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Research Article

Chiral analysis of amino acid neurotransmitters and neuromodulators in mouse brain by CE-LIF

Chiral CE method has been developed for quantitative determination of D-amino acid modulators of NMDA glutamate receptor; D-serine and D-aspartate along with L-glutamate and L-aspartate in biological samples. These ligands are suggested to be involved in regulation of NMDA receptor related brain functions, such as neurogenesis, neuronal plasticity, and memory formation. For sensitive determination of the amino acids LIF detection was chosen, and a fluorogenic reagent, 7-fluoro-4-nitro-2,1,3-benzoxadiazole was used for derivatization. An amino-modified β -CD, 6-monodeoxy-6-mono(3-hydroxy)propylamino- β -CD (HPA- β -CD) was applied as chiral selector. Determinations were accomplished in a polyacrylamide coated capillary and reverse polarity was used for the analysis of the negatively charged analytes. The method was optimized and validated; 6 mM HPA- β -CD in 50 mM HEPES buffer, pH 7 was appropriate to achieve baseline separation of the analytes. The limit of quantification with acceptable accuracy is 0.05 μ M for both D-amino acids. The method was used for the determination of D-aspartate and D-serine content in various brain regions of adult mice.

Keywords:

Amino acids / CE-LIF / Chiral separation / NMDA receptor modulators

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1 Introduction

Contrary to a long time belief recent results suggest that beside L-amino acids, smaller amount of their D-enantiomers are also formed in mammalian tissues. D-serine and D-aspartate are, for example, present in the brain and are suggested neuromodulators of the N-methyl-D-aspartate (NMDA) glutamate receptor [1, 2]. NMDA glutamate receptor plays a crucial role in higher brain functions, for example, in memory formation and learning processes, which are amongst the most complicated neuronal activities [3]. The receptor shows diverse subunit composition and their distribution in various brain regions results in different individual receptor activation and function. Simultaneous binding of an agonist and a coagonist is needed to activate the receptor [4]. The typical agonist is L-glutamate, while L-aspartate and D-aspartate can also bind to the primary binding site of the receptor [1, 5] however, their function has not been clarified so far. D-aspartate probably has an important role in neurogenesis, as its concentration has been found significantly higher in brain tissues of newborn animals compared to elder ones from various

species [6]. Its neuroendocrine modulator function has also been suggested [7].

Previously, glycine has been identified and regarded as the main ligand of the coagonist binding site. However, recent data suggest that D-serine plays an even more important role as a coagonist of the NMDA receptor [4, 8]. Its distribution correlates with NMDA receptor abundance in the brain [2], although its regulatory function is only partially understood.

Determination of the NMDA receptor agonist amino acids and the coagonist D-serine in biological samples can be problematic, since the sample volume is limited and the concentration of the D-enantiomers can be rather low compared to their L-enantiomers. Although there are some HPLC methods reported for the analysis of various D-amino acids [9–11], the outstanding separation power of CE makes this technique ideal for chiral resolution and analysis [12, 13]. The optical detection of the amino acids needs some chemical modifications, because of the lack of easily detectable group. Since LIF provides higher sensitivity compared to conventional UV absorbance detection, it could have significant advantage in the analysis of low level of D-amino acids in biological samples. There are several amine derivatization reagents suitable for amino acid analysis by CE. The most common laser source used in commercially available CE-LIF instruments is the 488 nm Argon ion laser. Labeling reagents with excitation

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Abbreviations: HPA- β -CD, 6-monodeoxy-6-mono(3-hydroxy)propylamino- β -CD; NBD-F, 7-fluoro-4-nitro-2,1,3-benzoxadiazole; NMDA, N-methyl-D-aspartate

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fitting this wavelength, like the fluorescein based labeling tags; FITC, carboxyfluorescein succinimidyl ester, etc. are thus widely used [14–16]. However, they possess fluorophore moiety, and the high excess of the reagent together with the many fluorescent side products formed in the derivatization reaction make the separation task rather complicated. Previously, another labeling reagent with excitation wavelength fitting the Argon ion laser, 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) was successfully used for CE analysis of amino acids and amines by our research group [15, 17, 18]. NBD-F is a fluorogenic tag and only a few fluorescent decomposition products are formed during derivatization, which is especially important in chiral separations, where the number of peaks to be separated is doubled. At a relatively high temperature (55–75°C) NBD-F reacts rapidly (5–20 min) with primary and secondary amines and because of these advantages it is getting to be a popular labeling reagent [11, 19].

