Effects of intravenous and intranasal P-glycoprotein modulation on the blood-brain barrier in young and aging rats

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Thesis of the PhD Dissertation

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1. Introduction

The blood-brain barrier (BBB) not only blocks the free distribution of molecules between brain and blood, but also protects the physiological environment of the brain by the effort of multiple transporter proteins that can be found in the endothelial cells of the brain capillaries. Some of these transporters provide the availability of the right amount of ions and nutrients while others protect the brain parenchyma from potentially neurotoxic blood-borne substances [1], [2]. The latter group is the efflux transporters, like the MDR1 or P-glycoprotein, which is part of the ATP-Binding Casette (ABC) protein family.

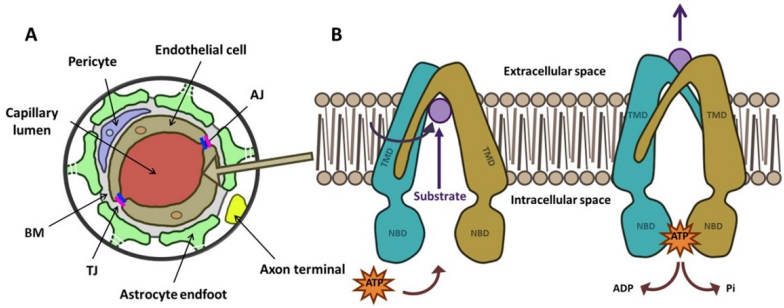

Figure 1 - The schematic structure of the blood-brain barrier (A) and its efflux transporters (B). *On Figure A) there are multiple type of cells that surround the capillaries of the brain: neurons, astrocytes, microglial cells and pericytes. The capillaries themselves are consist of distinctive endothelial cells with high number of tight and adherent junctions (TJ, purple and AJ, blue), which assure that paracellular transport through the brain capillaries are not permitted. The extracellular layer on the basal side of the endothelial cells called the basal membrane (BM) B) The P-gp (ABCB1) proteins of the BBB consists of two homologous subunits (teal and brown). In the presence of a substrate (bound to the transmembrane domains or TMD), an adenosine triphosphate (ATP) can be bound to the nucleotide-binding domains (NBD), which causes the protein to change the conformation and make way for the substrate into the extracellular space. The conformation change costs energy provided by the hydrolysis of the ATP, the reaction creates an adenosine diphosphates (ADP) and an inorganic phosphate (Pi) group. As the ADP detaches from the P-gp, the protein recovers its initial conformation.*

With aging the function of the BBB decreases: molecules, ions and immune cells can leak into the brain parenchyma and disturb the homeostasis

of the central nervous system (CNS) [3]. The altered extracellular environment and the extravasation of immune cells can induce the expression of various inflammatory cytokines that can progress the dysfunction of the BBB and increase the risk of neurodegenerative disorders. One of the main reason behind the increased leakage and the changes in the structure of BBB is the altered expression of multiple proteins [3]. For example the insufficient expression of the tight and adherent junction proteins can cause the disruption in the barrier integrity, also the decreased number of efflux transporter proteins can lead to the accumulation of neurotoxic agents in the brain parenchyma [4].

Occasionally, two drugs that have been administered simultaneously can interact with the same target in the body, inhibiting or increasing the effect of the other molecule (pharmakodynamic interaction) [5]. This phenomenon can happen at the BBB by inhibiting the efflux transporters, which can lead to increased influx of otherwise transporter-removed substrates. These kind of interactions can be a potential solution for the effective drug delivery into the CNS [6], but can also lead to harmfully increased concentration of toxic agents, especially in an elderly patient with decreased liver and kidney functions [5].

Intranasally administered drugs have been gaining ground in the field of CNS therapy. The main advantage over the traditional intravenous and oral administration methods is that it provides a direct pathway to the brain with minimal peripheral exposure [7]. The drug reaches its maximal concentration in the CNS relatively fast, while the administration itself not invasive (compared to the intracranial administration). In the last couple of years more and more intranasal drugs appear on the market, for example anticonvulsant [8] and hormonal treatments [9], [10].

