The mechanism of estrogen's effect in the hypothalamic regulation of energy homeostasis and circadian rhythm

Ph.D. Thesis

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Introduction

In addition to its well-described key role in the regulation of reproductive function, estrogen significantly modulates a variety of centrally regulated physiological functions. Within the central nervous system (CNS), estrogen plays a role in the mechanisms modulating processes such as learning and memory, motivation, mood, sleep, circadian rhythm and energy homeostasis. Cessation of ovarian function (natural or iatrogenic menopause) correlates with deregulation of these processes leading to learning and/or memory impairments, mood disturbances, unpredictable mood swings, anxiety, depression, temporal and erratic disturbances in thermoregulation (hot flushes, night sweats), altered sleep pattern and circadian rhythm as well as imbalanced energy homeostasis favoring obesity. This thesis is focusing on the effects of estrogen in the hypothalamic regulation of energy homeostasis and circadian rhythm.

Estrogen as a metabolic hormone. Obesity has become an enormous health problem in the developed world accounting for one of the largest financial burdens on the health care systems. Sustained obesity is a major risk factor of various diseases including metabolic disorders (diabetes, metabolic syndrome), cardiovascular diseases (atherosclerosis, hypertension, stroke, myocardial infarct), musculoskeletal diseases and as well as cancer. The demand (of the patients and of the pharmaceutical industry) for an efficient and safe anti-obesity drug is high. It has been known for a while, that the estrogen, namely its most potent form the 17-β-estradiol (E2), is a very potent antiobesity hormone. E2 reduces food intake and body adiposity and increases energy expenditure in animals and humans of both sexes. Menopausal women tend to gain body fat, which is thought to be a consequence of the decline in endogenous estrogen levels. In animal models ovariectomy induces an increase in food intake and decreases ambulatory and wheel running activities, leading towards prolonged positive energy balance and ultimately obesity. All these effects can be reversed with estrogen replacement. The effect of estrogen is thought to be mediated through the hypothalamus, but the mechanism is still poorly understood.

Leptin is thought to be a key player in regulating energy balance. It is a 16 kDa protein product of the *ob* gene and it is secreted to the blood stream by the adipocytes. Leptin functions as a fat reporter, an index of the body energy storage. The leptin's effect is mainly mediated by the ARC melanocortin system, which today is considered to be the key component in the regulation of energy balance. One set of these neurons expresses proopiomelanocortin (POMC); when stimulated they produce anorectic effects. The second group of cells in which increased activity leads to orexigenic responses contains neuropeptide

Y (NPY) and aguti-related peptide (AgRP). When the energy storages are full, the case of positive energy balance, the circulating leptin levels are high, leptin activates POMC neurons, triggers the release of α- and β-melanocyte-stimulating hormones (α- and β-MSH) from POMC axon terminals, which in turn activates melanocortin receptor subtypes 3 and 4 (MC3/4R), leading to suppressed food intake and increased energy expenditure. Simultaneously, leptin suppresses the activity of NPY/AgRP neurons of ARC, blocks their inhibitory effects, and thus further suppresses food intake and increasing energy expenditure. Mutations that disrupt the functions of leptin signaling result in obesity and diabetes. However, most obese people are not leptin or leptin receptor deficient, but rather, leptin resistant with elevated plasma leptin levels and poor responsiveness to leptin signal. Although leptin thought to carry out the most potent anorexigenic effect, numerous other metabolic signals are also known with significant anorexigenic or orexigenic effect on the central regulation of energy homeostasis, such as insulin, ghrelin, estrogen, prolactin, the glucocorticoids, resistin, interleukins, as well as nutrients such as glucose and free fatty acids.

Estrogen is also a potent antiobesity hormone and its effect thought to take place in the hypothalamic nuclei involved in the regulation of energy balance. The effect of estrogen is mediated by estrogen receptors (ERs), members of the nuclear hormone receptor family. Estrogen receptor α (ERα) has been suggested to mediate estrogen's antiobesity effect in both sexes, however other mechanisms that involve novel membrane-associated ERs cannot be ruled out.

Estrogen and leptin both act in overlapping neurons in the hypothalamus to regulate long-term energy balance. In fact, all the neurons that express ERα in hypothalamic regions related to energy regulation also express the long form (b) of the leptin receptor (LRb). The effect of estrogen appears independent of leptin, since estrogen reduces appetite and adiposity effectively in both leptin (*ob/ob*) and leptin receptor (*db/db*) mutant obese animals. Estrogen basically mimics the effect of leptin on the POMC and NPY/AgRP neurons. The similarity between leptin's and estrogen's effect raises the possibility that these hormones target common intracellular mechanisms. The signal transducer and activator of transcription 3 molecule (STAT3) is thought to have a central role in the intracellular signal transduction of leptin's antiobesity effect. The brain-specific STAT3 knockout mice were obese and diabetic, highly resembling leptin mutant phenotypes. The brain STAT3 knockout animal model, thus, provides us a unique experimental system for dissecting the interactive mechanism between leptin-STAT3 and estrogen signaling.

How is the estrogen's effect translated to appropriate neural responses in the hypothalamic feeding circuits? Until now, the focus of the research has been the intracellular transcriptional events of hormonal signaling in the hypothalamus to support overall cellular alterations. However, Cajal's neuronal doctrine predicts that functional outcomes of circuit alteration should stem from altered firing and/or connection (synaptology) of neuronal networks. Mounting evidence suggests that hypothalamus retains plasticity throughout life, immature synapses can frequently be found in the adult hypothalamus. Clear evidences are supporting the role of the synaptic plasticity in other hypothalamic regulatory processes. For example, the magnocellular system shows plasticity during changes in water homeostasis, in the ARC the synaptic organization of unidentified neurons exhibited changes in response to a varying gonadal steroid milieu, the synaptology of the perikarya of luteinizing hormone-releasing hormone neurons varies during changes in the gonadal steroid milieu or in photoperiod lengths. Nevertheless, synaptic plasticity was not considered an important aspect in daily energy regulation. Recent observations, however, suggest that it may be a regulatory component in the hypothalamic control of energy balance. First, leptin replacement in *ob/ob* mice indicated that synaptic rearrangement of feeding circuits is part of an ongoing general phenomenon. Furthermore, robust effects of peripheral ghrelin injections on the input organization of POMC neurons of mice led to wiring different from that induced by leptin. Based on the aforementioned evidences it is likely, that estrogen trigger similar neuronal adaptations in the hypothalamic melanocortin feeding circuits further supporting the notion that the plasticity is an inherent element of the hypothalamic regulation of energy control.