In order to achieve chiral separation addition of a chiral selector to the separation buffer is indispensable. CDs are the most preferred chiral selectors, due to their versatile complex forming ability [20, 21]. The most commonly used CDs for separation of amino acid enantiomers are β -CD and γ -CD and their methylated and hydroxypropylated derivatives [20]. However, CDs with ionizable groups have gained interest in electrophoretic separations, because they possess electrophoretic mobility and may form more stable complexes with oppositely charged analytes [22]. Previously our research group has successfully used a dual CD system containing an amino-CD derivative, 6-monodeoxy-6-mono(3-hydroxy)propylamino- β -CD (HPA- β -CD) providing chiral recognition in the separation of aspartate and glutamate enantiomers [17].

The aim of the present study is to extend our previous work for the analysis of D-amino acid modulators of NMDA glutamate receptor, including D-serine, and the simultaneous quantitative determination of NMDA receptor ligands: D-serine, D- and L-aspartate and L-glutamate by CE.

2 Materials and methods

2.1 Instrumentation

All the experiments were carried out on a P/ACE MDQ capillary electrophoresis system controlled by 32 Karat software version 5.0 (Beckman Coulter, Brea, CA, USA). For the determination of amino acids the system was coupled with LIF detector, containing an Argon-ion laser source, with excitation and emission wavelengths of 488 and 520 nm, respectively. For the separations fused silica capillaries of 75 μ m id and 365 μ m od (Agilent Technologies, Santa Clara, CA, USA) were applied. Before use the inner surface of the capillaries were coated with linear polyacrylamide according to the method of Hjertén et al. [23]. The effective and total lengths of the capillaries were 50 and 60 cm, respectively. Before each run the capillary was rinsed with water and separation buffer.

The samples were introduced with hydrodynamic injection (6894 Pa for 20 s). All separations were carried out at 25°C by applying constant voltage of –24 kV (400 V/cm).

2.2 Chemicals

L-Glutamate, D-serine, L-serine, L-aspartate, D-aspartate, cysteic acid (internal standard), NBD-F, HEPES, and boric acid were obtained from Sigma (St. Louis, MO, USA). The pH of the borate and the HEPES buffer was adjusted with 1 M sodium hydroxide solution. HPA- β -CD was provided by Cyclolab (Budapest, Hungary). All amino acids were dissolved in artificial cerebrospinal fluid, which was prepared at the Central Pharmacy of Semmelweis University (Budapest, Hungary). In all experiments ultrapure water from MilliQ Direct 8 water purification system (Merck Millipore, Billerica, MA, USA) was applied.

2.3 Samples

Five adult male C57BL/6 mice were decapitated and their brains were dissected immediately. A rostral slice containing the medial orbitofrontal cortex, and a caudal one containing part of the amygdala, hippocampus, and hypothalamus were cut using brain matrices. The rostrocaudal stereotaxic coordinates for the slices and the location of brain regions were chosen according to the Allen Mouse Brain Atlas [24]. Slices were approximately 1.5 mm thick. The bulbus olfactorius and the dorsal part of the cerebellum were dissected directly from the whole brain. The other regions of interest were dissected from the slices using a stereomicroscope. Tissue samples were weighed and put on dry ice immediately after dissection. They were stored at –80°C until further processing. Brain tissue samples were homogenized by sonication for 5 s in ACN: water 2:1 solution (10 μ L/mg). To remove precipitated proteins samples were centrifuged at 3000 $\times$ g for 10 min at 4°C. The supernatants were collected and diluted twofold with artificial cerebrospinal fluid.

2.4 Derivatization procedure

In order to derivatize amino acids 5 μ L sample was mixed with 5 μ L derivatization buffer of 20 mM pH 8.5 borate, containing 1 μ M cysteic acid as internal standard and 5 μ L 5 mg/mL ethanolic NBD-F solution. The mixture was heated at 65°C for 20 min and the derivatized samples were kept at –20°C until analysis.

2.5 Method validation

Validation procedure was completed according to the Food and Drug Administration Guidance for Industry [25]. Standard samples of six different concentrations (0.05, 0.1, 0.25, 0.5, 1, 1.5 μ M) of D-aspartate and D-serine were used to

construct the calibration curve with three parallels. To imitate the biological samples L-glutamate, L-aspartate, L-serine, taurine, and glycine were used in 50 times higher concentrations. The concentration of the internal standard in the samples was 1 μ M. 1/x Weighted linear regression was used to construct the calibration curves. Quality control samples at three concentration levels (0.08; 0.4; 1.25 μ M of D-aspartate and D-serine) were prepared to test intra- and inter-day accuracy and precision of the method. Intraday data result from five parallel measurements and interday data are from determinations performed in five consecutive days with three parallel runs. The 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 LOD was determined as the concentration where the S/N was 3:1.

