SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Study of pituitary adenylate cyclase activating polypeptide (PACAP) signaling in pathological processes of the reproductive tract

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Summary

PACAP is a neuropeptide that is important in the physiological processes of many peripheral tissues outside the central nervous system, including the reproductive organs. It protects tissues against the damaging effects of oxidative processes up to a certain threshold. These destructive processes are also involved in the pathogenesis of Alzheimer's disease and endometriosis, which are studied in this thesis.

In our experiments, we aimed to expand our knowledge of the role of PACAP in genital function. The research consists of three parts: to investigate neuropeptide signaling in the testes of Alzheimer's disease mice, to describe the problems of including surgically harvestable endometrium in molecular experiments, and to explore PACAP-induced mRNA expression changes in human endometrium and endometriosis samples.

The male gonads were studied by PCR, Western blot, immunohistochemistry and histological staining. The suitability of endometrial samples was assessed by clinical retrospective examination. Eutopic and ectopic endometrioid tissues were maintained and treated using a tissue culture method developed by us before RT-PCR studies were performed.

PACAP signaling is impaired in the testicle in neurodegenerative disease in a similar way to the brain. Alterations in neuropeptide signaling may contribute to the microscopic abnormalities observed in the male gonad. Our clinical criteria system based on inclusion and exclusion criteria increases the possibility of using endometrial samples in experimental research. Contrasting mRNA expression changes can be detected in endometrial and endometriosis samples when PACAP is administered in combination with female sex hormones.

The study of neuropeptide signaling provides evidence that the signaling abnormalities observed in Alzheimer's disease can be investigated in organs other than the brain. Using changes induced by PACAP administration, we have revealed that there may be fundamental differences between molecular processes in intact and abnormal endometrioid tissues. The selection of an appropriate model or tissue is essential when studying different pathological conditions.

I. Introduction

I.1. PACAP and its Signal Transduction, Physiological and Pathophysiological Effects

PACAP - pituitary adenylate cyclase activating polypeptide - is a neuropeptide that was first extracted from ovine hypothalamus in 1989. PACAP has been investigated in several species (including humans and mice in the present study) and has been found in a number of peripheral tissues outside the central nervous system (CNS). The neuropeptide is a member of the VIP-secretin-GHRH-glucagon superfamily. Two of the biologically active isoforms are known: PACAP38 and PACAP27.

Three of the neuropeptide-binding receptors have been identified. These are the PAC1, VPAC1 and VPAC2 receptors. Due to the sequence similarity of PACAP and VIP, all three receptors are able to bind the two peptides, however, PAC1R binds PACAP with a much higher affinity and specificity, whereas the binding affinity is the same for the other two receptors. PACAP-binding receptors bind overwhelmingly to transmembrane Gs proteins, but less frequently to Gi/0 and Gq/11 proteins. The canonical signaling cascade triggered by the binding of a neuropeptide to a (Gs-protein coupled) receptor is cAMP-mediated signaling. Protein kinase A (PKA) activated in this way can result in the expression of several genes by phosphorylation of several transcription factors (e.g. CREB) and thus their nuclear translocation. PACAP may also enhance the activity of other receptors (e.g. NMDA receptor) and other signaling pathways (e.g. MAPK, including ERK). Furthermore, the neuropeptide may affect several signaling pathways through a crosstalk mechanism. These include the BMP, Notch, Hedgehog and TGFβ signaling pathways.

In the central nervous system, PACAP is most abundant in the hypothalamus, while it is expressed in the testicle, the most abundant tissue in the peripheral nervous system. In the case of PACAP-binding receptors, a given brain area or peripheral tissue may express all three types of receptors, but also only two or one. A review of several publications suggests that the neuropeptide is primarily involved in the function of neurons, smooth muscle, endocrine and exocrine glands, but also affects the physiological processes of other tissues of epithelial origin (e.g. gonads and kidney) and other tissues of mesenchymal origin (e.g. cartilage and bone). The physiological role of PACAP can be inferred from the neuropeptide effects of its administration to tissues, from the signal transduction changes observed in various pathological processes, as well as from its absence (PACAP-KO and PACAP-receptor KO models). The overall importance of PACAP in tissues is cytoprotection, i.e. it acts against tissue damaging processes. This is due to its anti-apoptotic, anti-inflammatory and antioxidant

properties. Studies of various publications suggest that the role of the neuropeptide is important in physiological and pathological processes where increased metabolism leads to increased stress effects on tissues (e.g. increased oxidative stress). It is also important to note that with age, the amount of neuropeptide in the body decreases, thus PACAP is also involved in the ageing process.

Given that the knowledge base of the thousands of publications covering PACAP research is beyond the scope of this paper, we will mention, without claiming completeness, the organs in which its role has been described. These include many areas of the central nervous system, the sensory organs (eyes, inner ear), the hormone-producing tissues (e.g. pituitary gland, thyroid gland, pancreas), the exocrine glands and smooth muscle of the gastrointestinal tract, the parenchymal organs (heart, lungs, kidneys and liver), the skeletal system (bones and cartilage) and the reproductive tract. The role of neuropeptide is essential for the function of the latter. PACAP is expressed by almost all cell types in the testis, and can even be detected in the acrosome of male gametes. The neuropeptide is essential for the embryonic and pubertal development of the male gonad, is involved directly and indirectly in the processes of spermatogenesis through activating Sertoli and Leydig cells and the release of FSH and LH, and helps to maintain the blood-testis barrier intact. The absence of PACAP does not abnormally reduce fertility, but it impairs the process of spermatogenesis, damages the blood-testis barrier and may also cause morphological abnormalities in sperm shape. The role of the neuropeptide has also been described in various pathological processes. Examples include neurodegenerative diseases (e.g. Alzheimer's, Parkinson's, Huntington's diseases and amyotrophic lateral sclerosis), migraine, glaucoma, diabetogenic and hypertensive retinopathies, heart failure and allergic asthma.

I.2. The Tissues Studied in the Research: the Testis

In the animal kingdom, the testicle is the paired organ responsible for the production of sperm cells and the release of androgen hormones in males. The former is responsible for reproductive function, the latter for the maintenance of sexual behaviour, spermatogenesis and, in some species (including humans), the development of secondary sexual characteristics.

The testis is made up of two types of cells: germ cells and supporting somatic cells. The former include spermatogonia, the latter include Sertoli cells, interstitial cells (Leydig cells), peritubular cells (myoid cells) and cells of the supplying structures (e.g. endothelial cells of blood vessels).