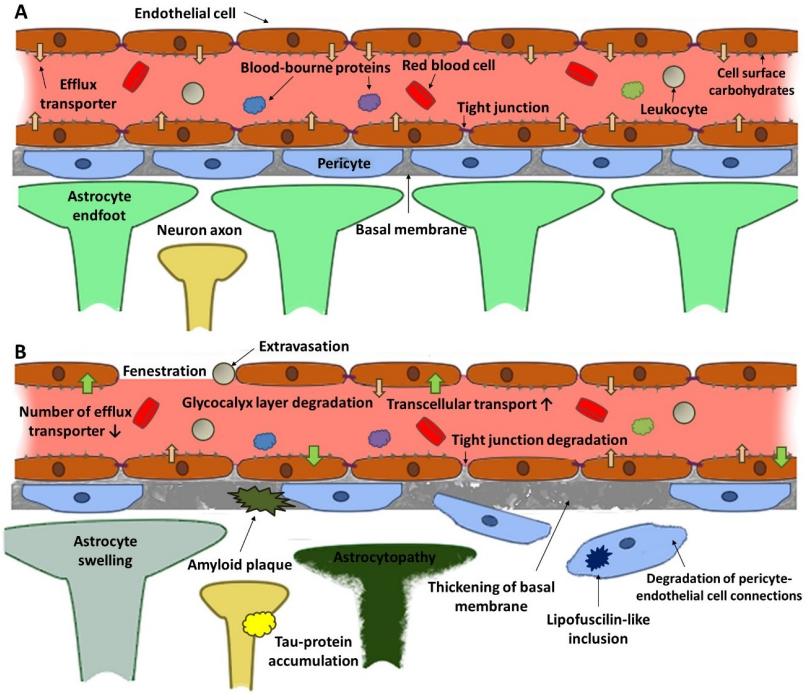

Figure 2. - Healthy (A) vs aged (B) BBB structure comparison.. *The age-related dysfunctions can be the results of multiple alterations: the tight junctions between endothelial cells loosen up, the surface glycoprotein layers degrade and the expression of efflux transporters expression decreases. These events cause increased paracellular (including the extravasation of leukocytes) and transcellular transport. Due to the altered expression profile of several proteins, the basal membrane grows thicker. The pericytes connection to the endothelial cells loosens up and lipofuscilin-like inclusions appear in the cell body. The surrounding astrocytes swell up, express increased amount of glial fibrillary acidic protein (GFAP) or even go through astrocytopathy. Amyloid and tau plaques accumulate in the brain parenchyma.*

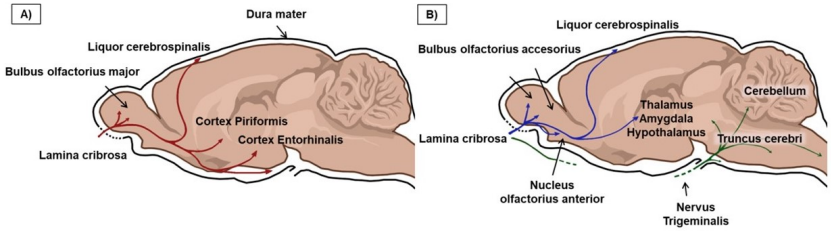

Figure 3. - Nose-to-brain pathway of intranasal drugs. *A) Red arrows show the route through the main olfactory pathway: entering through the lamina cribrosa into the bulbus olfactorius major, the drugs then can reach the pririformis and entorhinal cortices or enter the cerebrospinal fluid (liquor cerebrospinalis) which can further distribute it into other brain regions but mostly clear the substance out from the CNS. B) The green arrows show the path through the trigeminal nerve (nervus trigeminalis) which distributes the drugs in the hind regions of the brain, like the cerebellum and the brain stem (truncus cerebri). In animals like rats there is another pathway from the nasal cavity to the brain through the vormeonasal or accessory olfactory pathway. This route has been marked with the blue arrows: from the bulbus olfactorius accesorius the drug can reach regions like the nucleus olfactorius anterior, the thalamus, the amygdala and the hypothalamus. (The routes have been visualized by using the descriptions of publications [11] and [12])*

2. Aim of research

The first aim of this research was to investigate the age related changes in the permeability of the BBB, especially the function of P-gp proteins using Wistar rats. Furthermore the modulation of these efflux transporters was observed using intravenous and intranasal quinidine, combined with intravenous and intranasal P-gp inhibition and intranasal sympathomimetic treatments. The basic concept is that it would be very difficult to observe the permeability of the blood-brain barrier in humans, therefore the in vivo double and triple-probe microdialysis methods used for this purpose are of great importance.

3. Methods

3.1. Experimental animals

The examination was carried out mostly on male rats. Middle-aged, aged animals (550-900 g) were at least 13-21 months old, the young adult animals (weight about 250-400 g) were 3-4 months old male Wistar rats. The animals were anesthetized with intraperitoneal chloral hydrate during the experiments to reduce stress and pain.

