Chiral analysis of amino acid neurotransmitters acting on NMDA receptor by CE-LIF in biological samples

Doctoral thesis

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1. Introduction

It is known for a long time that glutamate and aspartate can be found in extremely high concentration in the central nervous system (CNS) compared to other tissues. The primer mediator is glutamate in sensory information transferring, motor coordination, memory formation and learning processes. NMDA receptor plays a crucial role in mediating the effects of glutamate. This receptor has a very complex regulation mechanism, as it is including several regulation steps. For the activation of NMDA receptor simultaneous binding of an agonist and a co-agonist is required. Formerly glycine was considered as the main agonist of NMDA receptor, however due to the improvement of analytical techniques it was found that D-serine has a higher affinity to the glycine binding site. An interesting finding is that L-aspartate and D-aspartate also can bind to the L-glutamate binding site however, the importance of this finding is not well established.

In the nature some of the compounds with the same molecular formula could be different from each other due to isomerism. Optical isomers have the same physical and chemical properties, only difference is the isomers rotate the plane polarised light in opposite way. For amino acids the central carbon binds to hydrogen, a carboxyl plus an amino group and to an R-substituent. Unlike in the past, where it was believed that only one amino acid enanitomer occurs naturally, today it is well established that both enantiomers can be found in living organisms. However the L-isomers can be found abudantly compared to D-isomers. Moreover recently more and more various functions of D-amino acids, like hormone, neurotransmitter or neuromodulator have been revealed.

D-serine and D-aspartate are the two main amino acids in the central nervous system. D-serine acts as a co-agonist and neuromodulator on the NMDA receptor. Moreover D-serine concentration correlates with the NMDA receptor distribution on different braing regions.

While the neuromodulator role of D-serine is well described in the literature, the role of D-aspartate is less understood. It was found that high concentration of D-aspartate can be found during nervous system development in chicks, rats and humans however, the concentration decreases during adulthood. Because of this finding it is suggested that D-aspartate plays a key role in the nervous system development. Higher level D-aspartate can be found in high proliferative brain regions. It was found that D-aspartate is capable to activate NMDA receptors. Several publications discuss the role of D-aspartate in cognitive function and also its agonist effect on the L-glutamate binding site of NMDA receptor. Because of these

findings the role of D-aspartate is suggested in learnning processes, memory formation and diseases connected with NMDA receptor disfunction.

The quantitative determination of enantiomers in biological matrix is difficult, due to one of the enantiomers is highly abudant compared to the other one. Moreover the enantiomers share common physical and chemical properties. Capillary electrophoresis is one of the most effective separation techniques with many advantages like few uL sample volume requirement, fast analysis time and low eluent consumption. For chiral separation mostly chiral selectors are used to distinguish enantiomers due to their complex-forming ability. These complexes are bearing different physical and chemical properties, thus they can be separated from each other. Recently for chiral separation mostly cyclodextrins are used in the field of drug analysis.

The two common modes of optical detection are UV and fluorescent detection. UV absorption is used most widely for detection. However the concentration sensitivity of UV absorbtion is very low, thus it has limited usage in combination with capillary electrophoresis. Fluorescent detection is very selective (one or two order of magnitude higher than UV) but only a few compounds possess natural fluorescence. It is very rare if the interested compound has fluorescent moiety, thus fluorescent compounds are created with derivatization reaction from molecule of interest. Lasers are used for proper excitation during fluorescent detection. Laser beam highly increases the sensitivity, moreover laser excites selectively in a defined wavelenght. Because of these advantages laser induced fluorescence is widely used for biological sample analysis in capillary electrophoresis.

2. Aims

I started my work in the Scientific Students' Association at Semmelweis University Department of Pharmacodynamics, where I was involved the development of a high efficacy capillary electrophoresis method capable of low concentration excitatory amino acid (D and L aspartate and glutamate) quantification relatively fast in biological samples. At this time a new interesting challenge arisen as simultanously determine the NMDA co-agonist D-serine next to the neuromodulator D-aspartate. Both D-amino acids can be found in very low concentration in biological samples.