The histology of the male gonad is made up of the seminiferous tubules and the interstitium. The latter fills the space between the tubules. The skeleton of the scrotal tubules is the lamina basalis, produced by epithelial cells. The main protein component of this layer is type IV collagen. Sertoli cells and spermatogonial cells rest on the lamina basalis. The distance between adjacent Sertoli cells is bridged by their lateral processes. Tight junction complexes are formed between them. This results in the formation of two compartments within the scrotal tubules: the basal compartment closer to the lamina basalis and the adluminar compartment closer to the tubule lumen. The former contains the spermatogonia, the latter the spermatocytes and spermatids. The testicular interstitium is divided into two subunits: the peritubular and the intertubular regions. The peritubular cells, which have smooth muscle properties and are capable of contracting, are located in the former, and their joint peristaltic contractions help to direct the spermatozoa in the tubular fluid towards the epididymal duct. The intertubular part is occupied by Leydig cells. The process of spermatogenesis, which is important for reproduction, is the formation of haploid sperm cells from diploid spermatogonia.

The DNA content of spermatocytes, spermatids and spermatozoa is essentially different from that of somatic cells. For this reason, the immune system would attack these cell types. To avoid this, the blood-testis barrier has evolved, which prevents the passage of white blood cells and many harmful substances from the basal compartment to the adluminar space, thus preventing the development of an immune reaction that threatens reproduction.

I.3. Tissues used in the Research: the Human Endometrium

The endometrium is the mucous membrane of the uterus, which is the innermost layer of the visceral organ. This tissue is responsible for containing and nourishing the embryo after fertilisation, providing a medium for its implantation and, after the placenta has formed, for the exchange of blood between the fetus and the mother's body through its bloodstream.

The uterine lining is a dynamically changing tissue in reproductive age, in terms of its microscopic structure: it follows hormonal changes of the menstrual cycle. Therefore, three phase patterns can be identified in the human endometrium: menstrual, proliferative and secretory. For the latter two, two histological layers can be defined, which are structurally and functionally distinct: the basal layer (stratum basale), which is attached to the smooth muscle layer of the uterus (myometrium), and the associated functional layer (stratum functionale), which is closer to the uterine cavity. In the absence of fertilisation and embryo implantation during the female reproductive cycle, the functional layer degrades and is shed from the

remaining basal layer with bleeding and is then evacuated from the uterine cavity through the vagina to the outside world. This process is called menstruation, which is a unique process among placental mammals. It is not observed in any mammal except some species (e.g. primates). The endometrium of most placental species follows the changes of the estrous cycle instead of the menstrual cycle.

At first glance, the human endometrium consists of two main units: endometrial glands and an endometrial stroma containing stromal cells and extracellular rich supply of cells. Towards the uterine lumen, the endometrium is covered by a single layer of epithelium, the endometrial epithelium. The glands are considered as invaginations of this epithelium embedded in the stroma. The endometrium (unlike the intestinal mucosa) is not separated from the smooth muscle of the uterus by a submucosa, the two layers being connected along an irregular wavy line: this is the endo-myometrial junction.

The endometrium contains several types of cells that create a complex tissue environment. In addition to epithelial cells, glandular epithelial cells and stromal cells, many stem cells and terminally differentiated cells are involved in the formation of the endometrium.

The menstrual cycle is regulated by the fine-tuned hormone production of the hypothalamic-pituitary-gonadal axis. Ultimately, hormones released in the ovaries regulate changes in the endometrium. Thus the concept of the menstrual cycle encompasses ovarian and endometrial cycles. The ovarian cycle consists of three main phases: follicular (about 11 days), ovulatory (about 5 days) and luteal (about 12 days). The two main hormones produced by the ovaries are estrogen and progesterone (P4). Estrogen is a mixture of the hormones estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4), of which oestradiol is the most important physiologically. The production of E2 is due to the activity of the ovarian aromatase enzyme, which converts androgenic hormones produced in the gonad into female sex hormones by aromatising androgenic steranase molecules.

The first day of the menstrual cycle is counted from the first day of menstrual bleeding of the endometrial cycle. The endometrial cycle consists of three phases: menstrual, proliferative and secretory. In the last days of the secretory phase, stromal cells begin to decidualise and form predecidual cells. The morphology of the endometrial glandular structure is best understood during the secretory phase. Recent research has revealed that the endometrial structure of human (and possibly menstruating) species is quite different from that of other placental mammals. In the stratum basale, instead of blind-ended glandular

chambers, there is a plexus network of interconnected glandular structures similar to rhizomes (interconnected root shoots of certain plants).

At the end of the secretory phase, if fertilisation and embryo implantation do not take place, the functional layer is shed due to processes that are still not fully understood (mainly a decrease in serum progesterone levels). Menstruation thus starts and the menstrual cycle resumes.

A healthy woman will menstruate around 400 times during her reproductive life. The first menstrual period is called the menarche. At the end of the reproductive life span, after the last menstrual period, if there is no bleeding within 12 months, we can mark the menopause.

I.4. Diseases Covered by the Research: Alzheimer's disease

Alzheimer's disease is the most common of the diseases that cause cognitive impairment and dementia in the elderly population (over 65). It is a slowly progressive neurodegenerative condition characterised by the presence of proteins with various abnormal structures or abnormal accumulation and neuronal damage, mainly in the brainstem.

Post-mortem studies have also revealed macroscopic, microscopic and molecularly verifiable lesions underlying the pathogenesis of Alzheimer's disease, which maintain a constant inflammatory process leading to toxicity and oxidative stress in the central nervous system. This leads to the degeneration or death of neurons.

The cause of Alzheimer's disease is still unknown. Over the years, different theories (e.g. cholinergic hypothesis, amyloid hypothesis, mitochondrial cascade theory) have been proposed for the development of the disease. Risk factors include genetic causes, gender (hormonal) differences, imbalance of the gastrointestinal microbiome, infections caused by certain pathogens, cardiovascular pathologies (e.g. angiopathy caused by obesity, diabetes, hypertension and atherosclerosis), sedentary lifestyles, different eating habits, toxicity caused by certain metals and ageing.

As Alzheimer's disease is a type of dementia, its symptoms include dementia symptoms, the clinical manifestation of which is based on three pillars: neuropsychological (cognitive) and neuropsychiatric (non-cognitive) symptoms, and changes in the performance of daily activities.

It is worth noting that animal studies have shown that Alzheimer's disease can affect organs other than the brain. These include the heart, liver and kidneys, which can also produce abnormal proteins. However, the symptomatic manifestations in peripheral tissues have been less studied.

Even today, Alzheimer's disease remains an incurable disease. Current treatment options can only slow down the progression of the disease and reduce the various symptoms. Physical activity has been shown to be beneficial. Drug treatment options have been and are being developed based on the molecular processes involved in the pathogenesis of the disease.

I.5. Diseases Covered by the Research: Endometriosis

Endometriosis is the endometrial tissue found anywhere in the body outside the uterine cavity, and which is similar in structure to the lining of the uterus (endometrial-like tissue).

The most common locations of endometriosis within the body are the ovaries the peritoneum and the infraperitoneal connective tissue space (mainly the septum rectovaginale, the connective tissue space between the vagina and the rectum). Three main types can be distinguished: ovarian (endometrioma, OMA), superficial peritoneal (SPE) and deep infiltrating endometriosis (DIE). In addition to these localisations, ectopic tissue may also be present in other areas of the body.