Estrogen and the biological clock. The capacity of the CNS to carry out higher brain functions, including learning, memory processing, and cognition, in general, is highly dependent on adequately organized autonomic and endocrine mechanisms. The synchronization of these functions with each other and with the environment is organized by the biological clock. The main biological clock resides in the suprachiamatic nucleus (SCN) of the hypothalamus and generates and maintains circadian and seasonal rhythms by integrating changes in photic conditions to the brain via direct retinal projections to its core region. Transduction of photic signals within the SCN involves a multi-signaling process that ultimately generates rhythms. The SCN via paracrine action and neural projections affects a wide variety of regions and related functions of the brain by providing synchronized rhythms for these CNS mechanisms. The SCN also receives processed visual input via a subdivision of the thalamic lateral geniculate nucleus (LGN), i.e., the intergeniculate leaflet (IGL) of the LGN. It has been suggested that the geniculohypothalamic tract exerts feedback action on the

SCN, modulating the entrainment of circadian rhythms to the light/dark cycle after phase-shifting.

Growing evidences support, that estrogen has significant effect on the biological clock during both development and adulthood maintaining the synchrony of endocrine and autonomic mechanisms. Estrogen has significant implications for a wide array of physiological functions through its effect on the master clock. Destruction of the SCN eliminates the preovulatory LH surge by blocking the positive gonadotropin feedback, and induces persistent estrus in female rats. GnRH neurons are receiving multiple inputs from the SCN and IGL. SCN and IGL efferents target and synapse on GnRH-containing neurons in the preoptic area and in the anteroventral periventricular nucleus (AVPV). Taking together, it is strongly suggested, that this sexually dimorphic, clock-dependent positive feedback of gonadotropins is due, in part, to gonadal hormone-dependent development and/or adult activity of the SCN and IGL. However, the mechanism by which estrogen exerts its effect in the neuronal circuits of the biological clock is still ill-defined.

Several areas of the brain exhibit gender differences, which thought to be due to gonadal steroid hormone-dependent development. During early development of the CNS, testosterone converted to estrogen by the key enzyme aromatase. The locally formed estrogen regulates the maturation and sexual differentiation of these sensitive brain areas through estrogen receptor β (ERβ). Aromatase activity was shown to be present in the limbic system and in certain nuclei of the hypothalamus, which are primarily participating in the regulation of reproductive functions. Its presence was also demonstrated in the circadian visual system as well, such as optic tract, SCN, ventral lateral geniculate body (most abundantly in the IGL), and superior colliculus. The aromatase activity in the SCN and IGL was mostly found during late prenatal and early postnatal periods. The SCN is sexually dimorphic. Three-dimensional images of the SCN of males and females showed that the volume of the SCN is larger in males and contains more nuclei. Furthermore, morphological studies demonstrated that SCN efferents, which contain vasopressin (AVP) or vasoactive intestinal peptide (VIP), develop in a gender specific manner.

The synaptology and efferent projections of the IGL and SCN start to develop from embryonic day 16 and 19, respectively. This coincides with the appearance of aromatase immunoreactivity in axons and axon terminals in the SCN and IGL with a peak at embryonic day 18. Plasma testosterone levels of males start to elevate at embryonic day 17 and peak between embryonic day 18 and 19. The presence of aromatase in the developing biological clock along with the results showing the existence of ERs in these same areas and the differential availability of substrate androgen in males and females indicate that parts of the

biological clock develop in a gender specific/hormone-dependent manner. However, the mechanism, by which of this developmental effect taking place is not well-understood.

The effects of ovarian hormones on the biological clock during adulthood have been extensively studied. Estrogens modulate circadian locomotor rhythms by shortening the free-running period and advancing the phase angle of entrainment. Ovariectomy also reduces the amount of daily activity in rodents; however, treatment with estrogen restores the activity levels to normal. Estrogen also modulates overall locomotor activity levels in males as well. Despite the extensive investigations, the effects of estrogen on mechanisms underlying the entrainment of SCN activity to changes in light/dark cycles remain undetermined. Advances in the onset of light increase SCN expression of transcription factors like c-fos, early growth response protein-1 (egr-1), and phosphorylated cAMP response element (pCREB). Also, light modulates the expression of the CREB binding protein (CBP) and calbindin-D (28K). These transcription factors thought to be the key players in the entrainment of biological rhythms within the SCN. Light-induced changes in SCN expression of these nuclear proteins correlate with light-induced changes in behavioral and physiological rhythms.

The estrogen's effect on the induction of transcription factors in the SCN may occur by a direct action of estrogen on ERβ located in the SCN and/or IGL. However, SCN appears to be minor target for estrogen. Alternatively, estrogen could act at other ER-rich brain sites that project to the SCN to modulate the expression of light-induced gene transcription in the SCN such as preoptic area, corticomedial amygdala, bed nucleus of the stria terminalis, and arcuate nucleus and the raphe nuclei. The serotonin (5-HT) neural system in the midbrain raphe may be a good candidate in modulating the effects of estrogen on the biological clock for many reasons. First, the dorsal raphe nucleus (DRN) receives direct projections from the retina. Second, the DRN and median raphe nucleus (MRN) contain both ERα and β. Finally, the raphe releases 5-HT into the SCN to regulate circadian rhythms. Based on these evidences, it is plausible that estrogen enhances light-induced transcription in the SCN indirectly by modulating 5-HT release from the raphe into the SCN.

Hypothesis

It is well known, that the estrogen has a significant role in the neural control of energy homeostasis and circadian rhythms. However, the mechanisms underlying these effects are poorly understood. The aim of our research, therefore, was to explore the inter- and intra-cellular mechanisms by which the estrogen's effect is translated to appropriate neuronal responses in the hypothalamus.

Hypothesis 1. The estrogen's anti-obesity effect is mediated through rapid synaptic changes of the hypothalamic feeding circuits independently from leptin. Thus, the synaptic plasticity is an inherent element in the hypothalamic regulation of energy homeostasis. We sought to test this hypothesis by determining the followings:

1. To test the effect of estrogen on synaptology of hypothalamic feeding circuits, whether the estrogen reorganizes the synapses of hypothalamic melanocortin cells.
2. To investigate the type of the excitatory synapses on POMC perikarya.
3. To study whether estrogen enhances the activity of POMC neurons.
4. To demonstrate the effect of estrogen on the synaptology of POMC neurons under physiological conditions as well.
5. To elucidate the relationship between synaptic changes and energy homeostasis by studying the dynamics of feeding behavior and synaptic rewiring induced by estrogen.
6. To determine whether estrogen-induced synaptic changes on POMC neurons are reflected at the electrophysiological level.
7. To test whether the estrogen-triggered anorexigenic behavior is independent of leptin.
8. To study whether estrogen mimics leptin's effect on the rewiring of POMC cells and enhancing their tone.
9. To demonstrate whether estrogen is able to acutely activate the downstream target of leptin signaling, STAT3, independent of leptin in the hypothalamus.
10. To test the role of ERα in the estrogen's antiobesity effect.