My aims were:

1. To develop and validate an efficient chiral capillary electrophoresis method capable of simultaneous quantification of D-aspartate and D-serine next to L-aspartate and L-glutamate in biological samples.

2. To examine the amount changes of NMDA receptor agonist L-glutamate, D- and L-aspartate, and the co-agonist D-serine in different experimental animals

- Examine the levels of D-aspartate and D-serine in different brain regions and age of experimental animals.

- Examine the extracellular concentration change of aspartate and glutamate due to different stimuli with microdyalisis sampling technique.

3. Methods

3.1 For the experiments Beckman Coulter P/ACE-MDQ (Beckman Coulter, Brea, CA, USA) capillary electrophoresis machine was used. For detection an argonion laser (Beckman Coulter) was applied with 488 nm excitation and 520 nm detection wavelenght. For separation 75 μm inner diameter (365 μm o.d.) fused silica capillary was used (Agilent Technologies Santa Clara, CA, USA). The inner capillary wall was coated with the method developed by Hjerten et. al.

3.2 For derivatization 5 μl sample or standard solution was mixed with 5 μl derivatization buffer containing internal standard and with 5 μl derivatization solution. After appropiate time and temperature for reaction the samples were stored on -20 °C. The sample mixed with 1.2 mg/ml concetration derivatization agent diluted in absolute ethanol, and with 20 mM pH 8.5 borate buffer. The reaction mixture was incubated on 60 °C for 20 minutes. The derivatization buffer contained 1 μM L-cysteic acid as internal standard. Before the analysis the mixture was diluted fourfold with destillated water.

3.3 The excitatory amino acids were separated in pH 8 100 mM borate buffer containing 5 mM HPA-β-CD and 8 mM DM-β-CD, the effective capillary lenght was 50 cm and the used voltage was 400 V/cm on 25^{o}C. The sample was injected via hydrodynamic injection (20 s – 6.89 kPa). 32 Karat software version 5.0 provided by the manufacturer and was used to control the device, for data collection and for evaluating electroferograms. Dual cyclodextrin system was used to achive the necessary chemical and chiral selectivity. DM-β-CD was used

to maitain chemical selectivity between aspartate and glutamate, while HPA-β-CD was responsible for chiral selectivity for each enantiomer. Our developed method is capable to quantificate D-aspartate, L-aspartate, D-glutamate and L-glutamate. The method's limit of detection (LOD) is 17 nM for D-aspartate and 9 nM for D-glutamate. The limit of quantification was 0.05 μM for both amino acids.

The developed method capable to separate D-serine and the excitatory amino acids contained 50 mM HEPES buffer with 6 mM HPA-β-CD chiral selector. The effective and total capillary lenght were 50 and 60 cm, repsectively. Prior to each run the capillary was rinsed with distilled water and running buffer. Hydrodynamic injection was used to sample introduction similarly to our previous method (20 s – 6.89 kPa). The separation was carried on 400 V/cm voltage and 25^{o}C. 32 Karat software version 5.0 provided by the manufacturer and was used to control the device, for data collection and for evaluating electroferograms.

3.4 Animal experiments were carried out at Semmelweis University, Department of Anatomy, Histology and Embryology, while our research group was responsible for the sample analysis. 2, 4, 8 and 62 days old chicks from Babolna Ltd. (Budapest, Hungary) were used for excitatory amino acid determination. 100-150 days old wistar rats were derived from the local breed (Semmelweis University, Department of Anatomy, Histology and Embryology, Budapest). Chicks were held in groups of ten in a 33 x 40 x 25 cm box until they became 8 days old. After that four chicks shared a larger cage until they have reached adulthood (62 days old). Rats were kept in standard laboratory cages.

C57BL/6 mice (n=5) were used in the simultaneous detection of D-serine and D-aspartate, which were derived from Semmelweis University, Department of Anatomy, Histology and Embryology. Mice were kept in standard laboratory cages.