The cause of the pathogenesis of endometriosis is unknown, but there are many theories about how it develops. The most widely accepted theory to date is Sampson's retrograde menstruation theory. Other theories worth mentioning are coelomic metaplasia, embryonic rest and transplantation (lymphovascular metastasis). Recent research has explained the development of endometriosis by stem cell theory, according to which the migration, proliferation and invasion capacity of stem cells for endometrial regeneration and bone marrow stem cells is increased for unknown reason(s), causing them to migrate to ectopic sites and form lesions. The literature describes increased expression of many "stem cell transcription factors" responsible for these changes. The most commonly studied transcription factors are Oct4, Nanog and Sox2, which are responsible for maintaining the pluripotency capacity of cells. These factors are expressed in response to activation of TGFβ signaling, and the increased presence of TGFβ-1 has been shown to play a role in ectopic tissue formation.

Abnormal immune processes, genetic abnormalities, environmental influences and even changes in the bacterial microflora of the endometrium are thought to be causative factors in the development of endometriosis. Endometriosis is a disease state maintaining a chronic inflammatory process.

Endometriosis is an estrogen-dependent tissue that, like the eutopic endometrium, can follow hormonal changes of the female menstrual cycle. Thus, it can shed and bleed during menstruation. Symptoms may include marked pain during menstruation (dysmenorrhoea),

chronic pelvic pain due to peritoneal adhesions and chronic inflammation, and painful sexual intercourse (dyspareunia). Other symptoms may include motility disorders, haematochezia and dyschezia (painful bowel movements) if intestines are involved, urinary disorders, dysuria (painful urination) and haematuria if the bladder is involved. The presence of endometriosis is often detected during the investigation of infertility and is a common cause of infertility.

The diagnosis of the disease is usually made in several steps, including both non-invasive and invasive procedures. The histological strucutre of the ectopic endometrium is similar to that of the eutopic mucosa: it is composed of endometrial gland and endometrial stroma cells. Tissue macrophages try to remove the accumulated blood that cannot be evacuated from the body, thus accumulating hemosiderin pigments. The presence of endometrial glands, endometrial stromal cells and hemosiderin laden macrophages in the removed ectopic tissue confirms the pathology and the diagnosis of endometriosis. Current research confirms the presence of stem cells (eEPs, eMSCs, SP and BMDCs) in endometrioid lesions, in addition to glands and stroma, which allow the proliferation of ectopic tissue.

The treatment of endometriosis is still an unresolved issue. There is no unanimous protocol to solve the problems associated with this condition, only therapeutic options and therapeutic (protocol) recommendations. Treatments can have two main goals: elimination of the ectopic tissue and elimination, resolution or reduction of the complications caused by it. The type of therapy depends on several factors, including the symptoms (e.g. infertility, pain) and the location and extent of the lesion. Treatment methods include conservative and surgical options, which can be combined. Essentially, all three main guidelines are: preservation and maintenance of fertility, pain relief and elimination or prevention of recurrence of ectopic lesion. It is also important that, because of the diversity of endometriosis, therapy should be tailored to the individual.

II. Goals

In this thesis, we aimed to investigate the role of PACAP in pathological processes of the reproductive organs. During the experiments, we investigated testicles from mice modeling Alzheimer's disease and endometriosis signaling. The research was divided into three parts and the materials and methods used in the experiments and the results obtained are described in this paper according to this structure.

In the first part of the study, we aimed to investigate whether lesions can be observed in the testis of Alzheimer's disease mice and, if so, whether impaired PACAP signaling is involved in this neurodegenerative disease. We also wondered whether physical activity, which is known to be beneficial in the disease, could influence any observed abnormalities.

When studying endometriosis, it is necessary to compare it with a similar control tissue, the human endometrium. The extraction and experimental use of physiological mucosa is difficult. Therefore, in the second part of the study, we aimed to investigate what proportion of endometrial samples harvested by surgical procedures are suitable for inclusion in molecular experiments and for comparison with endometriosis.

Once this was determined, the third part of the study aimed to investigate whether PACAP and neuropeptide-binding receptors are expressed in endometriotic tissues. In addition, we also aimed to investigate whether PACAP administration influences the molecular alterations observed during the development of endometriosis, in particular the abnormalities in TGFβ signaling.

III. Materials and Methods

III.1. Materials and methods used in Part I

III.1.1. Animals Used in the Research

The research compared Alzheimer's disease transgenic (B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J) and transgenically unmodified wild type male mice. Individuals were divided into three experimental groups: wild type mice (WT), Alzheimer transgenic mice (AD), and trained Alzheimer transgenic mice (TAD). 5-5 individuals were included in each experimental group.

III.1.2. HE Staining and Analysis of Tissues

The mice in the study were terminated at 3 months of age. Both testes removed from one animal were split in the horizontal plane. After fixation and paraffin embedding, the specimen was sectioned parallel to the sectioned surface on silanized slides. Subsequently, the sections were stained with hematoxylin-eosin (HE, Sigma-Aldrich, MO, USA) histological staining procedure. In the gonads, we measured the basement membrane thickness of the convoluted ducts and counted three cell types (spermatogonium, spermatocytes and spermatozoa) and Leydig cells in the interstitium. ImageJ 1.40 g freeware was used to measure the basal thickness of the basement membrane. The software was used to draw lines perpendicular to the longitudinal axis of the basement membrane. The pixel count of these lines was measured and the results were converted to micrometer values. The different cells were identified by identifying their morphological features under microscopy. Cell counting was performed independently by 3 investigators at 10× magnification on 10 convoluted seminiferous tubules in 5 separate fields of view.

III.1.3. Conventional RT-PCR

One half of the split gonads from one animal were tested by conventional RT-PCR. After being mechanically ground, the sample was dessolved in TRIzol reagent (Applied Biosystems, Foster City, CA, USA) and then the isolated total RNA was harvested in nuclease-free water after 30 min incubation at 4°C using the manufacturer's RNA isolation protocol. After dilution of all samples to the same RNA concentration, reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression of the following molecules was examined:

PAC1R, VPAC1R, VPAC2R, PKA, Sox9, Sox10, PP2A, type IV collagen, type IX collagen and testatin. Actin was found to be the most stable housekeeping gene.

The primers for the different genes were designed using PrimerBlast freeware software, produced by IDT (Integrated DNA Technologies, Interleuvenlaan, Belgium). Amplification of the genes tested was performed using a thermal cycler (Labnet MultiGeneTM 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA). PCR products were run on 1.2% agarose gel containing ethidium bromide. The gel bands were then photodocumented using UV light on a FluorChem E gel documentation system (FluorChem E, ProteinSimple, CA, USA). The integrated densitometry of the corresponding bands of the PCR products was determined using ImageJ 1.40 g freeware program. The integrated densitometry value of each gene was normalized to the integrated densitometry value of the respective housekeeping gene, followed by statistical evaluation.

