Dynamic changes of peptide-protein composition in aquatic animals underlying adaptation to environmental challenges

OTKA PD (#109099), **closing report**

Dr. Zsolt Pirger

Endocrine disrupting agents tend to be accumulated in various phase of natural ground waters due to the inadequate wastewater cleaning technologies, discharge of manufacturing waste effluents or unused drugs. Animals living under aquatic conditions are especially sensitive against these agents that affect their adaptive behaviour and homeostasis. Regrettably, environmental pollutants also accumulate in species of higher positions in the food chain. Humans are also facing the harmful effects of these pollutants, and the exposure itself is linked to a number of human reproductive, metabolic and cognitive diseases. Nowadays, the increasing concentration of progestogens and/or oestrogens in aquatic ecosystems, affecting the endocrine system and originating from synthetic oral contraceptives, is a priority issue of environmental protection. Nothing is more indicative of this than the fact that these agents have been put on the European Union watch list of emerging pollutants (Directive 2013/39/EU, European Parliament 2013).

Since we did not have information and data about the progestogen and oestrogen contamination of pilot area, first we need to measure their concentrations in the initiative period of my OTKA. We have described 0.23–13.67 ng/L progestogens (progesterone [PRG], drospirenone [DRO], levonorgestrel [LNG], gestaden [GES]) and 0.03-5.87 ng/L oestrogens (β-estradiol [E2], ethinyl-estradiol [EE2]) in natural waters on the catchment area of the largest shallow lake of Central Europe, Lake Balaton.

1) Avar P, Maasz G, Takács P, Lovas S, Zrinyi Z, Svigruha R, Takátsy A, Tóth LG, **Pirger Z**
   HPLC-MS/MS analysis of steroid hormones in environmental water samples
   DRUG TESTING AND ANALYSIS **8:**(1) pp. 124-128. (2016)
2) Avar P, Zrínyi Z, Maász G, Takátsy A, Lovas S, G.-Tóth L, **Pirger Z**
   β-estradiol and ethinyl-estradiol contamination in the rivers of the Carpathian Basin
   ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH **23**:(12) pp. 11630-11638. (2016)

Since the physiological effects of progestogens are still largely unknown in fresh water animals, I focused on this question in my investigations during the final period of my OTKA research program. Progestogen contaminants act as potent steroids with mixed progestagenic, androgenic and mild estrogenic effects through membrane and/or nuclear receptors that is why the general aim was to investigate the complex physiological (behavioural, development, neurobiological) effects of a mixture of progestogens (PRG, DRO, LNG, GES) in different exposure concentration (1, 10, 100 ng/L), using treated and control groups of pond snail (*Lymnaea stagnalis*) and common roach (*Rutilus rutilus*).

a) Behavioural tests were performed studying the effects of progestogens on feeding and respiration. Subsequently, intracellular electrophysiological recordings were carried out on various components (modulatory interneurons, motoneurons, central pattern generator [CPG] cells) of the identified neuronal networks of *Lymnaea* responsible for controlling feeding and respiration. At the behavioural level, snails systematically injected with mixture of progestogens displayed a significantly lower feeding (rasping) activity to sucrose that was

used as a feeding stimulus, compared to control animals. Mixture of progestogens also altered various characteristics of respiration. The start of respiration was significantly delayed in the 100 ng/L treatment group and the number of pneumostome openings showed a significant decrease in the 1 ng/L treated group. Additionally, the mean pneumostome opening time was prolonged in both treated groups. There were no significant differences in the total pneumostome time between the treated and control groups. At the cellular level, it was found that hormone treatments decreased the firing frequency of the feeding modulatory interneurons (cerebral giant cells – CGCs) via increasing the amplitude of their Ca^{2+} currents. This effect seemed to be cell type specific, since in the case of the respiratory CPG cells (right pedal-dorsal1 cells – RPeD1) there were no such changes. Feeding motoneurons (buccal cells – B1-B3) also showed decreased activity during fictive feeding induced, since their responsiveness to external sucrose stimulus was reduced.