3.5 For the excitatory amino acid analysis, samples were collected from 2, 4, 8 or 62 days old chick and from 100-150 days old rats. Chicks (n=61 and 84, depends on the experiment) and rats (n=8) had been anesthetised with ketamine-xylazine and were decapitated. Remove of interested brain regions was done under a stereomicroscope and the samples were stored on -80 °C for further processing. Following histological structures were separated in chicks: medial striatum (mSt), intermediate medial mesopallium (iMM), arcopallium (Arco) and cerebellum. On rats the after decapitation the following brain regions were separated: parietal cortex (Ptctx), nucleus accumbens (Ac), amygdala (Amy) and cerebellum. Brain regions from birds are equivalent with some parts of the mammalian brain like: Ptctx with iMM, nucleus accumbens with mSt and amygdala with arcopallium.

All tissue samples were homogenized in 10 μl acetonitrile-water solution (2:1) with ultrasonic for 5 sec. After homogezation the samples were centrifuged (3000×g, 10 min, 4 °C).

Supernatants are diluted tenfold with artificial celebrospinal fluid (ACSF) then samples were derivatized.

3.6 The insertion of the devices for the microdialysis experiments were performed on the day the animals has arrived. The animals were anaesthetized with intramuscular injection of ketamine-xylazine (40 and 8 mg/bodyweight (kg)). The microdialysis tubes were inserted in the left side of medial striatum and fixed with dental cement to the cranial bone. The chicks (n=8) were placed in glass holders with opened top. The following solution was prefunded in the tubes with 0.3 µl/min: (120 mM Na^+, 6 mM K^+, 2 mM Ca^{2+}, 125 mM Mg^{2+}, 129 mM Cl^-, 125 mM $H_2PO_4^-$, 21 mM HCO_3^-, pH 7.4). Collection of the samples was started after 18-20 hours of stabilization period in 200 µl polypropylene tubes with 20 minutes intervals. Samples were immediately placed on dry ice and stored in -80°C until evaluation. After three time sample collection (60 mins) the chicks could drink ad libitum. After 100 minutes 50 mM KCl ACSF was prefunded for 20 minutes which was followed by the standard ACSF for further sample collection. Sodium ions were reduced during the use of KCl for maintaining osmolality.

3.7 For the evaluation of the amount of D-serin and D-aspartate in the brain 5-5 newborn and adult C57BL/6 mice were decapitated and the brains were sectioned for appropriate segments. Sample preparation was performed with a rostral cut which contained the medial orbitofrontal cortex and with a caudal cut which contained the amygdala, hippocampus and the hypothalamus. The tissues were measured and placed on dry ice. Samples were stored on -80°C until evaluation. Sample preparation was performed the same way as for the evaluation of excitatory amino acids but with only two-fold dilution.

3.8 The evaluation procedure of amino acids and D-serin was validated according to the relevant FDA guidelines. Internal standard was 1 µM L-cysteic acid. The calibration curves were prepared with the 6-6 standard solutions of D-serin and D-aspartate (0.05; 0.1; 0.25; 0.5; 1; 1.5 µM). To imitate the amount of L-amino acids in biological environment the samples contained L-glutamate, L-aspartate, L-serine, taurine and glycine was used in fifty times higher concentrations (2.5; 5; 12.5; 25; 50, 75 µM). In quality control (QC) samples low, medium and high concentration D-aspartate and D-serine solutions were used (0.08; 0.04; 1.25 µM) to determine the intra- and inter-day precision and accuracy. Standard deviation was used to evaluate system precision, while accuracy was defined as the measured concentration expressed in percentage of nominal concentration. 5-5 parallel runs were used for intra-day measurements and 3-3 parallels for inter-day measurements for five consecutive days. The limit of quantification (LOQ) was determined as the lowest concentration of the amino acid enantiomers that could be derivatized and measured with precision and accuracy values below

20 % and within 80–120%, respectively. The limit of detection (LOD) was determined as the concentration where the signal-to-noise ratio was 3:1.