3.2. Microdialysis

The experiments were carried out with the use of quinidine (QND) as P-gp substrate and valspodar (PSC-833) as P-gp inhibitor [13] on anesthetized rats (in vivo). The venous probe (CMA/20 elite) was implanted into the right jugular vein; the brain probe (CMA-12) was inserted into the left striatum. The sample collection started at the -30 minute mark (the QND was administered at 0). The collector tubes were changed every 30 minutes until the end of the experiment (+240. minute mark).

For the intravenous administration, a Braun cannula was inserted into the femoralis vein. For the intranasal solution the animals were placed supine, and the treatments were administered before the implantation of the striatal probe, while the intranasal gel was administered into the left nostril with both probes already in place. This way the most important first sample is not missed out on like with nasal solution treatment.

The collected microdialysis samples were analyzed with HPLC-MS/MS. The age related changes of P-gp function was also examined with SPECT

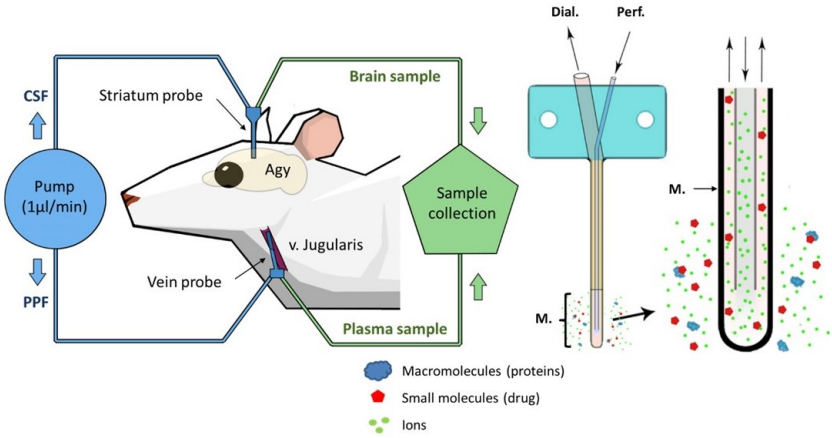

Figure 4. - Schematic setup of a microdialysis probe and a dual-probe microdialysis experiment. *Peripheral perfusion fluid (PPF) and CSF were perfused through the microdialysis probes (Perf.: perfusate). Small molecules, like the examined substrate can penetrate through the membrane (M.) therefore the collected samples (Dial.: dialysate) have the equivalent concentration of the free (non-binded) target drug (QND) as in the extracellular space of the examined tissue. The substrate concentration than can be compared and the distribution of the drug can be analyzed.*

imaging. For the tracing of the P-gp substrate distribution a radioactive P-gp [99m-Technetium]-2-methoxy-isobutyl-isonitrile was used. The P-gp inhibitor was PSC-833.

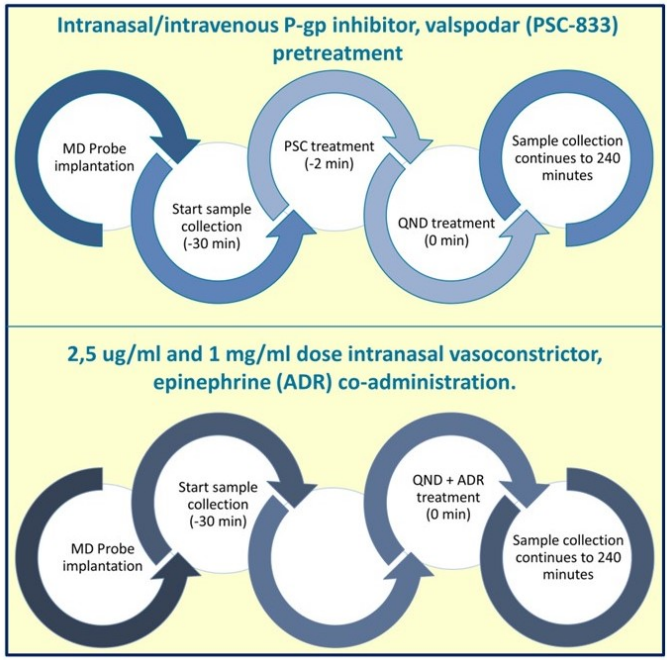

Figure 5. Flow chart of microdialysis experiments, investigating the penetration of intranasally administered P-gp substrate, quinidine. *The distribution of the substrate was tested in the presence of P-gp inhibitor (intranasal and intravenous formulations) and intranasal sympathomimetic. (MD: microdialysis; QND: quinidine; ADR: adrenaline.) MD: mikrodialízis; QND: kinidin; ADR: adrenalin.*

