Novel signaling mechanisms of GPCRs

Plasma membrane G protein-coupled receptors (GPCR) play an essential role in numerous hormonal and local control mechanisms, which are frequently affected in pathologic situations. In the present project, we have focused on the investigation of the type 1 angiotensin receptor (AT1R), type 1 cannabinoid receptor (CB1R), adrenergic receptors (AR), and V2 vasopressin receptor (V2R).

The AT1 angiotensin receptor (AT1R) mediates the most important effects of the octapeptide hormone angiotensin II, including the regulation of blood pressure and salt-water homeostasis, and development of pathological conditions, such as hypertension, heart failure, cardiovascular remodeling, renal fibrosis, inflammation, and metabolic disorders. The signal transduction of AT1R is mediated mainly by G proteins and β-arrestin proteins. In recent years, β-arrestins have become a main focus of pharmacological research. β-arrestins are central regulators and signal transducers of the G protein-coupled receptors (GPCRs), they mediate the desensitization of G protein signaling, initiate the endocytosis of GPCRs and, as scaffold proteins, regulate the activity of hundreds of signaling proteins. While G protein signaling of AT1R has been associated with the most of its adverse effects, such as pathological vascular and heart remodeling or hypertension, β-arrestins have been shown to play key role in beneficial effects of AT1R, including improvement of heart muscle contractility and protection of renal function, as it was further detailed in two review articles of our group **(Tóth AD et al, BPRCEM 2018*; Turu G et al, Front Endocrinol 2019*)**.

Some ligands of AT1R are able to discriminate between the activation of distinct signaling pathways, therefore they are called biased ligands. Several β-arrestin-biased peptide ligands have been developed, and one of them, TRV120027, was tested in clinical trials, however a phase II trial failed to show its benefit in acute heart failure patients. Nevertheless, biased AT1R ligands still have great potential in other diseases, such as chronic heart failure. Since the deeper knowledge of signaling of β-arrestin-biased ligands can aid the development of drugs with better side effect profiles, we established several novel experimental systems in order to study the mechanism of action of biased ligands. We hypothesized that biased ligands may differently affect the function of receptor heterodimers. To test this hypothesis, we examined the function of the β2 adrenergic receptor (β2AR)-AT1R heterodimer. Since there are controversial data about the existence of receptor heterodimers, first we verified the physical interaction between β2AR and AT1R with an approach that was developed in our laboratory (Szalai B et al PLoS One 2014). Using a BRET-based system, we found that β-arrestin binding of β2AR is significantly influenced by the AT1R. Simultaneous activation of AT1R and β2AR greatly increased the binding between β2AR and β-arrestins, and prolonged the lifetime of β-arrestin2 clusters at the clathrin coated pits. We also found different effects between conventional AT1R antagonists and β-arrestin-biased AT1R agonists. Whereas candesartan had no effect on the interaction between β2AR and β-arrestin2, the β-arrestin biased agonist TRV120023 significantly increased the binding. These results suggest that β-arrestin-mediated crosstalk between β2AR and AT1R may play an important role in the pharmacological effects of biased AT1R agonists **(Tóth AD et al, Mol Cell Endol 2017*)**.

We also investigated novel features of β-arrestin and GPCR interactions. We demonstrated that activation of protein kinase C by phorbol myristate acetate, stimulation of

Gq/11-coupled GPCRs or the tyrosine kinase receptor epidermal growth factor receptor (EGFR) induces β-arrestin2 recruitment to AT1R even in the absence of its agonist. We also showed that endogenous purinergic receptors can evoke the same effect, proving that the interaction can be triggered at physiological levels of PKC activation. We found that this heterologous mechanism of β-arrestin binding to AT1R did not need the active state of the AT1R, but was dependent on a structure, which we named as the stability lock. The stability lock is formed between phosphorylated C-terminal serine-threonine clusters on the receptor and two conserved lysines in the N-domain of β-arrestin2. We have generated improved FlAsH-based β-arrestin2 conformational BRET biosensors to follow the conformational transition of β-arrestin2 during its activation. We found that β-arrestin2 is stabilized in distinct active conformations upon homologous or heterologous activation, and we also showed that the stability lock not only stabilizes the receptor-β-arrestin interaction, but also governs the structural rearrangements within β-arrestins. This suggests that, besides C-terminal phosphorylation pattern, the conformational state of the receptor core has also a prominent role in governing the structural rearrangements in β-arrestins, thereby representing an important part of the receptor barcode. Several previous studies have demonstrated the central role of β-arrestin2 in mediating the MAPK signaling of AT1R. Interestingly, we found that the PKC-activated β-arrestin2 binding to AT1R also led to the recruitment of MEK1 and ERK2 to the AT1R, suggesting that the binding of β-arrestin to the receptor per se is necessary and sufficient for the AT1R-β-arrestin2-MAPK complex formation. We found that α1A adrenergic receptor stimulation also induced the assembly of AT1R-β-arrestin2-MAPK complexes. The formation of the AT1R-βarrestin2-MEK-ERK complex induced by another receptor represents an appealing mechanism by which a GPCR acts as a scaffolding protein rather than the initiator of the signal. These results demonstrated that β-arrestin2 can bind to the same GPCR in distinct active conformations, and revealed a not before known mechanism of β-arrestin activation and highlighted the key role of β-arrestin in receptor cross-talk **(Tóth AD et al, J Biol Chem 2018*)**.