III.1.4. Western Blot

One half of the split gonads from one animal were tested by Western blot. One sample was homogenized in 100 µL of RIPA buffer solution containing protease inhibitor after being mechanically disintegrated. Suspension was sonicated for 30 s at 40 A pulsation (Ultrasonic Processor, Cole-Parmer, IL, USA). Protein concentrations of samples were determined using BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The concentration determination based on the absorbance reaction was compared to the absorbance curve of a known standard concentration of BSA dilution series. The absorbance values were detected at 570 nm wavelength using a Hidex microplate reader (Hidex, Turku, Finland). Subsequently, denaturation of protein suspensions and uniform protein concentration were achieved by the addition of 1× Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA). Protein separation by weight of one sample was performed by running on 7.5% SDS-polyacrylamide gel. Subsequently, electrophoretic protein transfer onto nitrocellulose membrane was performed. The protein expression of the following molecules was investigated: PAC1R, VPAC1R, VPAC2R, PKA, Sox9, P-Sox9, Sox10, PP2A, type IV collagen, type IX collagen and testatin. Actin expression was detected as an internal control.

Nitrocellulose membrane protein bands were photodocumented using a chemiluminescence kit (Advansta Inc; CA, USA) according to the manufacturer's recommendation using a FluorChem E gel documentation system (FluorChem E, ProteinSimple, CA, USA). The integrated densitometry of the corresponding kDa bands of the proteins under study was determined using ImageJ 1.40 g freeware software. The integrated

density value of each protein was normalized to the integrated density value of its internal control (actin), followed by statistical evaluation.

III.1.5. Immunohistochemistry Staining

The following molecules were detected: P-Sox9 and type IV collagen. Sections of testis were prepared for staining after deparaffinization and hydration with alcohol and PBS solutions. The non-specific binding sites of the slides were blocked with PBS solution containing 1% BSA (Amresco, OH, USA). After washing in PBST solution, the binding reaction of the primary antibody produced against the respective molecule of interest was carried out overnight. The binding reaction of the fluorescently dye-labelled secondary antibodies (Alexa FluorTM antibody, Life Technologies Corporation-Thermo Fisher Scientific, Carlsbad, CA, USA) was carried out in a darkroom at room temperature. The dilution ratio of the antibodies in PBST solution was 1:1000. The preparations were then covered with DAPI-containing Vectashield mounting medium (Vector Laboratories, Peterborough, England). Immunofluorescence sections were photodocumented with an Olympus Bx53 microscope equipped with a DP74 camera (Olympus Corporation, Tokyo, Japan) using cellSense Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan).

III.1.6. Statistical Analysis

In this study, we compared the results from the three groups studied (WT, AD and TAD). Between 5 and 5 individuals per group were tested. The results of the AD group were compared with the WT group and the results of the TAD group were compared with the results of the AD group. In the PCR and WB experiments, all density values were normalized to the density value of the housekeeping gene and the housekeeping protein (in both cases, actin was found to be the most stable), respectively. Significant differences between the test groups and their controls were detected using a two-sample t-test. The significance threshold was set at $p<0.05$. For the groups tested, the degree of comparison with the control group was set at 0.0-1.0.

III.2. Materials and Methods Used in Part II

III.2.1. Collection of Clinical Data

The clinical data were collected from the DE KK E-MedSolution system based on a list provided by the Patient Documentation Department of the Health Financing and Controlling Directorate of the University of Debrecen. The list requested by us is from

January 2017 to March 2020 from Obstetrics and Gynecology Clinic to Department of Pathology "Irregular monthly bleeding, unspecified" (N92.60) ICD code and included data of patients who had undergone surgery. In total, the final reports of 1,162 cases and pathological histopathological findings of the harvested specimens were reviewed. In each case, the following data were collected in a table: age of the patient, type of surgery performed, indication for surgery, pathological diagnosis of the specimen, and whether the patient had a history of endometriosis or adenomyosis.

III.2.2. Classification of Pathological Diagnoses

For all the removed endometrial specimens, 21 different histopathological diagnoses were described. At the suggestion of a pathologist colleague, in order to provide a clearer evaluation of the results, pathological diagnoses with similar characteristics were grouped into larger categories. In this way, we were able to review the figures for 10 histopathological groups and draw conclusions. These groups were: proliferative phase endometrium, secretory phase endometrium, menstrual phase endometrium, endometrium showing effects of exogenous hormone action, menopausal endometrium, samples inadequate for analysis, endometrial polyp, endometrial hyperplasia, malignant tumors and endometritis.

III.2.3. Clinical and Pathological Exclusion and Inclusion Criteria

The pathological diagnosis of tissue removed from a patient during surgery is unknown at the time of surgery. To increase the chances of healthy endometrial tissue submitted to a laboratory for molecular research, it is useful to apply exclusion and selection criteria based on clinical criteria.

All conditions that could indicate an abnormal endometrial condition should be excluded. In view of this, cases where the operated patient was over 45 years of age or where preoperative investigations showed or suspected endometrial abnormality were excluded. Cases where the type of operation was not only aimed at the extraction of the endometrium and where the patient had a history of a confirmed endometrial lesion or endometriosis were also excluded. In addition, conditions that could alter or affect the physiological processes of the endometrium were excluded.

Taking into account the listed exclusion criteria, the following cases (inclusion criteria) were included for molecular testing based on clinical criteria: where the operated patient was younger than 45 years and the gynaecological surgery was performed exclusively to extract the endometrium from the uterine cavity. These surgeries were endometrial scraping

(D&C: curettage, fractionated curettage, abrasion, fractionated abrasion) and endometrial biopsy (HSC: hysteroscopy + endometrial biopsy and TCRE) surgeries. Cases where the indication for surgery was due to a bleeding disorder (e.g. prolonged heavy menstrual bleeding, irregular menses) were also included.

The histopathological diagnosis of the sample will only be known after pathological examination of the sample. Thus, only healthy tissues can be included for expreimental research by retrospective examination. On this basis, endometrium showing proliferative and secretory phase patterns was considered as a pathological inclusion criterion.

III.2.4. Mathematical Analysis

The figures for the cases in the 10 study groups mentioned above have been converted into percentages to make the results and their evaluation more transparent.

III.3. Materials and methods used in Part III

III.3.1. The Tissue Culturing Process

The research was performed in collaboration with the Obstetrics and Gynaecology Clinic, University of Debrecen, who provided us with two types of samples: ovarian endometrioma cyst wall fragments (ectopic endometrial sample) and endometrial tissue obtained from the uterine cavity during HSC surgery (eutopic endometrial sample). Both types of specimens were only involved to the research if the age of the operated patients was between 18 and 45 years, the patients had no chronic co-morbidities and were not chronically taking any medication. Tissues harvested during surgery were stored in sterile physiological saline solution until transfer. For each sample, information was requested on the age of the operated patient (date of birth), the 1st day of last normal menstrual period (LMP) and the type of sample sent.