4. Results

4.1 NBD-F was used in the Department of Pharmacodynamics as a derivatization agent to determine diamines and lysine and method optimization was completed. For excitatory amino acid quantification this optimized method was used, where the peak areas showed maximum using 20 mM pH 8.5 borate bufer for 15 minutes.

Time-dependence of derivatization was also examined for D-serine. After evaluation of electropherograms it was found that D-serine, D-and L-aspartate have higher peak areas, whereas L-glutamate showed no difference using 20 mM pH 8.5 borate buffer for 20 minutes. Therefore derivatization reaction time was elevated for 20 minutes.

4.2 a The derivatized amino acids possess one or two negative charge at alkaline or neutral pH, thus they have considerable electrophoretic mobility towards the anode. Their rapid analysis can be achieved by using anodic separation with suppression of the EOF. To achive EOF suppression capillary was coated with linear polyachrylamide similarly as in our developed method for excitatory amino acids. L-cysteic acid was used as an internal standard, because it has similar characteristic as L-aspartate and L-glutamate (bearing two negative charges on alkaline or neutral condition). The separation was performed on 50 cm effective lenght capillary. HPA-β-CD was appropriate for the chiral separation of aspartate and glutamate. It was planned to improve our method with extension of quantification the NMDA receptor co-agonist D-serine. D-glutamate was not found in biological samples originate from chick brain regions, therefore it was not necessary to use dual cyclodextrine system in our elaborated new method, which is capable to determine L-glutamate, D- and L-serine plus the serine enantiomers. The method development was started with 100 mM pH 8 borate buffer containing 5 mM HPA-β-CD as chiral selector. However, D-serine migrates just behind a broad tailing peak corresponding to glycine and decomposition products of NBD-F that may compromise its accurate quantification.

4.2 b Decreasing the pH of HEPES buffer between 8 and 7 both chemical and chiral selectivity improved. On the other hand below pH 8 changing the buffer component from borate to HEPES is required, which has proper buffer capacity in neutral condition. Glycine and the derivatization side products were succsessfuly separated from D-serine on pH 7. However, further pH decrease to pH 6.5 resulted in co-migration of aspartate and glutamate, thus 50 mM HEPES buffer pH 7 was chosen as background electrolyte.

4.2 c To continue method optimization the effect of chiral selector concentration on the separation was also studied. Increasing the concentration of HPA-β-CD between 3 and 7 mM resulted in enhanced chiral resolution and chemical selectivity: separation of D-serine from glycine and the decomposition product. Since no considerable difference in separations using 6 or 7 mM cyclodextrin concentration was found 6 mM HPA-β-CD was chosen as final chiral selector concentration.

4.2 d Several other amino acids were tested to avoid co-migration in biological samples, like GABA, taurine, glycine, methionine, alanine and valine. Taurine and glycine migrated before D-serine, while all other amino acids passed through later the detector window. GABA migrated at the closest to D-serine from the slower mobility components, but no resolution problem was occured (fig 1.).

