Final report

Alzheimer's disease (AD) is the most common form of dementia with gradual loss of memory and cognitive functions. Several studies reported damage to basal forebrain cholinergic system (BFC) neurons and concomitant memory deficits after injections of $A\beta_{1-42}$ in the nucleus basalis magnocellularis (NBM) of the rat and mouse. Among many different factors, for controlling the vulnerability of cholinergic neurons estrogen is an essential factor. Chronic administration of estrogen can restore synaptic connectivity in the cerebral cortex after the loss of BFC neurons against excitotoxic injury. However, this hormone replacement therapy used in post-menopausal woman is associated with detrimental side-effects such as increased risk of stroke and breast cancer, raising concern about its safety, and hence its usefulness as a potential treatment for AD. Preliminary data suggested that selective non-classical actions of estrogen via estrogen receptor alphas (ERα) alternative binding site (ABS) may be beneficial without the detrimental side-effects. Therefore, the aim of the proposal was to discover novel "Activator of Non-Genomic Estrogen Like Signaling" (ANGELS) molecules and test their efficacy in vitro and in vivo.

During the first two years of this project new ANGELS compounds were identified by *in silico* computer modelling, characterized in depths and were later also synthesized.

Bálint M, Jeszenői N, Horváth I, Ábrahám IM, Hetényi C. Dynamic changes in binding interaction networks of sex steroids establish their non-classical effects. Sci Rep. 2017 Nov 1;7(1):14847. doi: 10.1038/s41598-017-14840-9.

During the third year more details were added on the possible binding sites.

Bálint M, Jeszenői N, Horváth I, van der Spoel D, Hetényi C. Systematic exploration of multiple drug binding sites. J Cheminform. 2017 Dec 28;9(1):65. doi: 10.1186/s13321-017-0255-6.

Jeszenői N, Bálint M, Horváth I, van der Spoel D, Hetényi C. Exploration of Interfacial Hydration Networks of Target-Ligand Complexes. J Chem Inf Model. 2016 Jan 25;56(1):148-58. doi: 10.1021/acs.jcim.5b00638.

From the second year, in addition to in silico investigations, using in vivo and in vitro experiments, we tested whether ANGELS had neuroprotective effect on Aβ-induced cell death. The 4-estren-3α, 17β-diol (estren), one of the ANGELS compound, dose dependently restored the loss of cholinergic cortical projections after $A\beta_{1-42}$ injection into nucleus basalis magnocellularis in vivo. The behavioral investigations of these mice showed that estren also attenuated the learning deficits after $A\beta_{1-42}$ administration. Using acute brain slice and in vivo experiments we showed that estren rapidly and directly phosphorylates c-AMP-response–element binding protein (CREB) and extracellular-signal-regulated-kinase-1/2 (ERK) in BFC neurons. Experiments with estrogen receptor knock out mice demonstrated that estren restored the cholinergic fibers via estrogen ERα. These findings indicated that selective activation of non-classical intracellular estrogen signalling via ERα has a potential to treat the damage of cholinergic neurons in AD. Therefore, the in silico ANGELS selection was also based upon the non-classical ABS of the ERα.

Kwakowsky A, Potapov K, Kim S, Peppercorn K, Tate WP, Ábrahám IM. Treatment of beta amyloid 1-42 (Aβ(1-42))-induced basal forebrain cholinergic damage by a non-classical estrogen signaling activator in vivo. Sci Rep. 2016 Feb 16;6:21101. doi: 10.1038/srep21101.

During the third year the acute effect of estrogen as well as estrene was tested on blood flow of the somatosensory cortex after injection of $A\beta_{1-42}$ into the NBM using the laser speckle

technology and laser Doppler flowmetry. Our in vivo result did not show significant difference between E2 and vehicle treated animals, therefore, we did not make further testing on ANGELS compound in this model.