For both the eutopic and ectopic samples, 5 treatment groups were established in the study. In addition to the control group, samples were treated with female sex hormones, i.e. estradiol (E2) and progesterone (P4) solutions, PACAP solution, and hormones and PACAP in combination. Furthermore, it was necessary to establish an ethanol control group, as progesterone was dissolved in absolute ethanol. Accordingly, the sample received on the day of surgery was minced into 5 equal parts, and the tissue sections were placed in 60×15 mm cell culture petri dishes (Eppendorf, New York, NY, USA). In order to adhere the divided tissue pieces to the surface of the culture dish, 15 µL of Matrigel droplet (Cultrex® BME, Type 2) was dispensed into the bottom of the petri dishes. DMEM (HG-DMEM) solution

(Lonza, Bend, OR, USA) with a glucose concentration of 4.5 g/L was used as culture medium and changed every day during the treatment period. The treatment period lasted until the 24th day of the menstrual cycle, when LMP was known. The current cycle status of the samples (i.e., on which cycle day the tissue was removed) was also calculated based on the LMP.

For the groups treated with hormones, our aim was to mimic *in vitro* the hormonal changes observed *in vivo* during the menstrual cycle. To achieve this, we also aimed to match the final hormone concentrations in the culture medium to those measured in serum. For this purpose, four different hormone solutions were prepared according to the different hormonal states of the ovarian cycle. The final concentrations of the hormone solutions after administration to the medium were equal to the average of the serum limit concentrations measured in the early follicular phase, follicular phase, ovulatory phase and luteal phase.

For the hormone-treated groups, treatment was started with E2 and P4 solutions at concentrations corresponding to the respective cycle status of the sample. As the treatments progressed, when the sample reached a new cycle status day, treatment was continued with solutions containing the hormone concentration corresponding to the cycle status.

PACAP 1-38 solution was used for the PACAP-treated groups. Based on previous tissue culture experience, the final measurable concentration of PACAP in the medium was set at 1 ng/ml. In the present study, 6 ectopic samples and 3 eutopic samples were used.

III.3.2. HE Staining and Histopathological Analysis of Tissues

The basic premise of the present study was to confirm endometriosis by histopathological examination in ectopic specimens and to examine only pathology-free uterine endometrium (i.e. in proliferative or secretory phase) in eutopic specimens. Depending on the size of the specimen submitted to the laboratory, a small piece of tissue could be further examined using HE staining to confirm its pathological status. If the sample size did not allow this, we subsequently checked in the E-MedSolution system what was diagnosed during the pathological histopathological examination of the sample removed from a patient and sent for pathological examination.

One sample is further tested using HE staining. Histopathological analysis of HE stained tissue was performed with the assistance of a pathologist colleague.

III.3.3. Conventional RT-PCR

Using the tissue culture system detailed above, the last day of treatment for both ectopic and eutopic samples was cycle day 24. RNA isolation from the samples was

performed on cycle day 25. An InvitrogenTM PureLinkTM RNA Mini Kit (Thermo Fisher Scientific. Waltham, MA, USA) based on a spin-column principle was used for RNA extraction. Sample purity and RNA concentration were detected using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After dilution of all test groups to the same RNA concentration, reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression of prePACAP, PAC1R, VPAC1R, VPAC2R, TGFβ-1, TGFβR-I, TGFβR-II, Smad2, Smad3, Oct4, Nanog, Sox2, p21, VEGF and Aromatase was examined. As a housekeeping gene, GAPDH was found to be the most stable. The primers for the different genes were designed using PrimerBlast freeware software, produced by IDT (Integrated DNA Technologies, Interleuvenlaan, Belgium).

The amplification of the genes tested, as well as the running and gene documentation of the products, were carried out in the same way as described previously. The integrated densitometry of the corresponding bands of the PCR products was determined using ImageJ 1.40 g freeware. The integrated densitometry value of each gene was normalized to the integrated densitometry value of the respective housekeeping gene, followed by statistical evaluation.

III.3.4. Statistical Analysis

In this study, results from 6 ectopic samples and 3 eutopic samples were compared. For one sample, the PACAP treated group was compared to the control group within the treatment groups (ECT+PACAP group to ECT+0 group for ectopic samples, EUT+PACAP group to EUT+0 group for eutopic samples). Hormone-treated and hormone- and PACAP-treated groups were compared to the alcohol control group (ECT+E2+P4 and ECT+E2+P4+PACAP groups to ECT+ethanol group for ectopic samples, EUT+E2+P4 and EUT+E2+P4+PACAP groups to EUT+ethanol group for eutopic samples). The normalized density value of the control groups was considered to be 100%. The normalized density values of the different treatment groups were converted to % values, i.e. for a sample, the percentage of gene expression of the treatment groups compared to the control groups. Significant differences between treatment groups in each treatment group of the 6 ectopic and 3 eutopic samples compared to the control group were detected using a two-sample t-test. The significance threshold was set at $p<0.05$.

IV. Results

IV.1. Results of Part I of the Research

IV.1.1. Cell Density and Basement Membrane Thickness of the Convoluted Seminiferous Tubules

In AD mice, all cell types were significantly decreased in the testes compared to WT animals. In TAD mice, the number of all cell types was also lower compared to WT animals. However, it was found that the cell count values were normalized and the number of all cell types was significantly increased compared to the AD animals.

The thickness of the basement membrane in AD mice was significantly reduced compared to WT animals. Although the thickness of the histological layer in TAD animals increased significantly compared to AD mice, the thickness did not reach the same level as in WT mice.

IV.1.2. Expression of PACAP Receptors and PACAP Signaling Elements

The mRNA and protein expression of the three PACAP binding receptors, PAC1R, VPAC1R and VPAC2R, were examined. In AD mice, the expression of PAC1R was barely detectable and significantly decreased compared to WT animals. The expression of receptor mRNA in TAD mice was nearly identical to that in WT animals, with a significant increase in corrected density compared to AD animals. A similar correlated change was observed in Western blot analysis. The expression of PAC1R was significantly decreased in AD mice compared to WT animals. In the TAD animals, protein expression was significantly increased compared to AD animals, similar to that observed in WT animals.

VPAC1R mRNA expression tended to be decreased compared to WT individuals in both AD and TAD mice. However, the receptor protein expression was significantly increased in TAD animals compared to AD mice. In AD animals, VPAC1R protein expression was barely different compared to WT animals.

VPAC2R mRNA was barely detectable in the samples, and no difference in expression was observed in any of the study groups. However, when protein expression was examined, we found that the receptor was barely detectable in TAD animals, with significantly lower expression levels compared to the other study groups.

Among the PACAP signaling elements, the mRNA expression of PKA, Sox9, Sox10 and PP2A was examined. Using Western blot, the protein abundance of phosphorylated forms

of PKA and Sox9 was detected in addition to the same molecules. The localization of P-Sox9 was also investigated by immunohistochemical staining.