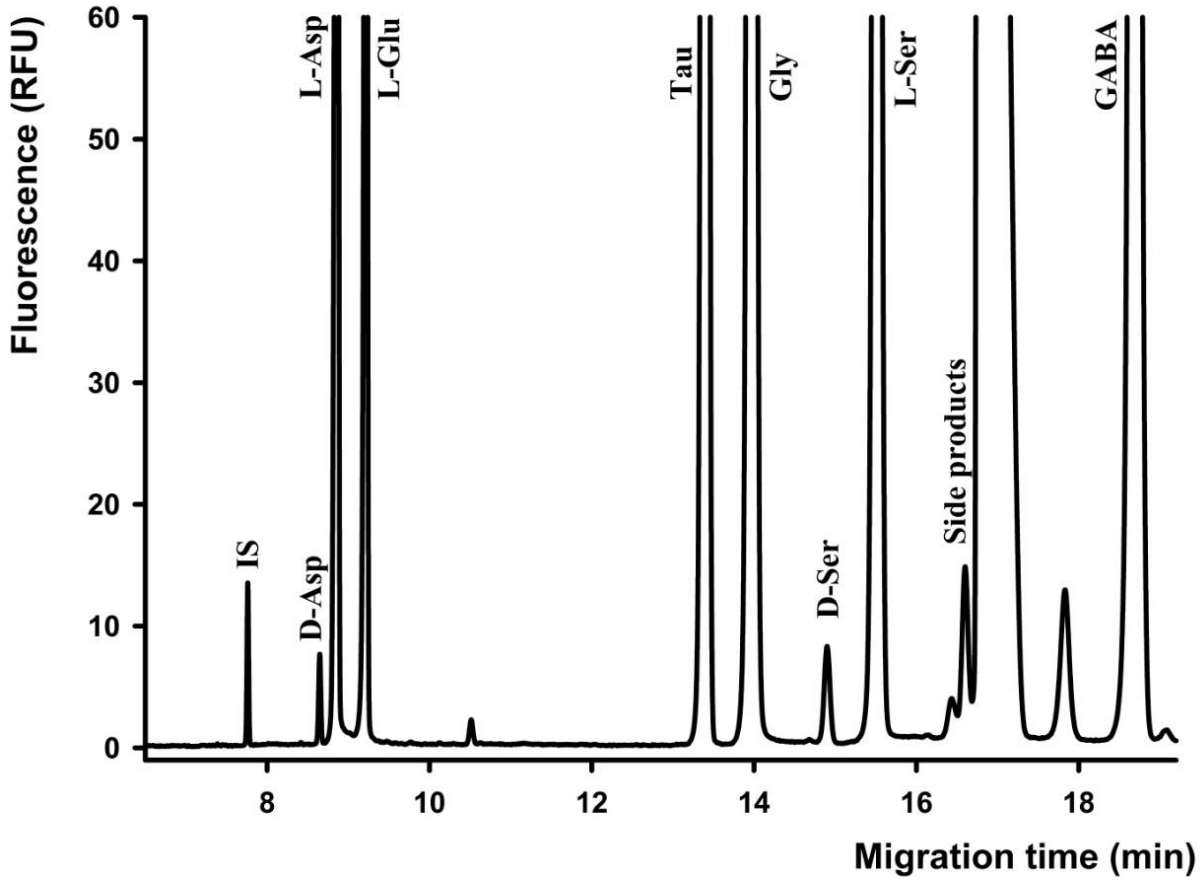

***Fig 1*: 1 µM L-cysteic acid internal standard (IS), standard sample containing 2.5 µM D-Asp and D-Ser, 25 µM L-Asp, L-Glu and L-Ser, 50 µM glycine (Gly), taurine (Tau) and GABA.**

Separation conditions: 50/60 cm x 75 µm fused capillary coated with polyachrylamide; injection: 6.89 kPa 20 s; 6 mM HPA-β-CD, 50 mM HEPES puffer pH 7; -24 kV.

4.2 e Because of the significant difference in the concentration of D- and L-amino acids in biological samples, a fifty-fold excess over D-enantiomers of L-aspartate, L-glutamate, taurine, glycine and L-serine was kept in all the samples used during the validation process. Calibration curves were set up between 0.05 and 1.5 μM concentracion intervall for D-aspartate and D-serine and between 2.5 and 75 μM for L-aspartate and L-glutamate and found linear correlation between peak area and concentration in these ranges. Intra- and inter-day precision and accuracy using quality control samples of 0.08; 0.4; 1.25 μM for D-aspartate and D-serine and 4; 20; 62.5 μM for L-aspartate and L-glutamate were within the required range. The intra-day accuracy for D-amino acids was between 85.10-105.94% and for L-amino acids between 85.05-112.11%. The intra-day precision was within 2.09-7.90 % for D-amino acids and 3.93-6.55 % for L-amino acids, respectively. The inter-day precision was within 0.44-9.47 % for D-amino acids and 0.95-14.90 % for L-amino acids, respectively. The LOQ values were determined for D-enantiomers on the basis of acceptable accuracy and found 0.05 μM for both D-aspartate and D-serine. The LOD values given as signal to noise ratio 3:1 were estimated 8 and 12 nM for D-aspartare and D-serine, respectively.