In a previous study (Szakadati G et al, Mol Pharmacol 2015) we demonstrated that distinct ligands of AT1R evoke β-arrestin binding to different extents, but the underlying molecular mechanism is not known. We speculated that ligand affinity, and especially the ligand dissociation rate constant, may contribute to the efficacy of the ligands, although efficacy and affinity are generally thought to be independent parameters. To precisely measure the ligand binding of AT1R, we have established a novel approach for sensitive quantification of receptor-ligand interaction. Although several methods were already available, all other approaches suffered from great limitations. Resonance energy transfer-based methods allow the detection of ligand binding to receptors with high temporal resolution and low signal-to-noise ratio, but they are hindered by the necessity of genetic modification of the receptor of interest, which can greatly alter the receptor function. To overcome this obstacle, we developed a novel system to measure ligand–receptor binding without the need of receptor tagging using a Gaussia luciferase (GLuc)-based cell surface biosensor. GLuc has outstanding brightness, and its proper substrate is the commonly applied and easily accessible coelenterazine, which makes the system cost-efficient. We deposited the GLuc-based cell surface biosensor in the Addgene plasmid depository, thus researchers from all over the world can easily access this novel tool. In our method, HEK 293 cells are transfected with the unmodified receptor of interest and the GLuc-

based biosensor. Thereafter, bystander BRET is measured between the biosensor and fluorescent ligands bound to their unmodified receptors in living cells. The method can be used both for equilibrium and kinetic ligand binding measurements, and allows the determination of the ligand binding parameters of both labeled and competitive unlabeled ligands. Furthermore, the assay can be used for any plasma membrane receptor, if a fluorescent ligand is available. We also demonstrated that the method can also be applied for high-throughput screening. In a screening for α1 adrenergic receptor ligands, we identified four novel α1 adrenergic receptor antagonists. In the future, we aim to exploit the assay for the identification of biased α1 adrenergic receptor ligands **(Tóth AD et al, J Biol Chem 2021*)**.

Endocannabinoids have been first identified in the neural tissue as serving retrograde synaptic modulation. These compounds have been revealed to play a role in several physiological and pathological mechanisms inside and outside the central neural system. We have previously reported that activation of Gq/11 protein-coupled receptors can cause the release of 2-AG endocannabinoid by the activation of diacylglycerol (DAG) lipase, which acts on CB1R in cell expression systems (Turu G, et al. J Biol Chem. 2009). As a next step we have identified these mechanisms in isolated vessels. This mechanism is activated in the vessel wall (aorta, isolated from rats and mice) by some GRCR agonist hormones (e.g. angiotensin II, phenylephrine) significantly modulating vascular functions by attenuating vasoconstriction via the activation of CB1R. We have also found that in response to Ang II, 2-AG endocannabinoid was released from rat aortic vascular smooth muscle cells, which effect was inhibited by tetrahidrolipstatin, a DAG lipase inhibitor (Szekeres M, et al. Mol Cell Endocrinol, 2015). We addressed the question, if the endogenous cannabinoid mechanism plays a role in the control of the tone and the contraction-relaxation responses of coronary arteries. Segments of the left anterior descending coronary artery (~100-150 μm in diameter) were isolated and subjected to pressure-microarteriography. CB1R protein was detected by immunohistochemistry and by Western blot, its mRNA was identified with qRT-PCR. Vascular contraction in the presence of agonists and antagonists affecting endocannabinoid production have been tested. Segments developed spontaneous, myogenic contraction, from which a substantial relaxation in response to WIN55212 (a specific agonist of the CB1Rs) has been found. Relaxation was blocked by O2050 and AM251 (neutral antagonist and inverse agonist of the CB1R, respectively). Spontaneous tone was enhanced by and by tetrahydrolipstatin (THL, a DAG lipase blocker). Ang II-induced contraction was augmented by O2050, AM251 and THL, while bradykinin (BK, an endothelium-dependent coronary vasodilator) or sodium nitroprusside (SNP, an NO donor) dilations were not affected. These observations provide evidence that endocannabinoid production is a significant modulator of the myogenic and agonist-induced contraction of intramural coronary arterial system. We suggest, that in addition to the known physiological mechanisms to reduce the substantial spontaneous/myogenic tone of coronary resistance vessels (metabolic factors, beta adrenergic stimuli, vasodilatory prostanoids and endothelial NO) the endogenous cannabinoids which may also have a therapeutic potential in coronary artery diseases **(Szekeres M et al. Prostaglandins Other Lipid Mediat., 2018*)**.