Hypothesis 2. The locally formed estrogen's organizational/developmental effect on the biological clock is responsible for the sexually dimorphism of the adult SCN and IGL, and it is taking place mostly late prenatally at the peak of aromatase activity in the brain. We sought to test this hypothesis by determining the followings:

1. To test, whether the number and the distribution of SCN and IGL neurons, which are newly originated during the late prenatal period, are gender specific.
2. To study, whether the testosterone treatment during the late prenatal period alters the number and distribution of the newly originated cells in the SCN and IGL of both genders through aromatisation.
3. To investigate the phenotypical characterization of these newly originated cells in the SCN and IGL.

Hypothesis 3. Estrogen modulates the light-induced activity of SCN and IGL neurons in adulthood either directly and/or indirectly through the raphe serotonin system and therefore, estrogen is important for optimal responses of the biological clock to changes of photic conditions. We sought to test this hypothesis by determining the followings:

1. To test, whether estrogen modulates the light-induced expression of various transcription factors in the SCN or in the IGL.
2. To demonstrate, whether light enhances the activity of the neurons in the raphe nuclei and whether this effect is modulates by estrogen.

Materials and methods

Experiment 1; estrogen's effect on the energy homeostasis. Numerous types of rodents were used in this experiment including wide type rat and mice, male rats with intracerebral ventricular cannula, male and female *ob/ob* and *db/db* transgenic mice, brain-specific STAT3-knockout mice, ERα-knockout mice and POMC-green fluorescent protein (GFP) mice.

To test the effect of estrogen on synaptology, we studied axosomatic synapse type and number on POMC perikarya. Ten-week-old wild type female rats were ovariectomized, and then two weeks after the procedure, rats were implanted with silastic capsules filled with crystalline 17-β-estradiol. Controls received empty implants (n = 6 per group). Animals were sacrificed three days after treatment and were used to investigate the synaptic changes in the ARC. Another set of ovariectomized female rats were received a single subcutaneous injection of either 50 mg E2 or only vehicle for control (n = 5 per group). These animals were sacrificed 4 hours (h) after the treatment and were examined for c-Fos immunoreactivity.

To elucidate the relationship between synaptic changes and energy homeostasis, we studied the dynamics of feeding behavior and synaptic rewiring induced by E2. Male rats with intracerebral ventricular cannula were used for these studies. Water-soluble E2 was infused into the third ventricle through the intracerebral ventricular cannula of these rats (n = 7 per group). Food intake was measured 4 h and 14 h after E2 infusion. Another set of these animals were used to study the parvalbumin expression 6 h after E2 infusion.

To address whether the estrogen-triggered plasticity of the anorexigenic melanocortin circuits is dependent on leptin signaling, we studied the effect of estrogen in leptin mutant animals. The male and female *ob/ob* and *db/db* mice (n = 6 per group) were also implanted with E2 capsules in a similar fashion as described above and were kept for four weeks to investigate the estrogen's effect on food intake and energy expenditure. Energy expenditure (EE) and respiratory quotient (RQ) were determined using a comprehensive mouse metabolic

monitoring system. The rates of oxygen consumption (VO2) and carbon dioxide production (VCO2) were determined. RQ was calculated as the ratio of VCO2 to VO2. Energy expenditure was calculated as EE = VO2 × (3.815 + 1.232) × RQ. The lean body mass was assessed after anatomical preparation of the torsos of the animals. The body composition was measured by standard MRI technology and reported in grams. Next, to test the effect of estrogen on synaptology in these *ob/ob* and *db/db* mice we studied axosomatic synapse type and number on POMC perikarya with and without E2 treatment.

The brain-specific STAT3-knockout and the ERα-knockout male mice were treated with subcutaneous E2 capsules in a similar fashion (n = 6 per group) to further elucidate the role of STAT3 and ERα in the mechanism of estrogen action.

Animals for light or electron microscopic analysis underwent tissue preparation for immunocytochemistry. Animals were first perfused transcardially with standard fixative solution under deep anesthesia. The brains were dissected out, and then 50 μm coronal sections were cut on a vibrotome. The brain sections containing the ARC were selected. Single labeling immunostaining for POMC and double labeling staining for Fos, Parvalbumin, GluR1, and GluR2 were carried out using standard techniques. Sections for electron microscopic analysis were flat embedded and the blocks were cut on an ultramicrotome. Postembedding immunostaining for GluR1, GluR2, Glutamate and GABA were carried out on selected sections using establishes protocol. The quantitative analysis of synapse numbers was performed in a double-blind fashion on electron micrographs using the dissector technique. For fluorescence microscopy the POMC-GFP mice brain slices were incubated with Alexa Fluor secondary antibody conjugates. All tissue sections were coded then the cell counts were performed without prior knowledge of the experimental treatments by two independent raters.

To determine whether E2-induced synaptic changes on POMC neurons are reflected at the electrophysiological level a set of POMC-GFP mice were also ovariectomized and treated with E2 capsules as described above (n = 6 per group). The frequency of miniature excitatory postsynaptic currents (mEPSCs) in E2-treated and control animals were recorded using standard electrophysiological techniques.

To test whether E2 is able to activate the downstream target of leptin signaling, STAT3, in the hypothalamus, we intraperitoneally injected a shot of water-soluble E2 or saline for control in both wild-type and *ob/ob* mice. Animals were sacrificed 0, 15, 30, 60, 90, 120 or 150 min after treatment respectively, and their hypothalami were used for the immunoblotting studies to investigate the E2-induced STAT3 phosphorylation (n = 2-4 per group). Immunoblotting was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-

PAGE) and antibody to STAT3 and horseradish peroxidase (HRP) -conjugated antibodies. The blots were visualized using chemiluminescent substrate.

Experiment 2; estrogen's effect on the biological clock during early development. Timed-pregnant female rats were purchased 10 days into their pregnancy (embryonic day 10). Animals were kept under standard laboratory conditions, in a 12-h light/dark cycle (lights on from 6:30 to 18:30). The pregnant rats were housed individually in plastic cages. A set of pregnant rats received daily subcutaneous injections of testosterone propionate (TP) from the 15th day of gestation until giving birth, others received injections with vehicle alone on the same days (n = 5 per group). All pregnant rats received a single intraperitoneal injection of a mitotic marker 5-bromo-2'-deoxyuridine (BrdU) on the 18th day of gestation (embryonic day 18). This particular treatment is used to label mitotic cells in developing fetuses given that BrdU crosses the placental barrier and incorporates into the mitotic cells. Sixty days following their birth, male and female pups treated with oil or testosterone during gestation were randomly selected to be killed. The fixation and tissue handling were performed as in Experiment 1. Subsequently immunostaining for BrdU was performed in all sections containing the SCN and IGL using well established protocol. Selected sections were processed for double labeling immunocytochemistry for AVP, GFAP, calbindin-D (28K) or VIP to investigate the phenotypical characterization of these newly originated BrdU-stained cells. These sections were then analyzed under light microscope. Additional sections from the same animals were processed for immunofluorescence detecting BrdU along with GFAP or VIP. These slides were analyzed under fluorescent microscope.