PKA mRNA expression was significantly decreased in AD mice compared to WT animals, whereas it was increased in TAD animals. The change detected in TAD mice was significant compared to AD animals. The protein expression of the molecule was also significantly decreased in AD mice, while in TAD animals it approached that of WT animals. PKA protein expression was significantly increased in TAD mice compared to AD animals. The amount of the more active, phosphorylated form of PKA in TAD animals was twice that detectable in WT mice and significantly higher than that in AD mice. The amount of P-PKA in AD animals was significantly lower compared to WT animals.

Sox9 mRNA expression was reduced in both AD and TAD groups compared to WT individuals. The change was not significant when comparing AD and WT or TAD and AD mice. Protein expression of the molecule was also higher in AD and TAD mice compared to WT individuals. The change was significant when comparing AD and WT and TAD and AD mice. The amount of the more active, phosphorylated form of Sox9 was nearly equal in WT and AD mice, whereas it was increased in TAD animals. The change was significant in TAD mice compared to AD animals.

Immunohistochemical staining showed that while in WT mice P-Sox9 immunopositivity was proportionally distributed in the seminiferous tubules and could be detected predominantly around the nuclei, in AD animals immunoreactivity was much lower and could be observed in the more peripheral parts of the seminiferous tubules. In TAD mice, immunoreactivity similar to that of WT mice was observed: the presence of P-Sox9 was predominantly detected around the nuclei.

Sox10 mRNA expression was significantly decreased in AD animals compared to WT animals. The expression of the molecule in TAD mice exceeded that detected in WT animals. This change was significant compared to AD animals. Protein expression of Sox10 was barely detectable in WT and AD mice, whereas TAD animals showed a significant increase in the expression of the molecule.

The expression of PP2A mRNA was nearly identical in WT and AD mice, whereas it tended to increase in TAD animals. In contrast, PP2A protein expression was significantly decreased in AD mice, while in TAD animals it was twice as high as that detected in WT animals. This increase was significant when compared to AD animals.

IV.1.3. Expression of Components of the Testicular Basement Membrane

The mRNA and protein expression of the following basement membrane component molecules were investigated: type IV collagen, type IX collagen and testatin. The presence of type IV collagen was also detected by immunohistochemical staining.

The expression of type IV collagen mRNA was significantly decreased in AD mice, whereas in TAD animals it exceeded the level detectable in WT mice. This increase was significant compared to AD mice. A similar change in protein expression of the molecule was observed: the expression of type IV collagen was significantly decreased in AD mice compared to WT animals, while it more than doubled in TAD animals. This increase was also significant compared to AD animals.

The immunoreactivity of type IV collagen in the basement membrane of the seminiferous tubules of WT mice showed a uniform distribution. In AD animals, little immunopositivity of the protein was observed. In contrast, in the TAD mice, the immunoreactivity of the protein was increased, with greater accumulation in the basement membrane.

The expression of type IX collagen mRNA was similar to that of type IV collagen mRNA: the expression of the molecule tended to decrease in AD animals, while that in TAD mice exceeded the density values measured in WT animals. This increase was also significant compared to AD animals. Interestingly, protein expression did not correlate with changes in mRNA expression. Protein expression of type IX collagen was also lower in AD and TAD animals than in WT animals. The change was significant when comparing both AD and WT and TAD and AD individuals.

There were no significant differences in testatin mRNA expression in either group compared to WT animals: it tended to decrease in AD animals and slightly increase in TAD mice. In contrast, testatin protein expression increased in AD and TAD animals compared to WT animals. This change was significant when comparing both AD and WT and TAD and AD groups.

IV.2. Results of Part II of the Research

Based on the histopathological reports, 17.64% and 10.67% of the total 1,162 cases were diagnosed as proliferative and secretory phase endometrium, respectively. The pathological diagnosis of the remaining cases were: 1.46% menstrual phase endometrium, 4.56% menopausal endometrium, 13.51% endometrial polyp, 24.01% endometrial hyperplasia, 4.04% malignant tumor and 1.72% endometritis. Of the endometrial specimens

removed, 16.18% were inadequate for further histopathological analysis and 6.20% showed signs of exogenous hormonal effects.

Of the total 1,162 cases aiming at the extraction of the endometrium, 949 surgeries were aimed solely at harvesting the tissue from the uterine cavity. In the remaining 213 cases, endometrial resection was only an additional part of another gynaecological operation.

Of the 949 cases, 833 endometria were extracted from the uterine cavity using the uterine scraping procedure. On histopathological examination, 14.17% and 9.84% of these were diagnosed as proliferative and secretory phase endometrium, respectively.

Of the 949 cases, 116 endometria were extracted by hysteroscopy with endometrial biopsy from the uterine cavity. On histopathological examination, 21.55% and 11.21% of these were diagnosed as proliferative and secretory phase endometrium, respectively.

Without knowing the histopathological diagnosis of the samples, using only the clinical exclusion and selection criteria described previously, 145 (15.28%) of the 949 cases were found to be suitable for inclusion in further experimental research. A retrospective review of the histopathological findings determined that 22.07% and 15.86% of these 145 samples were diagnosed as endometrial proliferative and secretory phase, respectively.

Of the 145 cases selected as suitable based on clinical criteria, 116 endometria were extracted from the uterine cavity using the uterine scraping procedure. On histopathological examination, 19.83% and 17.24% of these were diagnosed as proliferative and secretory phase endometrium, respectively.

Of the 145 cases selected as suitable based on clinical criteria, 29 endometria were extracted by hysteroscopy with endometrial biopsy of the uterine cavity. On histopathological examination, 30.03% and 10.34% of these were diagnosed as proliferative and secretory phase endometrium, respectively.

If the endometrium is to be studied as a control tissue, only the endometrium showing the proliferation and secretion phase pattern is suitable for further experimental studies. Out of a total of 949 samples, using only the clinical exclusion and inclusion criteria described previously and without knowledge of the histopathological diagnoses, 145 (15.3%) samples were found to be suitable for inclusion in experimental research studies. Of these, 116 endometria were extracted from the uterine cavity by the uterine scraping procedure and 29 endometria by hysteroscopy with endometrial biopsy. The actual cases suitable for molecular analysis can only be determined retrospectively once the pathology of the samples is known. Using the histopathological exclusion and selection criteria, a total of 32 samples were suitable for further *in vitro* experimental research. This is 3.37% of the total number of cases

(949 samples) and 22.07% of the clinically selected cases (145 samples). Of the 32 actually eligible samples, 28 endometria were removed from the uterine cavity by the uterine scraping method. This represents 3.36% of the total number of uterus scraping cases (833 samples) and 24.14% of clinically selected scraping cases (116 samples). 4 of the 32 actually eligible samples were obtained by hysteroscopy with endometrial biopsy from the uterine cavity. This represents 3.45% of the total number of biopsy cases (116 samples) and 13.79% of clinically selected biopsy cases.