In collaboration with Zsuzsanna Miklós, we have also investigated the role of Ang II-induced endocannabinoid signaling in the heart. In this study we aimed to investigate whether cardiac Ang II effects are modulated by 2-AG-release and to identify the role of CB1R in these effects. Expression of CB1R in rat cardiac tissue was confirmed by immunohistochemistry.

Thus, repeated Ang II-infusions were applied by increasing concentrations into isolated Langendorff-perfused rat hearts. Ang II infusions caused a decrease in coronary flow and ventricular inotropy. CB receptor agonist 2-AG and WIN55,212-2 administration to the perfusate enhanced coronary flow. The flow-reducing effect of Ang II was moderated in the presence of CB1R blocker O2050 and also by Orlistst, a DAG-lipase inhibitor. Our findings indicate that Ang II-induced cardiac effects are modulated by simultaneous CB1R-activation, likely due to 2-AG-release during Ang II-induced signaling. In this combined effect, the response to 2-AG via cardiac CB1R may counteract the positive inotropic effect of Ang II, which may decrease metabolic demand and augment Ang II-induced coronary vasoconstriction **(Miklos Z et al. Cells, 2021*)**.

In the next series of experiments, we were aiming to improve our delivery of GPCR and other sequences into cells using a combination of adeno-associated viruses (AAV) and extracellular vesicles (EVs), which are safe and efficient vectors for gene transfer. EV-associated AAVs are potentially efficient vectors considering that they carry the benefits of both AAVs and EVs. We tested vesicle-associated AAVs and vesicles mixed with AAVs on two major cell types: a neural cell line (N2A) and primary astrocyte cells. In contrast to previously published in vivo observations, the extracellular vesicle packaging did not improve the infection capacity of the AAV particles. Our results suggest that the *in vivo* observed improved efficacy of the EV-associated AAV particles is most likely due to the enhanced delivery of the AAVs through tissue barriers and/or to the shielding of AAVs from neutralizing antibodies **(Kovács OT et al. Int J Mol Sci, 2021*)**.

We have established collaborations to study vascular effects of GPCR ligands using methodologies involving myography and pressure microarteriography in our lab. In cooperation with professor Akos Koller (New York Medical College-Semmelweis University) we have investigated the vascular hormonal signaling functions in regular physical exercise on coronary arteries and the effect of an TxA2 GPCR receptor agonist. In a study on coronary arteries, we have performed functional studies concerning vascular responses to regular exercise. Exercise elicits early adaptation of rat coronary arteries enabling the coronary circulation to respond adequately to higher flow demands. We hypothesized that short-term daily exercise induces biomechanical and functional remodeling of the coronary resistance arteries related to pressure. We have performed 4-week treadmill experiments on male rats and coronary arterial function was investigated with pressure microarteriography. Arteries from exercised rats had thicker walls, higher myogenic response, reduced stress, less constrictor prostanoid modulation and higher NO-dependent vascular dilation abilities. These results indicate that daily exercise induces remodeling of rat intramural coronary arterioles, is likely resulting in a greater range of coronary autoregulatory function (constrictor and dilator reserves) **(Szekeres M et al. Journal of Vascular Research, 2018*)**.