Publication and conferences in this topic supported by OTKA:

3) Lovas S, Kiss T, **Pirger Z.**
   Behavioural and electrophysiological effects of chronic progestogen exposure on identified feeding and respiratory *Lymnaea* neurons
   eLIFE – under review (2016)
4) Lovas S, Kiss T, **Pirger Z**. - Changes of electrical characteristics induced by priority pollutant hormones in *Lymnaea* identified neurons – IBRO Workshop, poster presentation, 2016, Budapest
5) **Pirger Z**, Kiss T, Lovas S. - Changes of electrical characteristics induced by priority pollutant hormones in *Lymnaea* identified neurons – XII. International Congress of Neuroethology (ICN2016), poster presentation, 2016, Montevideo, Uruguay (partly supported my NAP project)
6) **Pirger Z.** - From neurons to behaviour: complex neuronal changes of *Lymnaea* to exposure of progestogen pharmaceuticals – XI East European Conference of the International Society for Invertebrate Neurobiology -Simple Nervous System, invited plenary speaker, 2016, Moszkva-Zvenigorod, Oroszország

b) Parallel with electrophysiological experiments, I have investigated the reproduction and development both at system level (such as mortality, laid egg production, vitellogenin [VTG] content, egg sac quality), and cellular/molecular level (such as time window of cell division, adenylate energy charge [AEC], hexose utilization, redox state, metabolites) on *Lymnaea* treated with mixture of progestogens for 21 days. Data were analysed using ELISA, stereo microscopy and capillary microsampling combined mass spectrometry, the latter as a novel technique developed and applied by us. According to our data, the treatment of adults caused elevated mortality, reduced egg production and low quality egg sacs on the first week, compared to the control. From the second week, the egg production and the quality of egg sac were similar in both groups. At the end of the third week, the egg production and VTG content of hepatopancreas were significantly elevated in the hormone treated group. At cellular level an accelerated cell proliferation was observed during early embryogenesis. In the course of these experiments, the metabolomic pattern of the single-cell zygote of *Lymnaea* was also investigated, and it was established the 26 identified metabolites were contained by the fertilized and newly laid oocyte. Based on our MS data, differences in molecular content of single-cell zygote were not observed between eggs obtained from control and treated adults. Out of the identified molecules, we focused onto AMP, ADP, ATP, UDP-hexNAc as well as UDP-hex. Their relative ratio in single-cell zygote stage the molecular endpoints

regarding AEC and hexose utilization were calculated from equations. The calculated and observed molecular ratios (elevated hexose utilization in cytoplasm, elevated adenylate energy charge in egg albumen) suggest that treated snails provided more glucose for eggs in order to improve the offspring viability. The metabolomic content of laid egg albumen was also examined and 21 metabolites were identified in them. Two developmental stages were investigated: 1 hour after egg laying (single-cell zygote) and after 96 hours (at the beginning of the metamorphosis, which is the halftime of the average hatching time). Differences in molecular pattern were not observed between 1-hour and 96-hour old egg albumen contents as well as between the control and the 10 ng/L treated groups. AEC as well as redox state was investigated which is the ratio of NADH and NAD+ molecules. The AEC did not alter in the treated group compared to the control within 1 hour after egg laying, but after 96 hours the difference was already significant. The redox state did not change in the treated group compared to the control either in 1-hour old, or in 96-hour old egg albumen. Modelling the environmental contaminations, we conclude that mixture of progestogens may perturb regulatory processes and development of freshwater species and *Lymnaea* can be indicative of it, based on the experimental evidences presented.