IV.3. Results of Part III of the Research

IV.3.1. Expression of PACAP Receptors and prePACAP mRNA in Endometrium Samples

Among the PACAP receptors, PAC1R and VPAC2R were expressed in all three samples tested, while VPAC1R was not detected in one of the samples. No significant change was observed in any of the treatment groups.

A trend towards a decrease in PAC1R expression was observed in the EUT+E2+P4+PACAP group compared to the control group, while in the EUT+E2+P4 and EUT+PACAP groups, little change was detected and the increase in expression was negligible.

For VPAC2R expression, all three treatment groups (EUT+E2+P4, EUT+PACAP, EUT+E2+P4+PACAP) showed a trend towards an increase in VPAC2R expression compared to control groups.

The mRNA of prePACAP was detected in only 1 sample, so the detected changes are statistically irrelevant for the changes.

IV.3.2 Expression of PACAP Receptors and prePACAP mRNA in Endometriosis Samples

Among the PACAP receptors, VPAC1R was expressed in all samples tested, while VPAC2R was detected in 5 out of 6 samples and PAC1R in 4 samples.

There is a trend towards a decrease in VPAC1R expression in the ECT+PACAP and ECT+E2+P4 groups, while the ECT+E2+P4+PACAP group shows a significant decrease compared to the control group.

No significant changes in the expression of VPAC2R were observed in either group: a small trend decrease in the PACAP-treated groups (ECT+PACAP and ECT+E2+P4+PACAP) and a small trend increase in the ECT+E2+P4 group.

For PAC1R, a significant change was observed in the ECT+E2+P4 group, where receptor expression increased. In the other treatment groups, a slight trend increase was detected compared to the control groups.

PrePACAP mRNA was detected in only 2 of the six samples. Since a statistically valid result requires at least 3 samples, no significance and standard deviation values were determined for prePACAP expression. However, in both samples, prePACAP expression tended to increase slightly in all treatment groups.

IV.3.3. mRNA Expression of TGFβ Signaling Elements in Endometrium Samples

TGFβ-1 expression was decreased in the EUT+E2+P4 group and increased in the EUT+PACAP group compared to the control groups. The co-administration of hormones and PACAP (EUT+E2+P4+PACAP) resulted in TGFβ-1 mRNA expression approaching the expression levels detectable in the control group.

TGFβR-I expression tended to increase in all three treatment groups (EUT+E2+P4, EUT+PACAP, EUT+E2+P4+PACAP). There was also an increase in the expression of TGFβR-II in the PACAP-treated groups (EUT+PACAP, EUT+E2+P4+PACAP), but a decrease in the expression of receptor mRNA in the hormone-treated group (EUT+E2+P4).

Similar changes to TGFβR-II expression were observed in Smad2 mRNA expression: the PACAP-treated groups showed an increase in expression (EUT+PACAP, EUT+E2+P4+PACAP), while the hormone-treated group (EUT+E2+P4) showed little change in mRNA expression compared to the control group.

Changes in the expression of Smad3 are also similar to those of TGFβR-II: a decrease in the hormone-treated group and a slight increase in the PACAP-treated group.

IV.3.4. mRNA Expression of TGFβ Signaling Elements in Endometriosis Samples

The expression of TGFβ-1 in ECT+PACAP and ECT+E2+P4 groups was barely different compared to the control groups, with a slight trend towards an increase in the former. However, a trend decrease was detected in the ECT+E2+P4+PACAP group.

For TGFβR-I, trend decreases were observed in all treatment groups compared to control groups.

The expression of TGFβR-II showed a slight trend increase in the ECT+E2+P4 group, whereas a decrease was detected in the PACAP-treated groups compared to the control groups. There was a significant decrease in ECT+E2+P4+PACAP.

Smad2 expression tended to decrease in all treatment groups compared to control samples.

Smad3 showed similar changes to Smad2, but the ECT+E2+P4+PACAP group showed a significant decrease in expression.

IV.3.5. mRNA Expression of Stem Cell Transcription Factors in Endometrium Samples

Oct4 expression was increased in the PACAP-treated groups (EUT+PACAP, EUT+E2+P4+PACAP), whereas it was slightly decreased in the hormone-treated group (EUT+E2+P4) compared to the control groups.

For Nanog, an increase in expression was observed in all three treatment groups (EUT+E2+P4, EUT+PACAP, EUT+E2+P4+PACAP), with a slight increase in the hormone-treated group compared to the control group.

Among all the molecules tested, Sox2 mRNA expression is one of the molecules for which a significant change in expression was observed in the eutopic samples: the expression level in the EUT+E2+P4+PACAP group was significantly decreased compared to the control group. A trend increase in Sox2 expression was detected in the EUT+E2+PACAP group and a decrease in the EUT+E2+P4 group.

IV.3.6. Expression of Stem Cell Transcription Factors mRNA in Endometriosis Samples

While Nanog and Sox2 were expressed in all samples, Oct4 was detected in only half of the samples.

For Nanog, a decrease in expression was observed in all treatment groups compared to control groups. While the decrease in the ECT+E2+P4 and ECT+PACAP groups was trend-like, the decrease in the ECT+E2+P4+PACAP group was significant.

Similar to Nanog expression, decreases in Sox2 expression were observed in all treatment groups compared to the control groups, but the significant change was not detected in the ECT+E2+P4+PACAP group but in the ECT+E2+P4 group.

In the 3 samples previously mentioned in which Oct4 was detected, the expression of the molecule mRNA tended to increase in the PACAP-treated groups, whereas in the ECT+E2+P4 group it tended to decrease compared to the control group.

IV.3.7. mRNA Expression of Other Molecules Important in the Development of Endometriosis in Endometrium Samples

Of all the molecules tested, aromatase mRNA expression is also one of the molecules where significant changes are observed in eutopic samples. In the hormone-treated groups (EUT+E2+P4, EUT+E2+P4+PACAP), a significant increase in aromatase expression was observed, whereas in the EUT+PACAP group, mRNA expression was barely different compared to the control group.

The expression of p21 was increased in all three treatment groups (EUT+E2+P4, EUT+PACAP, EUT+E2+P4+PACAP) compared to control groups.

Of all the molecules tested, the expression of VEGF mRNA is the last to show a significant change in eutopic samples: the EUT+E2+P4 group showed a significant increase in expression compared to the control group. In the PACAP-treated groups (EUT+PACAP, EUT+E2+P4+PACAP), a trend towards an increase in VEGF expression was observed.

IV.3.8. mRNA Expression of Other Molecules Important in the Development of Endometriosis in Endometriosis Samples

For all three molecules, expression decreases were observed in both the hormone- and PACAP-treated groups.

In the case of aromatase, while the ECT+E2+P4 and ECT+PACAP groups showed a small decrease in expression, the ECT+E2+P4+PACAP group showed a significant change compared to the control group.