Experiment 3; estrogen's effect on the biological clock during adulthood. Female wild-type rats were used in this experiment. Animals were kept under standard laboratory conditions, in a 12-h light/dark cycle (lights on from 06:30 to 18:30). The rats were ovariectomized under general anesthesia, and then two weeks later they were implanted with silastic capsules filled with crystalline E2. Controls received implants with cholesterol that time. The light shifting procedures were initiated 48 hours following the capsule implantations. Animals were randomly assigned to four groups according to the hormone treatment which they received and the manipulation of the light/dark cycle (n = 6 per group). The time at which the lights were turned on in the animal colony was termed Zeitgeber time (ZT) 0. A set of E2-treated and control animals was assigned to be sacrificed at ZT1 to detect the expression of transcription factors that is induced by the regular onset of light within the colony. A second set of E2-treated and control animals was exposed to light at ZT22, 2 h earlier then the regular

onset of light, and was sacrificed 1 h later at ZT23. The fixation and tissue preparation was performed in a similar fashion described above. Sections containing the SCN and the IGL from each animal were processed for immunocytochemistry using antibodies that detect the Fos, pCREB, egr-1, CBP, or calbindin-D (28K) proteins using standard protocols. Brain sections containing the DRN and MRN were processed with double labeling immunocytochemistry for Fos and 5-HT. After immunostaining, tissue sections were coded and analyzed using light microscopy. The DRN was divided into medial, lateral and caudal components for analysis. The medial region of the DRN was further divided into dorsal and ventral components.

Statistical analyses. All data are presented as mean ± s.e.m. The statistical confidence was set at $P \leq 0.05$ in all three experiments. The morphometric analysis was carried out in a blinded fashion.

Experiment 1. Statistical analysis was performed by unpaired t-test when applicable. F-test analysis was used with treatment (E2 vs. vehicle) as one factor. The Kruskal-Wallis one-way nonparametric analysis of variance was selected for multiple statistical comparisons. The Mann-Whitney U-test was used to determine significance of differences between groups.

Experiment 2. Longitudinal data were compared using factorial 2 x 2 ANOVA with treatment (testosterone vs. vehicle) as one factor and sex (female vs. male) as the second factor; or 2 x 2 x 2 ANOVAs with sex, treatment and SCN regions (core vs. shell). Fisher's LSD post-hoc analyses were performed as post-hoc tests when required.

Experiment 3. Factorial 2 x 2 ANOVA was used with treatment (E2 vs. cholesterol) as one factor and time of light onset (ZT0 vs. ZT22) as the second factor. Fisher's LSD post-hoc analyses were performed as post-hoc tests when required.

Results

Experiment 1; estrogen's effect on the energy homeostasis. First we studied axosomatic synapse type and number on POMC perikarya in ovariectomized wild-type female rats. We demonstrated that putative inhibitory synapses outnumbered excitatory contacts (35.29 ± 9.04 versus 10.75 ± 4.6 synapses per 100 μm of perikaryal membrane; $P < 0.05$), whereas estrogen treatment resulted in a dominance of excitatory contacts over inhibitory synapses (28.5 ± 7.25 versus 14.33 ± 3.65 synapses per 100 mm perikaryal membrane, $P < 0.05$). The shift in synaptic balance on POMC perikarya with a corresponding decrease in appetite and adiposity is similar towhat was previously observed in leptin-treated *ob/ob* mice. Almost all of the

asymmetric synapses on POMC perikarya were glutamatergic mediated by subunit 1 of the AMPA glutamate receptors (GluR1). Furthermore, we found that the estrogen regimen robustly elevated the number of arcuate parvalbumin-expressing cells, the vast majority of which were melanocortin cells. In parallel with the morphological effect on synapses, the number of c-Fos-positive POMC neurons in the ARC of wild-type animals nearly doubled 4 h after a single subcutaneous injection of E2 treatment.

Then we studied the dynamics of feeding behavior and synaptic rewiring induced by E2. Intracerebroventricular administration of E2 initiated the reduction of food intake 4 h after E2 treatment. In agreement with this, the induction of excitatory synapses on POMC perikarya was initiated 4 h (3.67 + 1.54 to 9.62 + 2.19 per 100 μm POMC perikaryal membrane) and peaked 6 h (20.65 + 3.35 per 100 μm POMC perikaryal membrane) after E2 administration. The number of symmetric, inhibitory synapses on POMC perikarya, however, was only slightly reduced with quicker response dynamics when compared to the change in asymmetric synapses (from 13.09 + 2.83, to 8.05 + 2.69 at 2 h and to 8.35 + 2.13 at 4 h, per 100 μm POMC perikaryal membrane after E2 treatment). This is consistent with previous observations on the temporal kinetics of leptin-induced arcuate synaptic plasticity.

To determine whether E2-induced synaptic changes on POMC neurons are reflected at the electrophysiological level, we studied miniature excitatory postsynaptic currents (mEPSCs) on the arcuate POMC neurons. POMC neurons from wild-type mice implanted with an E2 silastic capsule exhibited a 60% increase in mEPSCs when compared to those from control animals.

Next we studied the effect of estrogen in leptin mutant, *ob/ob* and *db/db*, animals. The mutant males and females were subjected to 4 weeks of estrogen treatment. We observed a strong, about 50% reduction in body weight gain in E2-treated mutants when compared to controls. The effect was similar in both sexes of *ob/ob* and *db/db* mice. The anorectic effect of E2 was detected within the first 16 h of E2 treatment, as was the reduction in the respiratory quotient, indicating that fat mobilization was increased. E2 replacement also increased energy expenditure. While fasting further reduced the energy expenditure of *ob/ob* mice, it had no significant effect on E2-treated mice. Consistent with this, E2 treatment increased basal body temperature and fully normalized the dysfunction of inducible thermogenesis in *ob/ob* and *db/db* mice: an overnight fast caused a >5°C reduction in body temperature in *ob/ob* and *db/db* animals, but E2 treatment reversed this phenotype. We also analyzed the synaptology of POMC neurons in the *ob/ob* and *db/db* mice with and without E2 treatment. POMC perikarya of *ob/ob* and *db/db* animals were dominated by symmetric, inhibitory contacts (11.93 ± 2.51 versus 3.42 ± 0.92 for *ob/ob*, and 12.12 ± 4.22 versus 3.3 ± 0.8 for *db/db*, per

100 μm POMC perikaryal membrane) with an overall low density of perikaryal synapses. The number of excitatory synapses on melanocortin perikarya, however, was significantly ($P < 0.01$) increased (from 3.42 ± 0.92 to 18.43 ± 4.24 for *ob/ob*, and from 3.3 ± 0.8 to 19.35 ± 2.45 for *db/db*, per 100 μm POMC perikaryal membrane), with the total number of synapses doubled in E2-treated animals. The increase in the number of synapses was due to changes in excitatory synapses only, presumably providing a high conductance 'hardware' that elevates the excitatory tone of POMC neurons. The number of inhibitory synapses, on the other hand, was not changed (11.93 ± 2.51 versus 12.66 ± 0.82 for *ob/ob*, and 12.12 ± 4.22 versus 13.45 ± 3.14 for *db/db*, per 100 μm POMC perikaryal membrane) by E2 treatment in obese mutants, which is different from the results of E2 treatment in ovariectomized wild-type animals.