3.3. Investigation of anatomical changes

The structural and protein expression changes in the BBB were examined with immunohistochemistry, electronmicroscopy and MRI recordings. The MRI images were also used to determine the coordinates of the striatum for the aged rats. The distribution of the intranasal solution in the nasal cavity was also checked with histology using Evans-blue. For the histological examinations the animals were perfused with saline (3 min), then a mixture

of 4% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer (PB) for immunohistochemistry and electronmicroscopy and with 10% formaldehyde for the intranasal investigation.

3.4. Behavioural tests

The effect of aging on the cognitive functions was investigated with Morris water maze and new object recognition (NOR) tests. These examinations helped to determine the state of special and recognition memory of the rats. The raw data of the Morris water maze test were analyzed with repeated measures ANOVA, for the NOR test a discrimination index (DI) was determined:

$$\mathrm{DI} = 100 * \frac{t_{2\acute{u}j} - t_{2ismert}}{t_{2\acute{u}j} + t_{2ismert}} \tag{1}$$

The DIs of the young and aged rats were analyzed using Student-test.

3.5. Sample preparation and ELISA

To measure the cytokine levels in the brain 3 young and 3 aged rats, the left striata were removed and homogenized with 1X cell lysis buffer. The total protein concentrations of the aliquots were determined with PierceTM BCA Protein test. The cytokine levels were measured with Chemiluminescence ELISA Array from Signosis.

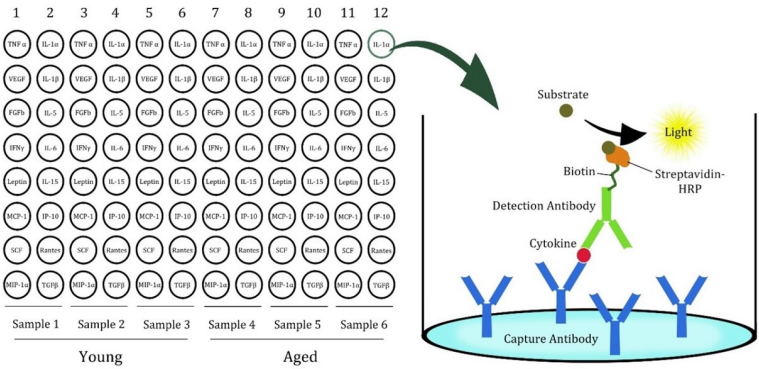

Figure 6. - Aliquot placement on the ELISA plate and a schematic figure of a plate cell. *The cytokines captured between their antibodies (the capturing ABs are cytokine specific while the detection ABs are species specific). Streptavidin-horseradish peroxidase (HRP) is bound to the detection AB that allows the presence of the cytokine to be detected, when the substrate of the peroxidase is added. Every aliquot (from 3 young and 3 aged animals) was tested for 16 different cytokines.*

4. Summary of results

Aging of the blood-brain barrier

- In the aged control group the concentration of quinidine was higher in the striatum with a slower elimination rate compared to in young animals. The higher levels of drug can be also be the result of decreased metabolism.

- The PSC-833 pretreatment equalized the differences of quinidine concentrations in aged and young animals, the elimination of the substrate decreased both in the brain and in the periphery.

- The result of PSC-833 treatment in young rats was more than 2 times more effective than in aged ones: the quinidine concentration measured in the striatum of treated young animals has increased 7-fold compared to control young group, while in the aged P-gp inhibited group had only 3,36 times higher concentration in the brain vs the control aged group.