Previous experiments demonstrated that ABS is responsible for the activation of non-classical estrogen action on intracellular signalling system and cytoprotection. Accordingly, we produced 20 ANGELS compounds with the best in silico binding characteristic on ABS of ERα. First, we validated classical genomic or non-classical action of these ANGELS compounds. In these experiments we used two human adenocarcinoma cell lines, MCF-7 and MDA-MB-231. The MCF-7 cell line expresses three estrogen receptors (ERα, ERβ, and GPER1), whereas MDB-MB-231 does not contain an estrogen receptor. To investigate the ERα dependence of the effect (gain and loss of function) we intended to generate a MDB-MB-231 cell line with hERα overexpression and the hERα gene was intended to be knocked out from the MCF-7 cell line using a genome sequence-specific CRISPR-Cas9 genome editing system. Unfortunately, this later attempt was not successful. Nevertheless, MCF-7 cells were treated with various concentrations of E2 and ANGELS (from 100nM to 10pM). In order to test whether the ANGELS compound acts on classical pathway, the activity of estrogen responsive elements (ERE) was measured after transfection of the ERE-luciferase plasmid. By detecting the luminescence of luciferase, we deduced the degree of ERE binding activity. To map the non-classical mechanism of action Western blotting technique was used. MCF-7 cells were treated with the ANGELS compound in a 100nM concentration for 5, 10 or 15 minutes. Cell lysates were tested for the phosphorylation of different second messenger molecules such as Phospho-kinase B (pAKT), pERK1/2, and pCREB. We also intended to perform a cyclic AMP (cAMP) measurements to map of non-classical action of ANGEL compounds on intracellular signalling system. We tried to transfect our cell lines with a plasmid containing a cAMP-sensitive protein, and intended to measure fluorescence resonance energy transfer to determine the change in cAMP level after treatment. Unfortunately, after one year of trying we had to give up these attempts. Eight ANGELS compounds were selected for further in vivo investigation based on their ERα mediated non-classical action on phosphorylated second messenger proteins level without ERE mediated transcriptional effect.

One of the most characteristic effects of E2 is that it causes hyperproliferation of cells in the uterine wall via ERα. To test the uterotrophic side effect of the eight selected ANGELS compounds female C57BL6 mice were ovariectomized (OVX), and they were treated by single subcutaneous injection of E2 or ANGELS. After 24 h, the uterus was removed and its weight was measured. Based on the lack of uterotrophic effect three out of eight ANGELS compounds was selected for further in vivo investigations.

In OVX adult female mice unilateral administration of $A\beta_{1-42}$ into the NBM induces one-sided cholinotoxicity with cholinergic fibre loss in the corresponding somatosensory cortex. Previously in this model estrene, the prototype of ANGELS, was effective. Therefore, we tested the three ANGELES compound and two of them was found to be effective (reduced the cholinergic fibre loss by around 50%). Importantly ANGELS also attenuated bilateral $A\beta_{1-42}$-injection-induced learning deficits. Taken together we identified two ANGELS compounds with non-classical estrogen action and remarkable neuroprotective potential without uterotrophic side-effect.

However, we recognised that $A\beta_{1-42}$-ijection is not a favourable AD model as it mainly resembles an acute neurotoxicity. Therefore, we tested our compounds also in a transgenic mice line, the 3xTg-AD mice, bearing the mutation of the presenilin 1, amyloid precursor protein as well as tau proteins. In 6-month-old 3xTg-AD mice a single injection was able to normalize some behavioural symptoms. However, to normalize the cholinergic fibre loss, chronic

treatment was necessary. Based upon extended behavioural characterization one ANGELS compound seemed to be especially beneficial.

Due to the unexpected die of the PI the publication of these results are hindered. However, three publications are in preparation.