Examination of the p21 molecule shows that the ECT+E2+P4 group mRNA expression barely differs from the control group, whereas the ECT+PACAP group shows a much greater trend of decreasing expression. In the ECT+E2+P4+PACAP group, the rate of expression decrease is significant.

No significant change in VEGF was detected in any treatment group compared to the control groups. While the ECT+E2+P4 group showed hardly any difference in mRNA expression levels, the trend was much more pronounced in the PACAP-treated groups.

Discussion of Results and New Scientific Findings of the Thesis

PACAP is not one of the endogenous substances that cause prominent changes in the body. Nevertheless, it plays an important role in many physiological and pathological processes: its presence or absence influences biochemical processes in tissues. In the present research, we investigated its role in Alzheimer's disease and endometriosis. An inflammatory pathogenesis involving oxidative stress processes is thought to underlie the development of both diseases. We have studied the effects of PACAP signaling in the male gonad in Alzheimer's disease and the effects of neuropeptide on eutopic and ectopic tissue in endometriosis.

Alzheimer's disease is a degenerative disease that can affect peripheral tissues other than the central nervous system, including the male gonad. PACAP signaling is known to be impaired in certain areas of the CNS. There are similarities between the brain and testis in terms of their intercellular connections (neuron-glial cell and Sertoli-germ cell) and blood-tissue barriers (blood-brain barrier and blood-testis barrier). It is not surprising, therefore, to assume that certain neurodegenerative diseases also manifest to some extent in peripheral tissues other than the central nervous system. Abnormal production or accumulation of Aβ-peptide is thought to underlie the pathogenesis of Alzheimer's disease. This molecule is formed from APP, a protein produced and found not only in the central nervous system but also in the testis. In intact tissues, amyloidogenesis is a controlled process. In contrast, pathological amyloid is formed in Alzheimer's disease. It has been shown that in Alzheimer's disease the tissue structure of the male gonad is impaired: the number of cells in the seminiferous tubules is decreased and the structure of the blood-testis barrier is impaired. Underactivation of PACAP signaling plays a role in the microscopically verifiable damage. Regular physical activity, which also slows down the progression of neurodegenerative disease in CNS, also has beneficial effects on testicular function: exercise normalises abnormalities in PACAP signaling in the male gonad. Neuropeptide signaling is most likely one of the biochemical processes that are activated during physical activity. We further hypothesize that the use of mice modeling Alzheimer's disease in experiments may be suitable for studying testicular degeneration processes. Furthermore, the male gonad may be a suitable organ for studying biochemical processes and tissue regeneration mechanisms activated by physical activity.

Endometriosis shares structural and behavioural similarities with the endometrium, and is also thought to originate from eutopic tissue. Comparison of abnormal and healthy

tissue may provide insight into the differences between their molecular processes. These differences may represent therapeutic options for the elimination of endometriosis. However, it is difficult to find an appropriate control for the study of eutopic tissue, and the study of cell lines and animal models does not cover all aspects of biochemical processes in human endometrial tissue. The endometrium is a complex tissue built up by many different cell types: the stem cells and terminally differentiated cells that make up the endometrium. This diverse population creates a tissue milieu that ensures the cyclical regeneration, renewal and intact function of the mucosa through finely tuned processes based on lively intercellular communication. The ectopic and eutopic tissue is composed of microscopically (histopathologically) identical cells. The cell lines used to study the endometrium are often isolated from pathological ectopic endometrium or menstrual blood and usually contain only one type of cell from the tissue. In addition, many cell lines are immortalised. The molecular processes of these cells, and those isolated from abnormal mucosa, differ in many respects from the function of intact tissue. The uterine mucosa of most mammals, including those most commonly used for experiments, undergoes an estrous cycle instead of a menstrual cycle during the reproductive life cycle. With the exception of some species (e.g. primates), only the human endometrium has a menstrual cycle. The disadvantages of experimenting with menstruating mammals are that they are extremely difficult to 'access', expensive to keep and raise serious ethical issues. In addition, endometriosis is also a 'human-specific' condition, as ectopic endometrium develops spontaneously only in humans and menstruating species. However, there are also serious obstacles for researchers studying the molecular mechanisms of the endometrium, which appears to be the most suitable. For only healthy endometrium is a true control tissue for comparison with endometriosis. However, most of the endometrium that can be harvested by surgical procedures is abnormal, so its molecular processes differ from those of healthy tissue in many respects. It is not an exaggeration to say that the results of most previous studies of the endometrium should be treated with due criticism. Our retrospective analysis has demonstrated that the use of clinical inclusion and exclusion criteria in the selection of endometrial specimens for testing increases the chances of working with intact endometrium in experimental research. Furthermore, histopathological examination of all samples is essential to accept reliable results. Histopathological confirmation of abnormal tissue is an exclusion criterion.

The role of PACAP in endometrium and endometriosis has not been previously investigated. In our study, we have demonstrated for the first time that PACAP-binding receptors are expressed in both types of tissue, despite the fact that the neuropeptide itself is

not expressed in all cases. We have also mimicked for the first time hormonal changes of the female menstrual cycle, which affects the function of both eutopic and ectopic tissues within the body. Administration of PACAP in hormonal media results in differential expression changes in endometrial and ovarian endometrioid samples, opposite to those of controls. Further studies are of course needed to confirm this. In view of the present experimental results, it appears that PACAP in endometriosis inhibits to some extent the mechanisms involved in the formation of ectopic tissue, while in the endometrium it acts towards the formation of abnormal tissue. In the latter case, the question is what is the limit between expression changes relative to the control that promote the remodelling of tissue that periodically differentiates and proliferates and those that cause the formation of ectopic tissue. It is unlikely that PACAP alone will be sufficient to eliminate endometrioid tissue in the future, however, its use in combination therapy may be a potential option in the management of this condition. The expression changes induced by PACAP administration provide evidence that there are fundamental differences in the biochemical processes of eutopic and ectopic tissue.

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List of publications related to the dissertation

1. **Szegeczki, V.**, Fazekas, L., Kulcsár, M., Reglődi, D., Török, P., Orlik, B., Laganà, A. S., Jakab, A., Juhász, T.: Endometrium as Control of Endometriosis in Experimental Research: assessment of Sample Suitability.
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List of other publications

3. Fazekas, L., Szabó, B., **Szegeczki, V.**, Fillér, C., Varga, Á., Godó, Z., Tóth, G., Reglődi, D., Juhász, T., Németh, N.: Impact Assessment of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) and Hemostatic Sponge on Vascular Anastomosis Regeneration in Rats.
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4. Józsa, G., Fülöp, B. D., Kovács, L., Czibere, B., **Szegeczki, V.**, Kiss, T., Hajdú, T., Tamás, A., Helyes, Z., Zákány, R., Reglődi, D., Juhász, T.: Lack of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Disturbs Callus Formation.
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