- SPECT imaging had similar results to microdialysis with the use of

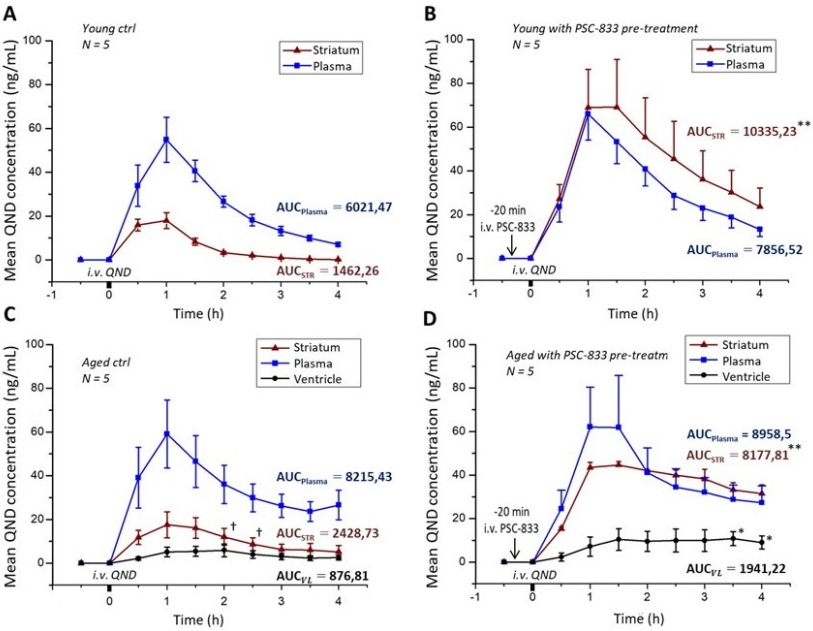

Figure 7. Concentration-time profiles of quinidine (5 mg/kg i.v.) in young (A, B) and middle aged (C, D) Wistar rats in absence (A,C) and presence (B,D) of PSC-833 (2x2 mg/kg i.v). *The values are mean concentrations ± standard error, N = 3–5/group. *: p < 0.05, **: p < 0.005 by T-test (control group vs PSC-833 treated group), †: p < 0.05 young controls vs aged controls. (Abbreviations: QND: quinidine, STR: striatum, LV: lateral ventricle, AUC: area under the curve, QND: quinidine)*

99m-Tc-MIBI as P-gp substrate.

- The chemilimunescence cytokine ELISA test showed significant increase in SCF and Rantes in aged animals compared to young ones. There were also elevated levels of multiple proinflammatory cytokines in the samples of the aged rats, like TNF and most of the examined interleukines. These results can be the sign of a chronic inflammation.

The modulation of intranasal P-gp substrate distribution

- Even though the nasal solution has been effectively administered into the caudal areas of the nasal cavity, it produced many unwanted

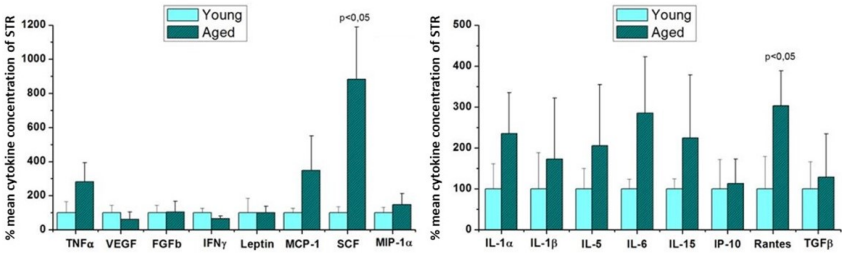

Figure 8. The relative levels of cytokines in the striatum of aged rats compared to young ones. *The 100% was set on the mean luminescence intensity of the samples from young animals.*

properties. The gel formulation offered solutions for most of these problems.

- The control group showed modest brain and systemic distributions.
- With local P-gp inhibitor treatment the distribution has not showed any significant changes compared to control.
- Local (nasal) adrenaline treatment has proven to enhance the CNS distribution of the P-gp substrate through the olfactory epithelium.
- The decreased peripheral concentration of quinidine has shown that the absorption from the mucosa has been successfully prevented by adrenaline.

4.1. New scientific results

The distribution of intravenously administered P-gp substrate quinidine depends on the age of the animal both in the control as well as in the P-gp inhibitor PSC-833 treated groups. The elimination of the drug in older animal is slower / the permeability of the blood-brain barrier is higher than in the young rats. This phenomenon can be explained by the aging of the blood-brain barrier; several structural and protein expression changes have been observed. A rise in proinflammatory cytokine levels, as a presence of escalating inflammatory processes in older animals was also detected. [J1, J2, J4]; [P1-P4]; [C1-C3]

Thesis I.

I.a. In the aged control group the concentration of quinidine was higher in the striatum with a slower elimination rate compared to in young animals.

I.b. The result of PSC-833 treatment in young rats was more than 2 times more effective than in aged ones: the quinidine concentration measured in the striatum of treated young animals has increased 7-fold compared to control young group, while in the aged P-gp inhibited group had only 3,36 times higher concentration in the brain vs the control aged group.

I.c. The chemilimunescence cytokine ELISA test showed significant increase in SCF and Rantes in aged animals compared to young ones. There were also elevated levels of multiple proinflammatory cytokines in the samples of the aged rats, like TNF and most of the examined interleukines.