4.3 D-amino acid levels of six different brain regions of adult and newborn mice were measured. The following brain regions were examined: amygdala, bulbus olfactorius, cerebellum, hippocampus, hypothalamus and praefrontal cortex. In all samples D-aspartate and D-serine were detectable and quantified. D-serine concentration was around one magnitude higher in adult mice (14-50 nmol/g wet tissue) than in newborn ones compared to D-aspartate levels (1.9-3.2 nmol/g wet tissue) in all brain regions, except cerbellum, where concentrations were comparable (0.9-1.2 nmol/g wet tissue). On the other hand in newborn mice the two amino acids are comparable (11-22 nmol/ g wet tissue for D-aspartate, while 11-26 nmol/g wet tissue for D-serine). In newborn mice the D-aspartate level is around one magnitude higher compared to adult mice, which supports the important role of D-aspartate in neurogenesis. The amount of D-serine can be highlighted in three brain regions in adult mice compared to new born ones, like amygdala, hippocampus and praefrontal cortex. These three brain regions play crucial role in neuronal plasticity, learning processes and memory formation. An interesting result was found in the cerebellum, where a significant D-serine decrease was measured in adult mice.

4.4 a During the determination of excitatory amino acids it was found that age has a significant effect on D-aspartate concentration. Significant decrease of D-aspartate concentration was seen in four examined chick brain regions between the second and the fourth day and stayed low from now one. If the L-and D-aspartate concentration is expressed

in percentage (L-aspartate correlated to summary of L-aspartate plus D-aspartate), the age-dependent D-aspartate concentration decrease can be clearly seen next to an eqilibrium L-aspartate level.

4.4 b Age-dependent transient decrease can be seen for L-aspartate and L-glutamate on the fourth day. L-glutamate concentration showed significant difference between brain regions. The lowest concentration of L-glutamate was measured in the mSt/Ac areas.

4.4 c No significant difference of L-aspartate concentration was found in adult chicks and rats, however, the effect of region and the interaction of the two factors were significant. As revealed by post hoc paired t-tests, chicks had lower L-aspartate concentration in the arcopallium but only when compared to the cerebellum whereas rats showed the lowest L-aspartate concentration in the cerebellum.

4.4 d Accordingly, the main interspecies difference in L-aspartate was due to a significantly lower value in the cerebellum of the rat. When comparing the ratios (L-aspartate/L-aspaspartate + L-glutamate), these did not differ between the two species but they did among brain regions. The two variables also interacted

4.4 e Rats displayed a slightly lower L-glutamate concentration than did chickens but only in certain regions: the mSt/Ac and iMM/Ptctx. In chicks, L-glutamate concentration was lower in the mSt and in the cerebellum than in the iMM, while in rats, the nucleus accumbens showed lower L-glu concentration than any other regions. As revealed by tests of within-subject contrasts, mSt contained less L-glu than any of the other three regions.

4.4 f Adult chicks displayed greater D-aspartate levels than rats did in three of the four regions measured. However, D-aspartate concentration in the cerebellum did not differ in the two species. D-aspartate concentration varied among brain regions, when both rats and chicks were taken into account. There was no significant difference between any two of the brain regions of chicks, however, in rats, D-aspartate was most abundant in the cerebellum and least concentrated in the Ptcx. Essentially the same results were evident when the ratios (D-aspartate/L + D-aspartate) were taken into account.