Farkas, Sz, Szabó A, Török B, Fazekas CsL, Correia P, Chaves T, Sólyomvári Cs, Zelena D Effect of ovariectomy induced hormone deprivation on cognitive functions in a triple transgenic mouse model of Alzheimer's disorder . Frontiers of Endocrinology In preparation

Farkas, Sz, Szabó A, Hetenyi Cs, Kovacs T, Zelena D, Abraham I Novel ANGELS compound with neuroprotective action. In preparation

Farkas, Sz, Szabó A, Török B, Fazekas CsL, Correia P, Chaves T, Abraham I, Zelena D, Chronic treatment with ANGELS compound in 3xTg-AD mice model of Alzheimer's disorder In preparation

We were not concentrating only on females as the neuroprotective effect of the compound might not be restricted to either sexes. Our previous work has shown that E2 rapidly phosphorylates CREB via ERα in female cholinergic neurons. Using this indicator, we examined whether nonclassical actions of E2 occur in a sexually dimorphic manner within BFC neurons in mice. In addition, we investigated the expression and intracellular distribution of ERα in cholinergic neurons in female and male mice. Animals were gonadectomized and treated 2 weeks later with E2. The number of CREB-expressing cholinergic neurons was not altered in any of the brain regions after E2 treatment in both males and females. However, E2 treatment rapidly (< 15 minutes) increased the number of cholinergic neurons expressing phosphorylated CREB (pCREB) in the NBM and medial septum, but not in the striatum in female mice. By contrast, E2 did not change pCREB expression in cholinergic neurons in male mice at any time point (15 minutes, 1 hour, 4 hours), irrespective of the neuroanatomical location. We also observed that, in females, more cholinergic neurons expressed nuclear ERα in all regions, whereas males showed more cholinergic neurons with cytoplasmic or both nuclear and cytoplasmic expression of ERα. Taken together, these results demonstrate a marked sex difference in the E2-induced nonclassical effect and intracellular distribution of ERα in BFC neurons in vivo. However, based upon these observation beneficial effect in both sexes might be expected.

Kim S, Barad Z, Cheong RY, Ábrahám IM. Sex differences in rapid nonclassical action of 17β-oestradiol on intracellular signalling and oestrogen receptor α expression in basal forebrain cholinergic neurones in mouse. J Neuroendocrinol. 2020 Jan;32(1):e12830. doi: 10.1111/jne.12830.

We also examined the impact of E2 on the function of nocisensor transient receptor potential vanilloid 1 (TRPV1) receptor in mice sensory neurons. Our study provided in vivo and in vitro evidence for E2-induced TRPV1 receptor upregulation and sensitization mediated by TrkAR via E2-induced genomic and nongenomic mechanisms. The sensitization and upregulation of TRPV1 receptor by E2 in sensory neurons may explain the greater pain sensitivity of female mice.

Payrits M, Sághy É, Cseko K, Pohóczky K, Bölcskei K, Ernszt D, Barabás K, Szolcsányi J, Ábrahám IM, Helyes Z, Szoke É. Estradiol Sensitizes the Transient Receptor Potential Vanilloid 1 Receptor in Pain Responses. Endocrinology. 2017 Oct 1;158(10):3249-3258. doi: 10.1210/en.2017-00101.

It is known that E2 alters glutamatergic neurotransmission and synaptic plasticity. We might assume that the neuroprotective effect of the ANGELS compounds is also connected to

neuronal sprouting and enhanced synaptic plasticity. As part of the molecular mechanism the surface movement of AMPA receptors (AMPARs) plays a critical role in excitatory neurotransmission and synaptic plasticity. We developed single molecule tracking and superresolution imaging method to follow AMPAR movement.

First, we analysed the rapid effect of estradiol (E2) on glutamate receptors using single-molecule tracking and super-resolution imaging techniques. In neurons differentiated from PC12 cells (dPC12), live-cell single-molecule tracking experiments revealed that E2 rapidly decreased the surface movement of AMPAR via membrane G protein-coupled estrogen receptor 1 (GPER1) in neurites in a dose-dependent manner. Activation of both GPER1 and ERβ by E2 was required to decrease the surface movement of AMPARs in somata. A super-resolution microscopy analysis and live-cell single-molecule tracking experiments showed that the cortical actin network plays a pivotal role via the Rho-associated protein kinase-cofilin and c-Jun-N-terminal kinase-cofilin pathways in the GPER1 mediated effects of E2 on the surface mobility of AMPAR. Live-cell single-molecule tracking experiments on cultured hippocampal neurons demonstrated that E2 decreases the surface movement of AMPAR both in synaptic and extrasynaptic regions on neurites. Furthermore, we demonstrated that E2 increases the synaptic dwell time of AMPARs. These observations show a novel regulatory mechanism for E2. Our results also provide evidence for understanding E2 action on neuronal plasticity and glutamatergic neurotransmission at the molecular level.