Intranasally administered P-gp substrate quinidine was successfully delivered to the central nervous system by selecting a suitable formulation. Subsequently, modulations of the brain distribution of intranasally administered P-gp substrate were performed with intranasal and intravenous P-gp inhibitor (PSC-833) and intranasal sympathomimetic (adrenaline) treatments. [J2, J3, J5]; [P5, P6]; [C4]

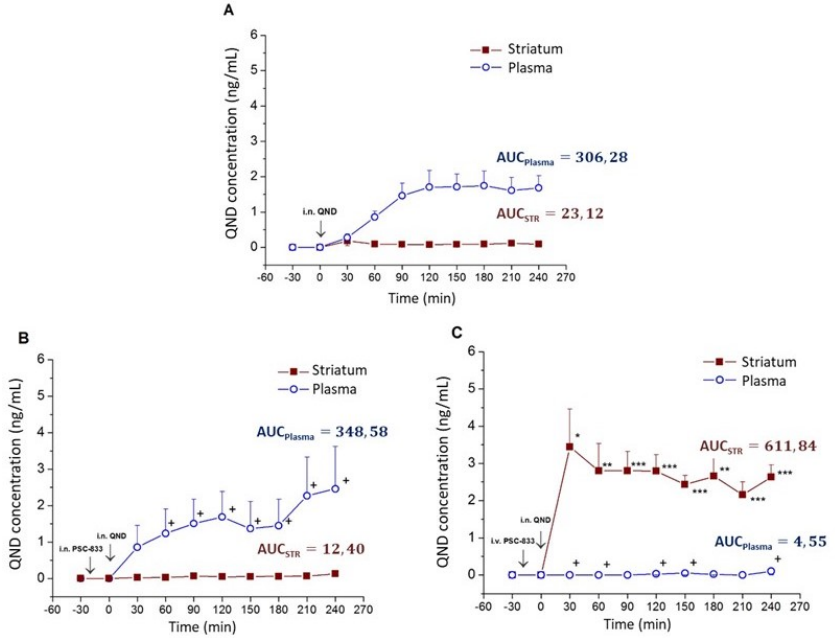

Figure 9. - Modulation of nose-to-brain delivery of intranasal (IN) quinidine (QND) by PSC-833, a dual-probe microdialysis study in rats. *A: control group: IN QND (1 mg) + IN vehicle; B: IN QND (1 mg) + IN PSC-833 (10 µg); C: IN QND + i.v. PSC-833 (4 mg/kg). *: $p < 0.05$, **: $p < 0.01$, ***:$p<0.005$ vs control striatum by Student t-test. (Data are given as means ± SE.; N = 5/group).*

Thesis II.

II.a. With local P-gp inhibitor treatment the distribution has not showed any significant changes compared to control. Intravenous P-gp inhibitor treatment increased brain distribution significantly, while the peripheral exposure of QND has decreased compared to control.

II.b. Local (nasal) adrenaline treatment has proven to enhance the CNS distribution of the P-gp substrate through the olfactory epithelium. The decreased peripheral concentration of quinidine has shown that the absorption from the mucosa has been successfully prevented by adrenaline.