4.5 We have tested the dynamic changes of extracellular L- and D-aspartate and L-glutamate in the Ac/mSt region, using in vivo microdialysis of freely moving chicks. Both L-amino acids showed simultaneous transient elevation on water administration (a reward stimulus for thirsty chicks). Similar but greater elevation of excitatory L-amino acid levels was evoked by the non-specific stimulus, high potassium. D-aspartate showed only a slight, non-significant elevation after water administration, but a robust (ca. tenfold) increase did follow potassium stimulation. Notably, the peak of D-Asp transient does not coincide with the period of high potassium-chloride administration, it merely follows the event with a delay. The L-aspartate

ratio proved to be remarkably stable throughout the microdialysis samples and also similar to the ratio observed in tissue extracts. Notably, however, the ratio of D-aspartate was almost doubled during potassium stimulation, amounting to 40% of total (both L and D) aspartate, whereas its concentration detected in tissue extract was considerably lower than that of L-aspartate by ca. two orders of magnitude.

5. Discussion

5.1 a I was involved in the development of chiral capillary electrophoresis methods capable to quantify excitatory amino acids (D- and L-aspartate, L-glutamate) and D-serine in biological samples. In collaboration with Semmelweis University, Department of Anatomy, Histology and Embryology with our elaborated method we have measured the amounts of excitatory amino acids from different chicken brain regions and as a validation of our method's precision and sensitivity we have compared our results with the results were found in the literature derived from biological samples. To achive appropriate chemical and chiral selectivity a dual cyclodextrin system was developed, where DM-β-CD was responsible to maintain chemical selectivity between L-aspartate and D-glutamate, while HPA-β-CD was used for ensuring chiral selectivity between enantiomers.

5.1 b High D-aspartate levels were measured in young chiks compared to adult ones. Our results showed no difference of L-aspartate and L-glutamate in different ages, but did for D-aspartate. The L-amino acid levels were the lowest on the fourth day, but later the concentration was constant. Expressing D-aspartate level in percentage correlated to the total aspartate amount was the highest on the second day. Three brain regions were examined, which have got crucial role in imprinting mechanism and passive avoidance test. These regions are the iMM, mSt and the arcopallium, which were analysed together with the known mamalian homologue brain regions (Ptcx, nucleus accumbens and amygdala). Cerebellum was used as a control for both species. According our results roboust correlation can be seen in the mSt/nucleus accumbens regions, where lower L-glutamate level was measured in both species compared to the other brain regions. The literature supports our results as in these areas mostly GABAergic innervations can be found with several excitatory terminals with glutamate and aspartate, but glutamatergic and aspartatergic perikaryon is less typical.

5.1 c Water intake only increased slightly D-aspartate amount in the extracellular matrix during leraning process examination. On the other hand significant amount of D-aspartate could be measured after KCl perfundation. Opposite to promt L-aspartate and L-glutamate elevation (which was also very roboust) D-aspartate liberation was sustained (ca. for one

hour) and only after returned to baseline level due to the effect of potassium. This plato could be explained by some kind of secondary mechanism after promt release of D-aspartate due to potassium, however further research is necessary to understand this complicated mechanism. According to our results it was found that tissue level of D-aspartate is lower ca. two magnitudes than L-aspartate while in the extracellular matrix suprisingly high amount of D-aspartate is released. Due to KCl around one third of total aspartate is the D-enantiomer. Significant L-aspartate and L-glutamate amount was measured due to learning mechanism (water drinking) and the effect of potassium.

5.2 a During my research period I have developed a validated chiral capillary electrophoresis method capable to quantify aspartate enantiomers, D-serine and L-glutamate. Using HPA-β-CD as a chiral selector baseline separation was achieved for D-and L-aspartate plus D-and L-serine. In the new method development dual cyclodextrin system was not necessary as biological samples are not containing D-glutamate, thus we have returned only HPA-β-CD containing system. During the method development the aspartate enantiomers baseline separation was achieved, however D-serine tended to co-migrate with glycine and derivatization side producs derived from NBD-F. The first goal of the method development is to increase the chemical selectivity between D-serine and the side products and glycine. Derivatized amino acids with NBD-F are bearing one or two negative charges in neutral pH, thus rapid analysis can be achieved by anodic separation. NBD-F has got relatively fast derivatization reaction kinetics and capable to reagate with primer and secunder amines forming relatively few side products. Biological matrix factors were also has taken into account. Standard samples were dissolved in artificial celebrospinal fludium, imitating the matrices of brain samples. Our method has been validated according to the FDA guidelines, moreover we have expected with the high amount of L-amino acids in the biological samples.