Publication and conferences in this topic supported by OTKA:

7) Zrinyi Z, Maasz G, Zhang L, Vertes A, Elekes K, **Pirger Z**
   Effect of progesterone and its synthetic analogues on reproduction and embryonic development of a freshwater invertebrate model
   SCIENCE OF TOTAL ENVIRONMENT – under review (2016)
8) **Pirger Z.** Szteroid szennyezők hatásának vizsgálata mikro-mennyiségű minták esetében. – XII. Környezetvédelmi Analitikai és Technológiai Konferencia, előadás, 2015, Balatonszárszó
9) Zrinyi Z, Zhang L, Vertes A, Maasz G, **Pirger Z** - Capillary-microsampling combined MS techniques: An easy way to giving answer to biological questions – 34. Informal Meeting on Mass Spectrometry (IMMS2016), poster presentation, 2016, Fiera di Primiero, Olaszország
10) Zrinyi Z, Zhang L, Maasz G, Vertes A, **Pirger Z** - Progesztogének hatása a nagy mocsári csiga szaporodására és embrionális fejlődésére – Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok Közös Tudományos Konferenciája (FAME2016), poszter, 2016, Pécs

c) As mentioned in the Introduction, in our experiments I have investigated the morphological and molecular effects of mixture of progestogens in different exposure concentrations in common roach. Based on literature data, steroids (e.g. progestogens) and the protein deglycase DJ-1 chaperon protein aim the same target molecules in cells, therefore, I hypothesized that a relationship may exist between progestogens and DJ-1. Furthermore, my aim was to follow the changes of signal molecules of different biological function due to progestogen treatments in serum and brain of fish. Adult roaches were exposed to 10, 50 and 500 ng/L of mixture of progestogen for 42 days and their somatic indices (brain-somatic, liver-somatic, gonadosomatic and kidney-somatic) were measured. VTG expression (oestrogen effect) or inhibition (androgen effect) in fish is a widely used biomarker so I measured its changes in liver by ELISA. To determine the quantity and to map the spatial distribution of DJ-1 chaperon protein the brain and liver tissues were analysed by ELISA and immunohistochemistry. Furthermore, we also studied molecular alterations a) in the serum by measuring cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and

triglyceride concentrations, and b) in brain homogenate using a cell stress array kit (26 proteins). The level of 26 proteins was measured parallel before and after progestogen treatment. These proteins are involved in various biological processes such as: metabolic (FABP-1; PON1; PON3), apoptotic (BCL-2; Cytochrome c; Phospho-JNK Pan; Phospho-p38a), antioxidative (PON2; Thioredoxin-1; SIRT2; SOD2), stress (HIF-1 alpha; HIF-2alpha; Phospho-HSP27; HSP60; HSP70; NFKB1; Phospho-p53), cell differentiation (ADAMTS1; Cited-2; Dkk-4; p21/CIP1; p27), catalytic (Carbonic anhydrase IX; IDO) or anti-inflammatory (COX-2) events. The somatic index of liver and kidney significantly changed in all the treated groups, whereas the gonadosomatic index of 500 ng/L treated group showed significant decrease compared to control animals. VTG level increased significantly in 500 ng/L progestogen treated group. Since the concentration of DJ-1 significantly increased in brain and liver in all progestogen treatment groups, the DJ-1 protein could be able to a more sensitive marker than VTG. Serum LDL and cholesterol levels of exposed fish were significantly decreased. DJ-1 was mediated through the stimulation of the expression of LDL-receptor which facilitates reuptake subsequently. The level of PON3, Phospho-p38a, SIRT2, SOD2, HIF-1 alpha, HIF-2alpha, HSP70 and p27 proteins were markedly increased. In summary, our observations provided new data about molecular alterations induced by the combined action of environmental progestogens. In addition, the DJ-1 chaperon protein as a possible biomarker helped trace the abiotic chemical environmental contaminations, like progestogens in the freshwater ecosystems.