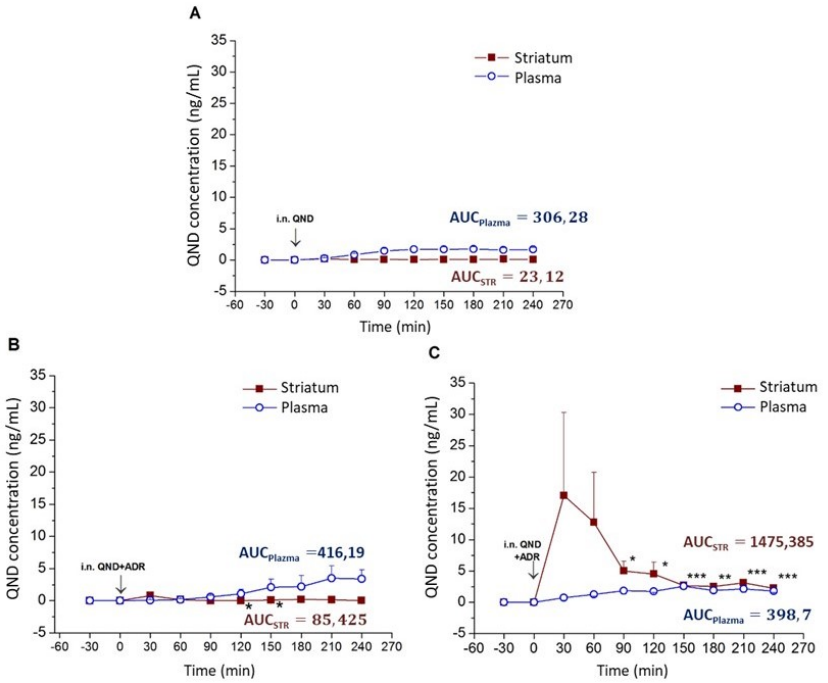

Figure 10. Modulation of nose-to-brain delivery of intranasal (IN) quinidine (QND) by adrenaline (ADR), a dual-probe microdialysis study in rats. *A: control group: IN QND (1 mg) + IN vehicle; B: IN QND + ADR 50 ng; C: IN QND + ADR 20 g. *: p < 0.05, **: p < 0.01, ***:p<0.005 vs control striatum by Student t-test. (Data are given as means ± SE.; N = 5/group).*

4.2. Results of additional investigations

With the help of additional experiments it was possible to identify the aging processes in the specimens, described in the literature:

- In young adult animals a moderate amount of GFAP expression is observed in the striatum in contrast, the astroglial-staining is increased in the brain tissue of old animals. An intense staining of the P-gp is visible in the striatum of young adult animals, indicating the high expression level of the transporter protein. However, a decreased expression level is found in case of the old animals.

- In case of the young adult animal, the capillary walls are thin, are sur-

rounded by astrocyte endfeet and several tight junctions are visible sealing the adjacent endothelial cells. Pericyte can be observed embedded in the basal membrane. In aged rats there are less tight junctions among the endothelial cells. The basal membrane is considerably thicker the capillary walls are thicker, and the extent of the astrocyte endfeet is considerably higher.

- The behavioral studies did not show any significant differences between young and aged rats. However, there were differences in the exploration time: most of the aged rats were “freezing” in the test box during the majority of the t2 test period, which can be the sign of anxiety or apathy. However, due to the small number of specimens, a reliable statistical evaluation could not be achieved.

5. Fields of application

The long-term goal of blood-brain barrier research is to find out more about the possible dysfunctions of this barrier system and also to develop drugs that can reach the CNS more efficiently by modulating it.

The results that I found out about the aging of the blood-brain barrier, especially about P-gp transporters suggest that while the barrier of the cerebral capillaries in the elderly is more vulnerable to P-gp substrate to get through (as a model to show the increased amount of unwanted substances that can cross the blood-brain barrier), an inhibitory drug interaction with P-gp transporters may have a more drastic effect in younger groups. However, chemical inhibition of P-gp can have an advantage in pharmacology for that the P-gp transporters prevent efficient drug delivery into the brain, and also a well-known cause of resistance of tumor cells to cytostatics.

Using the intranasally administered P-gp substrate, different P-gp modulation possibilities have been studied. Intranasal delivery is an effective drug delivery method to the central nervous system, while minimizing the peripheral exposure. The brain-blood ratio of the P-gp substrate is improved by systemic P-gp inhibition pretreatment. Local sympathomimetic treatment has also been successfully increased the cerebral distribution of intranasally administered P-gp substrates: intranasal adrenaline reduced absorption through the capillaries of the nasal mucosa, providing rapid and efficient cerebral penetration for the drug.

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List of publications

Journal publications:

[J1] **L. A. Bors**, K. Tóth, E. Zs. Tóth, Á. Bajza, A. Csorba, K. Szigeti, D. Máthé, G. Perlaki, G. Orsi, G. K. Tóth, F. Erdő „Age-dependent changes at the blood-brain barrier. A Comparative structural and functional study in young adult and middle aged rats," *Brain Research Bulletin*, vol.139, pp. 269-2775, 2018.

[J2] **L. A. Bors**, F. Erdő „Overcoming the Blood–Brain Barrier. Challenges and Tricks for CNS Drug Delivery," *Scientia Pharmaceutica* , vol. 87, no. 1, 2019.

[J3] Franciska Erdő, **Luca Anna Bors**, Dániel Farkas, Ágnes Bajza, Sveinbjörn Gizurarson , „Evaluation of intranasal delivery route of drug administration for brain targeting," *Brain Res Bull.*, vol.143, pp. 155-170, 2018.