5.2 b During the separation of D-serine, glycine and NBD-F derivates originates mainly from hydrolysis of NBD-F it was found that pH has notable effect on chemical selectivity. It was known from the literature, that the main decomposition products of NBD-F were claimed to be its phenolic derivatives and as the phenolic hydroxyl group is considerably less acidic compared to the carboxylic moiety of the amino acids, the charge and migration characteristic of these compounds are pH dependent in the neutral and slightly alkaline range. At lower pH the charge and mobility of the phenolic compounds decrease, while those of the amino acids are largely unaffected. On the other hand the charge of the chiral selector increases resulting in a stronger electrostatic interaction with the amino acids and slower mobility of the complexes. This latter provides a more considerable difference between the mobility of the free analytes and their complexed forms. Reducing the pH thus may have significant effect on

both chiral and chemical selectivity. Since below pH 8 the buffer capacity of borate declines, 50 mM HEPES buffer was used in the further experiments. Decreasing the pH of HEPES buffer between 8 and 7 both chemical and chiral selectivity improved. As further pH decrease to 6.5 resulted in compromised separation of aspartate and glutamate due to the increasing of non-enantiomer specific ionic interaction between the cyclodextrine and the acidic excitator amino acids, thus both chemical and chiral selectivity was ceased.

5.2 c During method development all amino acids were examine, which occur in biological samples and there is a possibility to co-migrate with D-serine. The following amino acids migrated through the detector window after method optimization: GABA, taurine, glycine, methionine, alanine, treonine and valine. Taurine and glycine migrated before D-serine, while all the other substances arrived later to the detector window. From these slower substances GABA was the closest to D-serine, but no resolution problem was occured.

5.2 d Limit of quantification (LOQ) was 0.05 μM for examined compounds. Our developed method has been tested on newborn and adult C57BL/6 mice (n=5-5). In different brain regions, where high number of NMDA receptors can be found we have measured high amount of D-serine. These brain regions are playing crucial role in memory formation like hippocampus, praefrontal cortex and amygdala. Both D-aspartate and D-serine showed age-dependent difference. In young mice one magnitude higher amount of D-aspartate can be found compared to adult mice, independently from brain regions. Similar results were obtained from chicken brain measurements. On the other hand D-serine amount in adulthood is significant higher in brain regions involved in memory formation, while similar amount can be found in the other brain regions compared to young mice (except cerebellum). In cerebellum we have measured lower D-serine amount, which correlates with the brain concentration has been described in the literature. Our developed method is capable to quantify in one run these two very important D-amino acid concentrations from various biological samples.

6. Publications

***Publications in the topic of dissertation*:**

Wagner Zs, Tábi T, **Jakó T**, Zachar G, Csillag A, Szökő É.
Chiral separation and determination of excitatory amino acids in brain samples by CE-LIF using dual cyclodextrin system
Anal. Bioanal. Chem., 2012; 404, 2363-2368. (IF: 3,778)

Jako T, Szabo E, Tabi T, Zachar G, Csillag A, Szoko E
Chiral analysis of amino acid neurotransmitters and neuromodulators in mouse brain by CE-LIF.
ELECTROPHORESIS 35:(19) pp. 2870-2876. (2014) (IF: 3,028)

Gergely Zachar, **Tamás Jakó**, István Vincze, Zsolt Wagner, Tamás Tábi, Eszter Bálint, Szilvia Mezey, Éva Szökő, András Csillag
Age-related and function-dependent regional alterations of free L- and D-aspartate, and L-glutamate in postembryonic chick brain
Acta Biol. Hung. 69(1):1-15. (2018)