To test whether E2 is able to activate the downstream target of leptin signaling, STAT3, in the hypothalamus, we intraperitoneally injected a water-soluble E2 in both wild-type and *ob/ob* mice. We observed an acute increase in p-STAT3 levels in the hypothalamus upon E2 administration. The phosphorylation levels were induced within 30 min and peaked at about 60 min after E2 injection. Chronic E2 administration with silastic capsule implants in a mouse model of brain STAT3 knockout had no effect on body weight reduction, whereas it had a potent effect in control mice. This later observation clearly suggests that the STAT3 activation is crucial for estrogen's effect in homeostasis.

We tested what effect, if any, elevated E2 levels had on POMC perikaryal synapses in ERα-null mice. Despite elevated levels of circulating E2 in ERα-null mice, the number of excitatory synapses on POMC perikarya in these mice (2.35 ± 0.25 per 100 μm POMC perikaryal membrane) was significantly lower than that in E2-treated *ob/ob* or E2-treated *db/db* mice (18.43 ± 4.24 and 19.35 ± 2.45, respectively, per 100 μm POMC perikaryal membrane; $P < 0.01$). In addition, the number of observable POMC-immunopositive neurons in the arcuate nucleus was significantly reduced in ERα-null animals compared to controls (32.6 versus 50.7 per section). The number of POMC neurons in the arcuate nucleus in ERα-null mice was comparable to that in *ob/ob* and *db/db* mice.

Experiment 2; estrogen's effects on the biological clock during early development. BrdU-labeled cells were observed across the rostro-caudal extent of the SCN. In the IGL, however, BrdU staining was sparse and appeared to label small nuclei, suggesting that BrdU labeled glia cells. To determine sex differences in the number of SCN cells that originate at the peak of the aromatase activity and that survive to adulthood, we counted the BrdU-labeled cells in the medial, caudal and rostral portion of the SCN. The SCN was further subdivided into core and the shell portions.

The counts from the medial portion of the SCN revealed that females had an overall higher number of BrdU-labeled cells than males ($F_{1,21}$ = 4.23, P = 0.05), and that TP decreased the overall number of BrdU-labeled cells ($F_{1,21}$ = 13.3, P < 0.05). A significant interaction effect ($F_{1,21}$ = 4.176, P = 0.05) suggested that the sex difference in BrdU staining was dependent on the prenatal presence of testosterone. Statistical analyses of cells counts within the core or shell produced similar results, in which oil-treated females had a significantly higher number of BrdU-labeled cells in both core and shell, and prenatal TP treatment reduced this number.

Similarly, the caudal portion of the SCN of oil-treated female rats contained significantly more BrdU-labeled cells than the same portion of SCN in male or in testosterone-treated animals (P < 0.05). As for other portions of the SCN, TP decreased the number of BrdU-labeled cells dramatically. Although testosterone decreased BrdU-labeled cells in the caudal SCN of both males and females, it appeared to have a stronger effect in females than in males. These overall effects were not differentially expressed within the SCN core or shell.

Interestingly, in contrast with the medial and caudal SCN, there were no significant sex differences in the number of BrdU-labeled cells per section of the rostral SCN. TP treatment, however, did significantly reduce the overall number of BrdU-labeled cells in this subregion of the SCN, and these effects were more evident in females than in males. Using a 2 x 2 x 2 ANOVA, with sex, treatment and SCN regions (core vs. shell), it was observed that the SCN core of males had a higher number of BrdU cells per section than females ($F_{1,17}$ = 8.79, P < 0.05), and that testosterone reduced this number overall ($F_{1,17}$ = 10.034, P < 0.05). A post-hoc one-way ANOVA showed that females exposed to testosterone in utero had a significantly lower number of BrdU-stained nuclei in the SCN than animals in the other groups. No significant differences were observed in the rostral SCN shell.

In order to determine the nature of the cells tagged with BrdU on embryonic day 18, tissue sections were processed for double immunocytochemistry for BrdU and other neurochemicals that are known to be expressed in the SCN. These assays demonstrated that BrdU-labeled cells within the SCN were heterogeneous in nature, with no specific group of cells proliferating at the time of BrdU injection. First, the location of BrdU-positive cells appeared to overlap that of previously reported AVP-immunoreactive cells in the SCN shell. Nevertheless, few AVP cells appeared to co-express BrdU. Second, calbindin-D (28K) immunoreactivity was found in the core and shell portions of the SCN, particularly in its medial portion across the anterior–posterior plane. We have shown that several calbindin-D (28K)-immunoreactive cells that co-expressed BrdU in their nuclei. However, not all calbindin-D (28K)-immunopositive cells expressed BrdU. Third, cells labeled for VIP were found in the ventrolateral portion of the SCN. These were particularly evident in the ventral

portion, embedded in the optic chiasm. A number of VIP-immunoreactive cells also appeared to express BrdU-immunoreactive nuclei. The majority of double-labeled VIP cells were found in the ventrolateral portion of the SCN core, and in VIP cells embedded in the optic chiasm. Fourth, BrdU immunoreactivity was not found within astrocytes that produce GFAP. In fact, GFAP-immunopositive astrocytes were more abundant in the ventromedial portion of the SCN, whereas BrdU-immunolabeled cells were located mostly on the dorsolateral portion of the SCN. Nevertheless, some GFAP-positive cells were seen surrounding cells labeled with BrdU.

Experiment 3; estrogen's effect on the biological clock during adulthood. First, we examined the ability of a phase shift to induce Fos immunreactivity in the SCN. Animals presented with light at ZT22, 2 h prior to the regular onset of light within the colony room, showed a significant increase in Fos-immunoreactivity in the SCN than those sacrificed after the regular onset of light (ZT0) ($F_{1,20} = 13.9$, $P < 0.05$).

Then we examined the E2 effect during this phase shift. E2-treated animals had an overall higher number of Fos-immunoreactive nuclei than animals given cholesterol. A significant interaction effect suggested that E2-treated rats presented with light at ZT22 had more Fos-immunreactive cells in the SCN than animals in the other groups ($F_{1,20} = 4.15$, $P = 0.05$). This was confirmed by a one-way ANOVA followed by a Fisher's LSD test ($F_{3,20} = 7.86$, $P < 0.05$).