3 Results and discussion

The derivatized amino acids possess one or two negative charge at alkaline or neutral pH, thus they have considerable electrophoretic mobility toward the anode. Their rapid analysis can be achieved by using anodic separation with suppression of the EOF. Linear polyacrylamide coated capillary was thus used for the separations. Cysteic acid was chosen as an internal standard, because of its similar characteristics of charge and size to those of aspartate and glutamate.

Previously we have found that a positively chargeable amino CD, HPA- β -CD was appropriate for the chiral separation of aspartate and glutamate [17]. In those previous experiments we studied the D-aspartate and D-glutamate content in various brain regions in experimental animals. Only D-aspartate has been found and quantified, while D-glutamate could not be detected. In the present experiments we aimed to extend our work to separate serine enantiomers, as well. Since detectable amount of D-glutamate was not expected in the biological samples, the method development was started with 100 mM pH 8 borate buffer containing 5 mM HPA- β -CD as chiral selector. As amine derivatized aspartate and glutamate bear two negative charges they migrate ahead of the other amino acids, while serine is more similar to the majority of the amino acids having one negative charge. Various amino acids were thus included in the standard mixture sample to study their separation from serine enantiomers. Taurine and glycine have been found to migrate in their close proximity. Most of the other amino acids, for example, alanine, threonine, valine, and γ -aminobutyric acid have slower velocity. Using these separation conditions baseline separation of analytes of interest; D-aspartate, L-aspartate, L-glutamate, D-serine, and L-serine (Fig. 1) was achieved in less than 14 min. However, D-serine migrates just behind a broad tailing peak corresponding to glycine and some decomposition products of NBD-F that may compromise its accurate quantification. The improvement of their resolutions was thus aimed. Further increase in the CD concentration or incorporation of methanol as organic modifier to the BGE could not improve

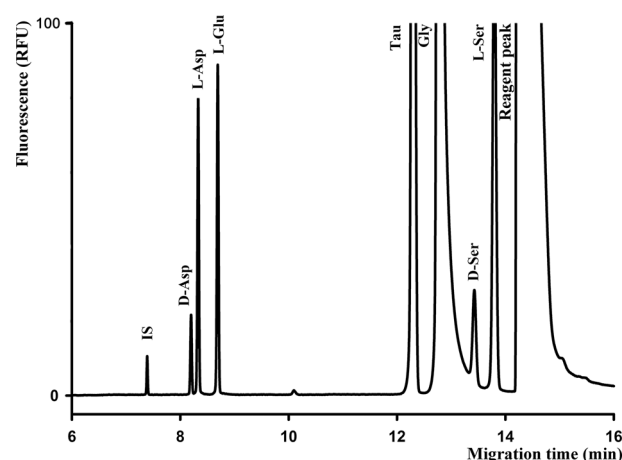


Figure 1. An electropherogram of standard mixture containing 25 μ M L-glutamate (L-Glu), L-aspartate (L-Asp), and L-serine (L-Ser); 5 μ M D-aspartate (D-Asp), and D-serine (D-ser); 50 μ M glycine (Gly) and taurine (Tau) with 1 μ M cysteic acid internal standard (IS). Separation conditions: BGE:100 mM pH 8 borate buffer with 5 mM HPA- β -CD; 50/60 cm $\times$ 75 μ m id coated fused-silica capillary, –24 kV. Sample introduced by 6894 Pa pressure for 20 s.

the chemical selectivity. The main decomposition products of NBD-F were claimed to be its phenolic derivatives [26] 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. Moreover the charge of the chiral selector ($pK_a = 8.7$ [27]) also alters in this pH 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. At pH 8 in HEPES buffer altered chemical and chiral selectivity were observed compared to borate. Besides decreased chiral resolution the migration time of the main decomposition products changed considerably resulting in more pronounced comigration of D-serine with glycine. This may be explained by the complex forming property of borate with sugars [28].

Decreasing the pH of HEPES buffer between 8 and 7 both chemical and chiral selectivity improved (Fig. 2A and Table 1). According to our expectations the migration time of decomposition products increased considerably and at pH 7 all migrated behind L-serine. At the same time the chiral resolution enhanced likely due to the greater mobility difference between the complex and free forms of amino acids. As further pH decrease to 6.5 resulted in compromised separation of aspartate and glutamate, 50 mM HEPES buffer of pH 7 was chosen to use.