Publication and conferences in this topic supported by OTKA:

11) Maasz G, Zrinyi Z, Takacs P, **Pirger Z.**
Complex molecular changes induced by chronic progestogens exposure in roach, *Rutilus rutilus*
ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY – under review (2016)

12) Maász G, Zrínyi Z, Takács P, Lovas S, Fodor I, Kiss T, **Pirger Z** - Krónikus progesztogén terhelés indukálta molekuláris, szövettani és szomatikus index változások az őshonos búzaszemű keszegen (*Rutilus rutilus*) – Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok Közös Tudományos Konferenciája (FAME2016), poszter, 2016, Pécs

Overall, some of the progestogens have also been found in freshwaters and drinking water, which is a warning sign that the current handling of pharmaceuticals may lead to future health and environmental problems. My analysis showed that the mixture of progestogens directly affects the physiological function both at the system and the cellular levels in invertebrate and vertebrate freshwater species. It is suggested that the currently low (few ng/L) progestogen concentrations represent already a biological risk in the catchment area of the largest shallow lake in Central Europe both presently and future.

Others:

During my OTKA period, I have built up a new neuro-analytical laboratory, I have won a National Brain Research Project (No. KTIA_NAP_13-2-2014-0006) and Bolyai Foundation (No. BO/00952/16/8), I have written a review about the occurrence, distribution and physiological role of PACAP in invertebrates (*Pirger et al., 2016*) and two research paper about its neuroprotective function in dopamine-based neurodegeneration (*Maasz et al., 2016*; *Maasz et al., 2014*). In addition, I have investigated the relevance of PACAP and insulin-like

growth factor-1 (IGF-1) as neuropeptides in aging. Due to the evolutionarily conserved nature of these polypeptides and their established role in memory and synaptic plasticity, there is a very high probability that they could also act as "memory rejuvenating" agents in humans (*Pirger et al., 2014*). As well as, based on my general aim of my OTKA research program the role of hibernation in neurophysiology and adaptation of snail (*Helix pomatia*) were also investigated and described (*Kiss et al., 2014*).
Furthermore, I have participated as a key group member in the preparation of GINOP and KEHOP research proposals (under decision). I also performed tutorial activity in this third period, including 2 postdocs, 2 PhD students and 2 MsC students (Gábor Maasz, Péter Avar, Sándor Lovas, Zita Zrínyi, Réka Svigruha, István Fodor) who were also involved in the above mentioned experimental works.

The number of my OTKA research program is also list in relevant part of these publications:

Pirger Z, Krajcs N and Kiss T: Occurrence, Distribution, and Physiological Function of Pituitary Adenylyl Cyclase-Activating Polypeptide in Invertebrate Species – Chapter 2 in *Springer International Publishing Switzerland* 2016, D. Reglodi, A. Tamas (eds.), Pituitary Adenylate Cyclase Activating Polypeptide - PACAP – ***Current Topics in Neurotoxicity 11***, ISBN: 978-3-319-35133-9, DOI 10.1007/978-3-319-35135-3_2

Maasz, G., Zrinyi, Z., Reglodi, D., Petrovics, D., Kiss, T., Tamas, A., **Pirger, Z**: Pituitary adenylate cyclase-activating polypeptide (PACAP) has neuroprotective function in dopamine-based neurodegeneration developed in two parkinsonian models – ***Disease Models and Mechanisms*** – under minor revision, (2016) **IF=4.978**

Maasz, G., **Pirger Z**, Reglodi D, Petrovics D, Schmidt J, Kiss P, Rivnyak A, Hashimoto H, Avar P, Jambor E, Tamas A, Mark L: Comparative protein composition of the brains of PACAP-deficient mice using mass spectrometry-based proteomic analysis – ***Journal of Molecular Neurosciences***, 54(3):310-9 (2014) **IF=2.534**

Pirger Z, Naskar S, László Z, Kemenes G, Reglődi D, Kemenes I.: Reversal of age-related learning deficiency by the vertebrate PACAP and IGF-1 in a novel invertebrate model of aging: the pond snail (*Lymnaea stagnalis*) – ***Journals of Gerontology Series A: Biological Sciences and Medical Sciences***, 69(11):1331-8 (2014) **IF=4.984**

Kiss T, Battonyai I, **Pirger Z**.: Down regulation of sodium channels in the central nervous system of hibernating snails – ***Physiology and Behavior,*** 131:93-8 (2014) **IF=3.150**