The phase advance of 2 h increased the overall number of p-CREB-immunoreactiv cells ($F_{1,20} = 41.88$, $P < 0.05$). Although there was no overall E2 effect ($F_{1,20} = 4.49$, $P > 0.05$), there was a significant interaction effect ($F_{1,20} = 4.91$, $P < 0.05$). The post-hoc testing revealed a significant group effect ($F_{3,20} = 16.43$, $P < 0.05$), where E2-treated animals exposed to light at ZT22, had more p-CREB-positive cells than E2-treated animals exposed to light at ZT0, or cholesterol treated rats in either light condition.

The 2-h phase advance in the onset of light increased the number of calbindin-D (28K) ($F_{1,20} = 23.8$, $P < 0.05$), and egr-1 ($F_{1,20} = 11.22$, $P < 0.05$) immunoreactive cells in the SCN. E2 treatment, however, had no effect in the expression of either of these proteins in the SCN. Neither the phase advance nor E2 affected the expression of CBP in the SCN.

In contrast to the SCN, Fos-immunoreactivity was expressed at comparable levels in the IGL of OVX rats regardless of the hormone treatment, or the time at which they were presented with light on the day of sacrifice. The numbers of p-CREB- ($F_{1,20} = 40.52$, $P < 0.05$), and calbindin-D (28K)- ($F_{1,20} = 10.36$, $P < 0.05$) immunoreactive cells in the IGL were increased significantly by the 2-h phase advance in the onset of light. The expression of egr-1 and CBP

in the IGL was not altered by the phase advance. Finally the E2 treatment did not affect the expression of any of these proteins in the IGL.

We examined the ability of a phase shift to induce Fos immunreactivity in the DRN and in the MRN. Statistical analyses revealed that animals presented with light at ZT22, 2 h before the regular onset of light within the colony room, showed a significant increase in Fos expression in the medial, lateral and caudal regions of the DRN compared with those killed after the regular onset of light (ZT0). E2 treatment, however, had no effect on the expression of Fos protein in any regions of the DRN. In contrast to the DRN, neither the 2-h phase advance nor the E2 treatment affected the number of Fos-immunoreactive nuclei in the MRN.

To test whether estrogen enhances light-induced transcription in the SCN indirectly by modulating 5-HT release from the raphe into the SCN, we examined the ability of E2 to increase the proportion of 5-HT cells expressing Fos after a phase shift. Neither the phase shift nor the E2 treatment altered the number of 5-HT-immunolabelled cells in the DRN. However, double-labeling staining revealed that exposure to an advance in the onset of light (ZT22) significantly increased the percentage of 5-HT cells co-expressing Fos in the medial ($F_{1,20}$ = 12.54, $P < 0.05$), lateral ($F_{1,20}$ = 11.50, $P < 0.05$) and as well as in the caudal ($F_{1,20}$ = 4.74, $P < 0.05$) regions of the DRN compared with those killed after the regular onset of light (ZT0). E2-treated animals had an overall higher percentage of double-labeled 5-HT cells in the lateral DRN ($F_{1,20}$ = 6.03, $P < 0.05$) and a near significant higher percentage in the medial DRN ($F_{1,20}$ = 3.52, $P = 0.07$) but not in the caudal DRN ($F_{1,20}$ = 0.07, $P > 0.05$) compared with animals given cholesterol implants.

Conclusions

Our findings provided key evidences to better understand the mechanism of estrogen's effect on the central control of homeostasis and circadian rhythm, which might be highly relevant in the clinical setting. The followings are our most important conclusions.

Estrogen's effect on energy homeostasis.

1. We were first able to demonstrate that estrogen increases excitatory synapses on the hypothalamic POMC perikarya. This effect is similar what was seen after leptin administration to leptin deficient animals.
2. Almost all of these excitatory synapses are glutamatergic, the vast majority of them mediated by GluR1. These receptors are thought to be responsible for the specific induction of the calcium binding protein, parvalbumin.

3. In parallel with the morphological effect on synapses, estrogen enhances the activity of POMC neurons in the ARC.
4. Estrogen is able to change the synaptology of POMC neurons under physiological conditions as well during the rodent estrus cycle further supporting the notion, that synaptic plasticity has an element role in the hypothalamic regulatory systems.
5. We also clearly demonstrated, that the temporal kinetics of the synaptic rewiring induced by estrogen correlate well with the dynamics of the feeding behavior.
6. The estrogen-induced synaptic changes on POMC neurons are reflected also at the electrophysiological level. This finding is further supporting our hypothesis.
7. Furthermore, we demonstrated that estrogen-triggered anorexigenic behavior is completely independent of leptin.
8. One of our major discoveries is that estrogen mimics leptin's effect on the rewiring of POMC neurons and enhancing their tones. This finding further support the notion that the synaptic plasticity has a major role in the hypothalamic control of energy homeostasis.
9. In term of intracellular mechanism we have presented evidence, that estrogen activates the downstream target of leptin signaling, STAT3, independent of leptin.
10. And finally, our data strongly suggest, that estrogen's antiobesity effect on synapses of melanocortin system is mediated mostly by the ERα.

These observations unveiled, that the estrogen's antiobesity effect is mediated through a rapid synaptic changes of the hypothalamic feeding circuits independent of leptin, supporting the notion that the synaptic plasticity of ARC feeding circuits is an inherent element in the hypothalamic regulation of energy homeostasis. Our findings may offer alternative approaches to reducing adiposity under conditions of failed leptin receptor signaling.

Estrogen's effect on the biological clock during early development.

1. The number and the distribution of SCN neurons, which are newly originated during the late prenatal period, are gender specific. Female animals have more BrdU-labeled cells in the medial and caudal SCN than males. However, no sex differences are seen in the IGL.
2. Testosterone treatment during the late prenatal period reverses this sex difference in the SCN suggesting that the sex difference was dependent on the prenatal presence of testosterone and the degree of aromatisation. No such an effect was observed in the IGL.

3. By investigating the phenotypical characterization of these newly originated cells in the SCN, we showed that these cells expressing calbindin-D (28K), VIP and, to a lesser degree AVP, suggesting that these cells are primarily involved in the sex dimorphic functions of the circadian clock.

We have shown that the locally formed estrogen's developmental effect on the biological clock is, at least in part, responsible for the sexually dimorphism of the adult SCN, and taking place mostly late prenatally at the peak of aromatase activity in the brain. Our results unveiled a previously unknown effect of gonadal steroids on the developing SCN, which may contribute to the emergence of gender-specific circadian rhythms.

Estrogen's effects on the biological clock during adulthood.

1. Estrogen enhances light-induced expression of transcription factors in the SCN but not in the IGL.
2. Changes in the onset of light enhances the activity of the neurons in the raphe nuclei and this effect is modulated by estrogen.

Our observations demonstrated that estrogen modulates the light-induced activity of SCN neurons directly or indirectly through the raphe serotonin system. These date further support the notion, that estrogen is important for optimal responses of the biological clock to changes in environmental light.