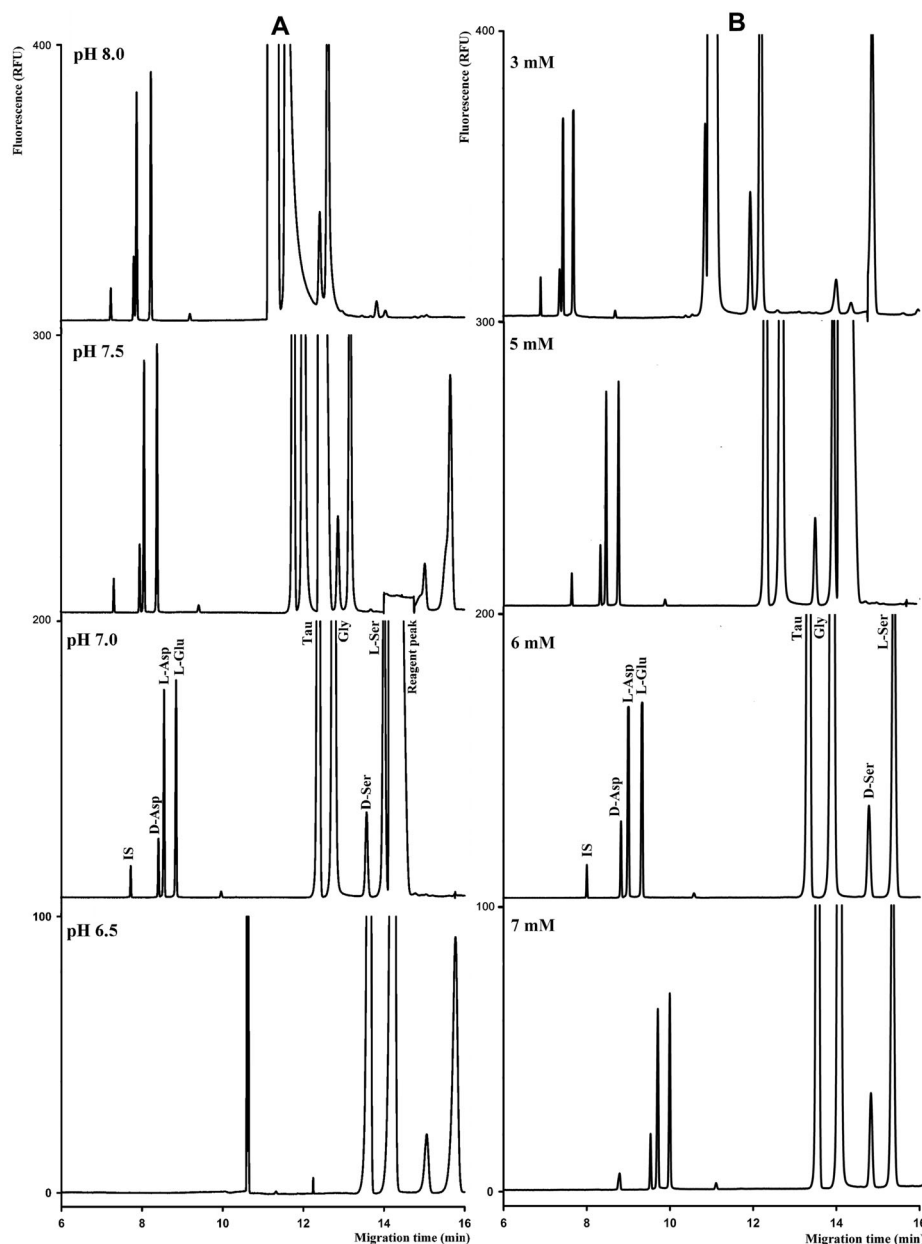


Figure 2. Electropherograms of standard mixture containing 25 μM L-Glu, L-Asp and L-Ser; 5 μM D-Asp and D-ser; 50 μM Gly and Tau with 1 μM cysteic acid internal standard (IS). Separation conditions: (A) BGE: 50 mM pH 8, 7.5, 7, 6.5 HEPES buffer with 5 mM HPA- β -CD, (B) BGE: 50 mM pH 7 HEPES buffer with 3, 5, 6, 7 mM HPA- β -CD; 50/60 cm $\times$ 75 μm id coated fused-silica capillary, -24 kV. Sample introduced by 6894 Pa pressure for 20 s.