Godó S, Barabás K, Lengyel F, Ernszt D, Kovács T, Kecskés M, Varga C, Jánosi TZ, Makkai G, Kovács G, Orsolits B, Fujiwara T, Kusumi A, Ábrahám IM. Single-Molecule Imaging Reveals Rapid Estradiol Action on the Surface Movement of AMPA Receptors in Live Neurons. Front Cell Dev Biol. 2021 Sep 23;9:708715. doi: 10.3389/fcell.2021.708715. eCollection 2021.

Neurotrophin receptors such as the tropomyosin receptor kinase A receptor (TrkA) and the low-affinity binding p75 neurotrophin receptor p75NTR play a critical role in neuronal survival and their functions are altered in AD. Changes in the dynamics of receptors on the plasma membrane are essential to receptor function. However, whether receptor dynamics are affected in different pathophysiological conditions is unexplored. Using live-cell single-molecule imaging, we examined the surface trafficking of TrkA and p75NTR molecules on live neurons that were derived from human-induced pluripotent stem cells (hiPSCs) of presenilin 1 (PSEN1) mutant familial AD (fAD) patients and non-demented control subjects. Our results show that the surface movement of TrkA and p75NTR and the activation of TrkA- and p75NTR-related phosphoinositide-3-kinase (PI3K)/serine/threonine-protein kinase (AKT) signalling pathways are altered in neurons that are derived from patients suffering from fAD compared to controls. These results provide evidence for altered surface movement of receptors in AD and highlight the importance of investigating receptor dynamics in disease conditions. Uncovering these mechanisms might enable novel therapies for AD.

Barabás K, Kobolák J, Godó S, Kovács T, Ernszt D, Kecskés M, Varga C, Jánosi TZ, Fujiwara T, Kusumi A, Téglási A, Dinnyés A, Ábrahám IM. Live-Cell Imaging of Single Neurotrophin Receptor Molecules on Human Neurons in Alzheimer's Disease. Int J Mol Sci. 2021 Dec 9;22(24):13260. doi: 10.3390/ijms222413260.

Barabás K, Godó S, Lengyel F, Ernszt D, Pál J, Ábrahám IM. Rapid non-classical effects of steroids on the membrane receptor dynamics and downstream signaling in neurons. Horm Behav. 2018 Aug;104:183-191. doi: 10.1016/j.yhbeh.2018.05.008.

Several further review paper was published on the beneficial effect of estrogen.

Kovács T, Szabó-Meleg E, Ábrahám IM. Estradiol-Induced Epigenetically Mediated Mechanisms and Regulation of Gene Expression. Int J Mol Sci. 2020 Apr 30;21(9):3177. doi: 10.3390/ijms21093177.

Kövesdi E, Szabó-Meleg E, Abrahám IM. The Role of Estradiol in Traumatic Brain Injury: Mechanism and Treatment Potential. Int J Mol Sci. 2020 Dec 22;22(1):11. doi: 10.3390/ijms22010011.

In relation to this project we recently published a review paper on the beneficial effect of estrogen and/or estrogen system modulating agents in postmenopausal women with the first authorship of the PhD student worked on the project before.

Farkas S, Szabó A, Hegyi AE, Török B, Fazekas CL, Ernszt D, Kovács T, Zelena D. Estradiol and Estrogen-like Alternative Therapies in Use: The Importance of the Selective and Non-Classical Actions. Biomedicines. 2022 Apr 6;10(4):861. doi: 10.3390/biomedicines10040861.