The author's publications associated with the thesis

1. Abizaid A, **Mezei G**, Horvath TL. (2004) Estradiol enhances light-induced expression of transcription factors in the SCN. **Brain Res**, 1010: 35-44. **IF: 2.389**
2. Abizaid A, **Mezei G**, Sotonyi P, Horvath TL. (2004) Sex differences in adult suprachiasmatic nucleus neurons emerging late prenatally in rats. **Eur J Neurosci**, 19: 2488-2496. **IF: 3.820**
3. Abizaid A*****, **Mezei G***, Thanarajasingam G, Horvath TL. (2005) ***Co-first authors**. Estrogen enhances light-induced activation of dorsal raphe serotonergic neurons. **Eur J Neurosci**, 21: 1536-1546. **IF: 3.949**
4. Gao Q, **Mezei G**, Nie Y, Rao Y, Choi C.S, Bechmann I, Leranth C, Toran-Allerand D, Priest CA, Roberts JL, Gao XB, Mobbs C, Shulman GI, Diano S, Horvath TL. (2007) Anorectic estrogen mimics leptin,s effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. **Nat Med**, 13: 89-94. **IF: 26,382**

5. **Mezei G**, Rigo J Jr, Horvath TL. (2009) Estrogen as metabolic hormone. The role of estrogen in the hypothalamic regulation of energy homeostasis. **Metabolizmus**, 7: 111-114. Hungarian.

Abstracts

1. Abizaid A, **Mezei G**, Horvath TL. (2003) Estradiol enhances light induced fos immunoreactivity (Fos-IR) within the suprachiasmatic nucleus (SCN) of ovarectomized (OVX) female rats. Program No. 511.3 Neuroscience Abstract, New Orleans, LA, USA: Society of Neuroscience, 2003. Online IF: 8.306
2. Abizaid A, **Mezei G**, Horvath TL. (2003) Estradiol enhances neuronal activation in the SCN of female rats after an advance in the onset of light. Horm Behav, 44: 36. IF: 3.222
3. Abizaid A, Sotonyi P, Borok E, **Mezei G**, Horvath TL. (2003) Sexually dimorphic proliferation of cells in the suprachiasmatic nucleus (SCN). ENDO 2003. The Endocrine Society's 85th Annual Meeting, Philadelphia, PA, USA.
4. Qian G, **Mezei G**, Diano S, Horvath TL. (2004) Estradiol mimics leptin's effect on synaptic plasticity of POMC cells. ENDO 2004, The Endocrine Society's 86th Annual Meeting, New Orleans, LA, USA.

The author's publications not associated with the thesis

1. Marton T, Tanko A, **Mezei G**, Papp Z. (2001) Diagnosis of an unusual form of iniencephaly in the first trimester of pregnancy. **Ultrasound Obstet Gynecol**, 18: 549-551.
2. Papp C, Beke A, **Mezei G**, Toth-Pal E, Papp Z. (2002) Chorionic villus sampling: a 15-year experience. **Fetal Diagn Ther**, 17: 218-227. **IF: 1.053**
3. Papp C, Beke A, Ban Z, **Mezei G**, Toth-Pal E, Papp Z. (2003) Chorionic villus sampling: a 15-year experience. **Magy Noorv L**, 66: 271-282. Hungarian.
4. Szigeti Z, Toth-Pal E, Papp C, Beke A, Joo JG, Ban Z, **Mezei G**, Papp Z. (2004) Cytogenetic investigation of fetuses conceived by intracytoplasmatic sperm injection. **Prenat Diagn**, 24: 579-580.
5. **Mezei G**, Papp C, Toth-Pal E, Beke A, Papp Z. (2004) Factors influencing parental decision making in prenatal diagnosis of sex chromosome aneuploidy. **Obstet Gynecol**, 104: 94-101. **IF: 3.512**

6. Beke A, Papp C, Toth-Pal E, **Mezei G**, Oroszne NJ, Joo JG, Csaba A, Papp Z. (2004) Cytogenetic exploration of fetal ultrasound anomalies. **Orv Hetil**, 145: 2123-2133. Hungarian.
7. Joo JG, Beke A, Csaba A, **Mezei G**, Papp Z. (2004) Obstetric, prenatal diagnostic and phenotypechracteristics of holoprosencephaly. **Magy Noorv L**, 67: 3-10. Hungarian.
8. Szigeti Z, Toth-Pal E, Papp C, Beke A, Joo JG, Ban Z, **Mezei G,** Papp Z. (2004) Cytogenetic investigation of fetuses conceived by intracytoplasmatic sperm injection. **Magy Noorv L**, 67: 137-142. Hungarian.
9. Beke A, Papp C, Toth-Pal E, **Mezei G**, Oroszne NJ, Joo JG, Csaba A, Papp Z. (2005) Trisomies and other chromosome abnormalities detected following positive sonographic findings. **J Reprod Med**, 50: 675-691. **IF: 0.835**
10. Papp Z, **Mezei G**, Hidvegi J, Hupuczi P. (2005) Abdominal partial resection of the vagina and colpopexy: experiences with the procedure for posthysterectomy vault prolapse in 74 cases. **Orv Hetil**, 146: 2641-2645. Hungarian.
11. Papp C, Hajdu J, Beke A, **Mezei G**, Szigeti Z, Ban Z, Papp Z. (2006) Prenatal diagnosis of Turner syndrome. A report on 69 cases. **J Ultrasound Med**, 25: 711-717. **IF: 1.189**
12. Papp Z, **Mezei G**, Gavai M, Hupuczi P, Urbancsek J. (2006) Reproductive performance after transabdominal metroplasty: A review of 157 consecutive cases. **J Reprod Med**, 51: 544-552. **IF: 0.835**
13. Papp C, Ban Z, Szigeti Z, Hajdu J, Beke A, Toth-Pal E, Csaba A, Joo JG, **Mezei G**, Papp Z. (2006) The role of ultrasound examination in second trimester screening for fetal aneuploidies. **Orv Hetil**, 147: 2131-2137. Hungarian.
14. Papp C, Hajdu J, Beke A, Ban Z, Szigeti Z, Toth-Pal E, Csaba A, Joo JG, **Mezei G**, Papp Z. (2007) Prenatal diagnosis of Turner syndrome. A report on 69 cases. **Magy Noorv L**, 70: 37-43. Hungarian.
15. Papp C, Hajdu J, Beke A, Ban Z, Szigeti Z, Toth-Pal E, Csaba A, Joo JG, **Mezei G**, Papp Z. (2006) Prenatal diagnosis of trisomy 13: a report on 28 cases. **Magy Noorv L**, 69: 555-561. Hungarian.
16. Beke A, Barakonyi E, Belics Z, Joo JG, Csaba A, Papp C, Toth-Pal E, **Mezei G**, Ban Z, Papp Z. (2007) The risk of aneuploidies in cases of unilateral and bilateral choroids plexus cysts.. **Magy Noorv L**, 70: 29-36. Hungarian.
17. Szigeti Z, Hajdu J, Csapo Z, Toth-Pal E, Joo JG, Csaba A, **Mezei G**, Papp Z, Papp C. (2007) Correlation of prenatal ultrasound diagnosis and pathological findings

following induced abortions, in fetuses with trisomy 21. **Magy Noorv L**, 70: 219-227. Hungarian.

