h), and next with carbocyanine 3 (CY3)-conjugated streptavidin (dilution: 1:9000, cat. no. 016160084, Jackson ImmunoResearch Labs, West Grove, PA, USA) (at 21 °C, for 1 h). The sections were also incubated (at 21 °C, for 18 h) with antibodies against the PGF9.5 (dilution: 1:800, polyclonal rabbit, cat. no. 104004, Abcam, UK) and CGRP (dilution: 1:1600, polyclonal guinea pig, cat. no. T-5027, BMA Biomedicals, Augst, Switzerland). Following rinsing (as given above), the sections were incubated with biotinylated anti-rabbit IgG (dilution: 1:1000, cat. no. AP132B, Chemicon International, Temecula, CA, USA) (at 21 °C, for 1 h), and then with CY3-conjugated streptavidin (the same as given above) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-guinea pig IgG (dilution: 1:800, cat. no. 706095148, Jackson ImmunoResearch Labs, West Grove, PA, USA) (at 21 °C, for 1 h) to visualize the antibody combinations: PGF9.5/CGRP. Next, the washed sections wereoverslept in carbonate-buffered glycerol (pH 8.6). To perform the negative controls the primary antibodies were omitted. As a positive control, sections of the porcine duodenum were used. Immunoreactivity was assessed using the microscope with epi-fluorescence and appropriate filters (Olympus BX51, Olympus Consilio Sp. z o. o., Warsaw, Poland). Immunostained structures were analyzed and photographed using an Olympus BX51 microscope (Olympus Consilio Sp. z o.o., Warsaw, Poland) equipment with epi-fluorescence and the appropriate filter sets for FITC (B1 module, excitation filter 450–480 nm, barrier filter 515 nm) and CY3 (G1 module excitation filter 510–550 nm, barrier filter 590 nm). The density of nerve fibers in the myometrium was evaluated according to the method given earlier using computer software (Image Processing and Analysis in Java, v.1.53 m). In brief, the counting of these structures was performed in five randomly chosen microscopic observation fields (each: 0.1 mm²) in the myometrium of six sections of each uterus. To prevent double-counting the same fibers, the uterine sections to be assessed were at a distance of at least 100 μm.

Preparation of uterine strips and contractility measurement. The strips (approximate size 3 × 5 mm) of myometrium and endometrium/myometrium were used to study contractile function. After rinsing in saline, they were mounted between two stainless steel hooks in an organ bath with a capacity of 10 ml (Radnoti Unit Tissue Organ Bath System type 159920, Germany) under 5 mN tension. The Krebs–Ringer solution (composition / mM/l/: NaCl, 120.3; KCl, 5.9; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 15.5; glucose, 11.5; pH 7.4) placed in the bath was warmed to 37 °C. The strips were under the influence of ACh (doses: 10⁻⁷, 10⁻⁶, 10⁻⁵ M, cat. no. A6625, Sigma, St. Louis, MO, USA) which was reported earlier. To estimate the hαCGRP and hαCGRP8–37 doses, the initial ACh dose of 10⁻⁷ M was measured for 10 min. Subsequently, the strips were under the influence of CGRPR antagonist—hαCGRP8–37 (dose: 10⁻⁷ M, cat. no. ab142492, Abcam, UK) for 2 min and hαCGRP (doses: 10⁻⁹, 10⁻⁸ M) was then added, and the effects of both substances were registered for 10 min. After each measurement, the strip was washed (in PBS, three times). After completing the measurements, the viability of strips was determined again using ACh (doses as given above). Only results registered from strips in which the discrepancies under the influence of ACh at the beginning and end of the study were less than 20% were included in the statistical analysis.

Amplitude (the difference between the minimum and maximum values for a single contraction /mN/) and frequency (the number of peaks) of the strips were registered by a force-displacement transducer and analyzed in a computer with PowerChart software (Chart v5, scope v5, AD Instruments). The uterine strip treatments are depicted in Fig. 7. To estimate the viability of strips and their usefulness for further study, strips were influenced by ACh (doses: 10⁻⁷, 10⁻⁶, 10⁻⁵ M, cat. no. A6625, Sigma, St. Louis, MO, USA) which was reported earlier. Following this, hαCGRP (doses: 10⁻⁹, 10⁻⁸ M, cat. no. ab142458, Abcam, UK) was used. The action of a particular dose of hαCGRP was measured for 10 min. Subsequently, the strips were under the influence of CGRPR antagonist—hαCGRP8–37 (dose: 10⁻⁷ M, cat. no. ab142492, Abcam, UK) for 2 min and hαCGRP (doses: 10⁻⁹, 10⁻⁸ M) was then added, and the effects of both substances were registered for 10 min. After each measurement, the strip was washed (in PBS, three times). After completing the measurements, the viability of strips was determined again using ACh (doses as given above). Only results registered from strips in which the discrepancies under the influence of ACh at the beginning and end of the study were less than 20% were included in the statistical analysis. The ACh doses were used previously. To estimate the hαCGRP and hαCGRP8–37 doses, the initial research was performed in which healthy pig uteri were treated with CGRPR (doses: 10⁻¹⁰, 10⁻⁹, 10⁻⁸ M) alone and together with an antagonist (doses: 10⁻⁹, 10⁻⁸, 10⁻⁷ M). As a result, it was found that hαCGRP at doses of 10⁻⁹ and 10⁻⁸ M more effectively influenced the contractile parameters and that hαCGRP8–37 at a dose of 10⁻⁷ M statistically significantly changed hαCGRP-affected the contractile parameters (data not present).

Statistical analyses. Mean (± SEM) total numbers of PGF9.5- and CGRP-like IR nerve fibers, the numbers of these fibers around particular myometrial structures, as well as mRNA and protein CLR levels were counted for particular groups. To determine differences in the frequency of CGRPR-like IR nerve fibers as part of the total population of PGF9.5-like IR nerve fibers, the total number of PGF9.5-like IR fibers in each group was set to 100% and the number of CGRP-like IR fibers was expressed as a percentage (mean ± SEM) of the total population of PGF9.5-like IR fibers. The mean (± SEM) values of amplitude and frequency counted for a particular group before the addition of substances (pre-treatment period) were accepted as 100%. The influences of substances were expressed as the percentage (mean ± SEM) values of these parameters measured before their use. The analysis of contractile function concerned the comparisons between mean values before and following...
each treatment in each group, as well as the mean values between groups in response to the same treatment. The statistical significances between the obtained data were evaluated by the Bonferroni test (ANOVA, InStat Graph Pad, San Diego, CA). Three thresholds (*P < 0.05, **P < 0.01, ***P < 0.001) were used to indicate statistically significant differences.

**Ethical approval.** The studies presented in the manuscript were carried out in accordance with the ARRIVE guidelines. All study procedures were approved by the Local Ethics Committee for Experiments on Animals (University of Warmia and Mazury in Olsztyn, Poland; Consent no. 65/2015). The guidelines in EU Directive 2010/63/EU for animal experiments were included.

**Data availability**
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions
B.J.: contributed to the conception and design of the study, performed surgical procedures, analyzed and interpreted data, written draft, reviewed and edited the manuscript. J.C.: participated in the analysis and interpretation of acquired data and reviewed the manuscript. M.S.: participated in the laboratory analyses and edition of the manuscript. K.P.: helped in surgical procedures, participated in the laboratory analyses and edition of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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