[J4] **L. A. Bors**, K. Tóth, E. Zs. Tóth, Á. Bajza, A. Csorba, K. Szigeti, D. Máthé, G. Perlaki, G. Orsi, G. K. Tóth, F. Erdő , „Corrigendum to "Age- dependent changes at the blood-brain barrier. A comparative structural and functional study in young adult and middle aged rats," *Brain Research Bull.*, vol.155, pp. 211-212, 2020.

[J5] **L. A. Bors**, Á. Bajza, M. Mándoki, B. J. Tasi, Gy. Cserey, T. Imre, P. Szabó, F. Erdő, „Modulation of nose-to-brain delivery of a P- glycoprotein (MDR1) substrate model drug (quinidine) in rats," *Brain Research Bull.*, vol.160, pp. 65-73, 2020.

Conference posters:

[P1] **L. A. Bors**, Á. Bajza, B. Hutka, L. Dénes, K. Szigeti, N. Hegedűs, D. Szöllősi, D. Máthé, A. Csorba and F. Erdő, "Investigation of the impact of aging on blood-brain barrier function in rats – Does P-glycoprotein (P-gp) have any role in changing BBB permeability?", 10th SFB35 - Transmembrane Transporters in Health and Disease, *Bécs, Ausztria, 2017*

[P2] Á. Bajza, **L. A. Bors**, B. Hutka, A. Csorba, D. Máthé, G. Orsi, G. Perlaki, K. Tóth and F. Erdő, "Investigation of the effect of aging on blood-brain barrier morphology and function in wistar rats.", *FENS Regional Meeting, Pécs, Magyarország, 2017*

[P3] **L. A. Bors**, K. Tóth, Á. Bajza, E. Tóth, A. Csorba, D. Máthé, K. Szigeti, G. Perlaki, G. Orsi and F. Erdő "Age-related changes in P-glycoprotein function at the blood-brain barrier - A comparative preclinical study", *Meet the*

Experts Transporter Conference Budapest, Magyarország, 2018

[P4] **L. A. Bors**, Á. Bajza, K. Tóth, A. Csorba, K. Szigeti, D. Máthé, I. Gyertyán and Erdő F. "In vivo model of the aging blood-brain barrier: observations of the functional and structural changes with multiple methods", *FENS Regional meeting, Belgrád, Szerbia, 2019*

[P5] **L. A. Bors**, F. Erdő "How to deliver a p-gp substrate into the central nervous system? - Intranasal formulations of quinidin", *4th Hungarian Neuroscience Meeting for Undergraduate Students, Graduate Students, and Junior Post-Docs (HuNDoC), Szeged, Magyarország, 2020*

[P6] **L. A. Bors**, Á. Bajza, T. Imre, P. Szabó and F. Erdő "Method development for investigation the blood-brain barrier permeability with intranasally administrated p-gp substrate", *IBRO Workshop, Szeged, Magyarország, 2020*

Conference publications:

[C1] **L. A. Bors** "Investigation of age-related functional changes and membrane transporter interactions at the blood-brain barrier in rodents." *in PhD Proceedings Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics, Pázmány Péter Catholic University* – 2017. G. Prószéky, P. Szolgay Eds. Budapest: Pázmány University ePress, 2017, pp 12

[C2] **L. A. Bors** "Altered protection against xenobiotics in the aged brain? - Functional changes of P-glycoprotein at the blood brain barrier." *in PhD Proceedings Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics, Pázmány Péter Catholic University* – 2018. G. Prószéky, P. Szolgay Eds. Budapest: Pázmány University ePress, 2018, pp 9

[C3] F. Erdő, K. Tóth, Á. Bajza, E. Tóth, A. Csorba, **L. A. Bors**, D. Máthé, G. Perlaki, G. Orsi, J. Molnár, I. Wilhelm, I. Gyertyán, „Effetct of healthy aging on blood-brain barrier morphology and function. Does it have any impact on the memory and the protein expression? A comparative study in aged and young rats." *Annual Meeting of SFN, San Diego, USA*, 2018

[C4] **L. A. Bors** "Overcoming the blood-brain barrier by different intranasal formulations of quinidine, a P-gp model substrate." *in PhD Proceedings Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics, Pázmány Péter Catholic University* – 2019. G. Prószéky, P. Szolgay Eds. Budapest: Pázmány University ePress, 2019, pp 11

Other publications

1. F. Farner, **L. A. Bors**, Á. Bajza, G. Karvaly, I. Antal, F. Erdő, “Validation of an in vitro-in vivo assay system for evaluation of transdermal delivery of caffeine”, *Drug Delivery Letters* vol. 9, no.1 pp. 15-20, (2019)

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