In cooperation with Dr. Szabolcs Várbíró (Department of Obstetrics and Gynecology, Semmelweis University) we have performed vascular studies in a polycystic ovary disease (PCOS) model and in vitamin D-deficient rat model to investigate the vascular effects of vitamin D-therapy. In these studies, the contraction induced by phenylephrine (Phe), a GPCR alfa-adrenergic receptor agonist, and the modulatory effect of Vitamin D-state and sex hormones were studied. We have performed vascular functional studies by myography on renal arteries investigating the role of vitamin D deficiency on vascular functions. Since Vitamin D

deficiency shows positive correlation to cardiovascular risk, which might be influenced by gender specific features. Our goal was to examine the effect of Vitamin D supplementation and Vitamin D deficiency in male and female rats on renal artery. Rats were fed with Vitamin D reduced chow for eight weeks to induce hypovitaminosis. Another group of animals received normal chow with further supplementation to reach optimal serum vitamin levels. Isolated renal arteries of Vitamin D deficient female rats showed increased Phe-induced contraction. Inhibition of cyclooxygenase by indomethacin decreased the Phe-induced contraction. Ang II-induced contraction was pronounced in Vitamin D supplemented males. In both Vitamin D deficient groups, Ach-induced relaxation was impaired. Increased elastic fiber density and decreased eNOS immunostaining was observed in Vitamin D deficient females. The density of AT1 receptor staining was the highest in the male Vitamin D deficient group. These results suggest that vitamin D deficiency induces renal vascular impairment in both sexes, though in males AT1 receptor-upregulation, in females NO-depression is dominant. GPCR receptor function can s modified by the VitD status and sex hormones **(Sipos M et al. Nutrients, 2021*)**.

We have also investigated segmental remodeling of coronary resistance arteries, inhibition of angiogenetic processes, their rarefaction induced by angiotensin II-infused hypertension on the alterations in resistance artery network geometry. Female rats were infused with 100 ng/kg/min Ang II with osmotic minipumps for four weeks that raised mean arterial blood pressure. Geometry of the left coronary artery system was studied on plastic casts and on in situ microsurgically prepared, saline pressure-perfused video-microscoped networks. We have found that bifurcation angles increased with increasing asymmetry of daughter branches but not in hypertensives. Dividing the whole network peaked at 200μm diameter in normal networks. This peak diminished and was replaced by a peak at 300μm in hypertensives. Higher blood flow routes were needed to cover the same distance from the orifice. Shrinkage of many parallel connected 200μm segments, concomitant enlargement of many larger segments accompanied with morphological deformities can be expected to contribute to elevated vascular resistance. This observation indicates significant alterations of coronary network properties in Ang II-induced hypertension in addition to the earlier described processes of hypertrophic and homeotrophic wall remodeling and simple rarefaction **(Monori-Kiss A et al. Heliyon, 2020*)**.

The urine concentrating function of the kidney is essential to maintain the water homeostasis of the human body. It is mainly regulated by the arginine vasopressin (AVP), which targets in the kidney the type 2 vasopressin receptor (V2R). The V2R is a member of the GPCR superfamily The inability of V2R to respond to AVP stimulation leads to decreased urine concentration and congenital nephrogenic diabetes insipidus (NDI). NDI is characterized by polyuria, polydipsia, and hyposthenuria. In the present project, we identified a point mutation (S127F) in the AVPR2 gene of an NDI patient, and we characterized the impaired function of V2R in HEK293 cells. Based on our data, the S127F-V2R mutant is almost exclusively located intracellularly in the endoplasmic reticulum (ER), and very few receptors were detected in the cell surface, where the receptor can bind to AVP. In line with the minimal plasma membrane localization, the overexpressed S127F-V2R mutant receptor has negligible cAMP generation capability compared to the wild-type receptor in response to AVP stimulation. Since certain misfolded mutant proteins, that are retained in the ER, can be rescued by pharmacological chaperones, we examined the potential rescue effects of two pharmacochaperones on the S127F-V2R. Although, a significant increase in cell surface expression was not detectable using

cytometry or microscopy, we found that pretreatment with both tolvaptan (a V2R inverse agonist) and MCF14 compound (a cell-permeable high-affinity agonist for the V2R) were capable of partially restoring the cAMP generating function of the receptor in response to vasopressin stimulation. According to our data, both cell permeant agonists and antagonists can function as pharmacochaperones, and serve as the starting compounds to develop medicines for patients carrying the S127F mutation **(Erdélyi L et al. Endocrine Abstracts, 2018*).**

* Highlighted publications are listed in the "Publications" section of the report.