18. Szigeti Z, Hajdu J, Csapo Z, Toth-Pal E, Joo JG, Csaba A, **Mezei G**, Pete B, Papp Z. (2007) Quality control of prenatal sonography in detecting trisomy 18. The value of perinatal autopsy. **Magy Noorv L**, 70: 281-288. Hungarian.
19. Papp C, Hajdu J, Beke A, Ban Z, Szigeti Z, Joo JG, Csaba A, Toth-Pal E, **Mezei G**, Papp Z. (2007) Prenatal sonographic findings in 207 fetuses with trisomy 21. **Magy Noorv L**, 70: 83-90. Hungarian.
20. Papp C, Szigeti Z, Hajdu J, Csapo Z, Lazar L, Toth-Pal E, Joo JG, Csaba A, **Mezei G**, Papp Z. (2007) Correlation of prenatal ultrasound diagnosis and pathological findings following induced abortions, in fetuses with trisomies. **Gyermekgyogy**, 58: 422-428. Hungarian.
21. Joo JG, Beke A, Szigeti Z, Csaba A, **Mezei G**, Toth-Pal E, Rab A, Papp Z, Papp C. (2007) Role of fetopathological investigations in the clinical assessment of urinary tract malformations. **Magy Noorv L**, 70: 355-362. Hungarian.
22. Joo JG, Beke A, Toth-Pal E, Papp C, Szigeti Z, Csaba A, **Mezei G**, Lazar L, Papp Z. (2007) Major diagnostic and pathological features of iniencephaly based on twenty-four cases. **Magy Noorv L**, 70: 211-218. Hungarian.
23. Joo JG, Beke A, Szigeti Z, Csaba A, **Mezei G**, Toth-Pal E, Papp C. (2008) Craniospinal malformations in a twelve-year fetopathological study; the efficiency of ultrasonography in view of fetopathological investigations. **Early Hum Dev**, 84: 115-119. **IF: 1.850**
24. Joo JG, Beke A, Szigeti Z, Csaba A, **Mezei G**, Toth-Pal E, Papp Z, Papp C. (2008) Craniospinal malformations in a twelve-year fetopathological study; the efficiency of ultrasonography in view of fetopathological investigations**. Orv Hetil**, 149: 23-27. Hungarian.

Book chapters

1. Szigeti Z, Toth-Pal E, Papp C, Beke A, Joo JG, Ban Z, **Mezei G**, Papp Z. Cytogenetic investigation of fetuses conceived throught intracytoplasmatic sperm injection. In: Eds. Papp Z, Rodeck C. **Recent Advances in Prenatal Genetic Diagnosis**. Medimond, Bologna, 2004: 153-156.
2. Beke A, Barakonyi E, Belics Z, Joo JG, Csaba A, Papp C, Toth-Pal E, **Mezei G**, Ban Z, Papp Z. The risk of aneuploidies in cases of choroids plexus cysts. In: Eds. Kurjak A, Chervenak FA, **Perinatal Medicine**. Medimond, Bologna, 2005: 237-240.

Abstracts

1. Beke A, Papp C, Toth-Pal E, **Mezei G**, Oroszne NJ, Joo JG, Csaba A, Papp Z. (2002) Chromosomal abnormalities detected following positive ultrasound finding. Fetal Diagn Ther, 17 (Supp. 1): 47. IF: 1.053
2. Jenei K, Beke A, Csabay L, Sipos Z, Barakonyi E, Ban Z, Sziller I, **Mezei G**, Papp C, Toth-Pal E. (2002) Incidence of Toxoplasma and CMV infection in cases with positive ultrasound findings. Fetal Diagn Ther, 17 (Supp. 1): 47. IF: 1.053
3. Joo JG, Papp C, Toth-Pal E, Beke A., Csaba A, **Mezei G**, Papp Z. (2002) Successful enucleation of a necrotizing fibroid causing oligohydramnios and fetal postural deformity in the 25th week of gestation. Fetal Diagn Ther, 17 (Supp. 1): 52. IF: 1.053
4. **Mezei G**, Beke A, Toth-Pal E, Papp Cs, Papp Z. (2002) Factors influencing parental decision-making in prenatal diagnosis of sex chromosome aneuploidy: 12-year-experience. Fetal Diagn Ther, 17 (Supp. 1): 98. IF: 1.053
5. Beke A, Papp C, Toth-Pal E, **Mezei G**, Oroszne NJ, Joo JG, Csaba A, Papp Z. (2002) Cytogenetic exploration of fetal ultrasound anomalies. Nogyogyaszati és Szuleszeti Tovabbkepzo Szemle, 4 (Supp.): 25. Hungarian.
6. Jenei K, Beke A, Csabay L, Sipos Zs, Barakonyi E, Ban Z, Sziller I, **Mezei G**, Papp C, Toth-Pal E. (2002) Incidence of Toxoplasma and CMV infection in cases with positive ultrasound findings. Fetal Diagn Ther, Nogyogyaszati és Szuleszeti Tovabbkepzo Szemle, 4 (Supp.): 28.
7. **Mezei G**, Papp C, Toth-Pal E, Beke A, Papp Z. (2002) Factors influencing parental decision-making in prenatal diagnosis of sex chromosome aneuploidy: 12-year-experience. Nogyogyaszati és Szuleszeti Tovabbkepzo Szemle, 4 (Supp.): 29. Hungarian.
8. Joo JG, Papp C, Toth-Pal E, Beke A, Csaba A, **Mezei G**, Papp Z. (2002) Successful enucleation of a necrotizing fibroid causing oligohydramnios and fetal postural deformity in the 25th week of gestation. Nogyogyaszati és Szuleszeti Tovabbkepzo Szemle, 4 (Supp.): 110. Hungarian.
9. Papp C, Toth-Pal E, Beke A, **Mezei G**, Ban Z, Papp Z. (2003) Chorionic villus sampling: our experience. Ultrasound Obstet Gynecol, 22 (Supp. 1): 137. IF: 1.973
10. Beke A, Barakonyi E, Belics Z, Joo JG, Csaba A, Papp C, Toth-Pal E, **Mezei G**, Ban Z, Papp Z. (2005) The risk of aneuploidies in cases of choroids plexus cysts. J Perinat Med, 33 (Supp): 162. IF: 0.713

Number of peer-reviewed publications in international journals:	12 (**IF: 45.814**)
From these as a first author:	2 (**IF: 7.461**)
Number of peer-reviewed publications in Hungarian journals:	17
Number of book chapters:	2
Number of abstract presentations:	14 (IF: 18.426)
Cumulative impact factor:	**ΣIF: 64.440**