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 (Fig. 2B and Table 1). Since no considerable difference in separations using 6 or 7 mM CD concentration was found 6 mM HPA- β -CD was chosen as final chiral selector concentration.

3.1 Validation

Because of the significant difference in the concentration of D- and L-amino acids in biological samples, a fifty-fold ex-

cess over D-enantiomers of L-aspartate, L-glutamate, taurine, glycine, and L-serine was kept in all the samples used during the validation process. The calibration curves for D-aspartate, D-serine, and L-aspartate, L-glutamate were constructed in the 0.05–1.5 μM and 2.5–75 μM concentration range, respectively, and found linear. The equations of calibration curves for D-aspartate and D-serine were $y = 0.7009 (\pm 0.08969) - 0.0151 (\pm 0.03524)$ and $y = 0.2689 (\pm 0.03196) - 0.0076 (\pm 0.03644)$ with R^2 values 0.9988 and 0.9985, respectively, ($n = 5$). The equations of calibration curves for L-aspartate and L-glutamate were $y = 1.1053 (\pm 0.13167) - 0.8281 (\pm 1.51156)$ and $y = 1.1036 (\pm 0.14967) - 0.1076 (\pm 1.49164)$ with R^2 values 0.9972 and 0.9972, respectively ($n = 5$). Intra- and inter-day precision and accuracy using quality control samples of 0.08; 0.4; 1.25 μM for D-aspartate and

Table 1. The resolution and selectivity data of D/L-aspartate, D/L-serine, glycine/D-serine at different separation conditions

Conditions	Peaks	Resolution (Rs)	Selectivity (α)
100 mM pH 8 Borate buffer 5 mM HPA- β -CD	D/L-Asp	1.71	1.016
	Gly/D-Ser	1.39	1.052
	D/L-Ser	1.71	1.027
50 mM pH 8 HEPES buffer 5 mM HPA- β -CD	D/L-Asp	1.15	1.009
	Gly/D-Ser	1.44	1.072
	D/L-Ser	1.17	1.016
50 mM pH 7.5 HEPES buffer 5 mM HPA- β -CD	D/L-Asp	1.52	1.013
	Gly/D-Ser	2.53	1.072
	D/L-Ser	1.52	1.023
50 mM pH 7.0 HEPES buffer 5 mM HPA- β -CD	D/L-Asp	1.63	1.017
	Gly/D-Ser	2.68	1.064
	D/L-Ser	1.84	1.032
50 mM pH 6.5 HEPES buffer 5 mM HPA- β -CD	D/L-Asp	NR	NR
	Gly/D-Ser	2.21	1.057
	D/L-Ser	2.00	1.048
50 mM pH 7.0 HEPES buffer 3 mM HPA- β -CD	D/L-Asp	1.38	1.011
	Gly/D-Ser	2.61	1.088
	D/L-Ser	1.49	1.021
50 mM pH 7.0 HEPES buffer 6 mM HPA- β -CD	D/L-Asp	1.76	1.020
	Gly/D-Ser	2.73	1.063
	D/L-Ser	2.21	1.040
50 mM pH 7.0 HEPES buffer 7 mM HPA- β -CD	D/L-Asp	1.79	1.020
	Gly/D-Ser	2.89	1.057
	D/L-Ser	2.32	1.037

D-serine and 4; 20; 62.5 μ M for L-aspartate and L-glutamate were within the required range. The intraday accuracy for D-amino acids was between 85.10 and 105.94% and for L-amino acids between 85.05 and 112.11%. The intraday precision was within 2.09–7.90% for D-amino acids and 3.93–6.55% for L-amino acids, respectively. The interday 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 μ M for both D-aspartate and D-serine. The LOD values given as S/N 3:1 were estimated 8 and 12 nM for D-aspartate and D-serine, respectively.

3.2 Biological samples

The method has been applied for the determination of D-amino acid distribution in mouse brain. A representative electropherogram of brain sample is shown in Fig. 3. Six different brain regions; amygdala, bulbus olfactorius, cerebellum, hippocampus, hypothalamus, and prefrontal cortex were analyzed. In all examined brain areas D-aspartate and D-serine were detected and quantified. The concentration of D-serine (14–50 nmol/g wet tissue) was about one order of magnitude higher compared to that of D-aspartate (1.9–3.2 nmol/g wet tissue) in all brain areas but cerebellum, where their concentrations were comparable (0.9 and 1.2 nmol/g wet tissue, respectively), as shown in Fig. 4. Both D-serine and D-aspartate seemed to be most abundant in brain regions known as important in memory and learning. Amyg-

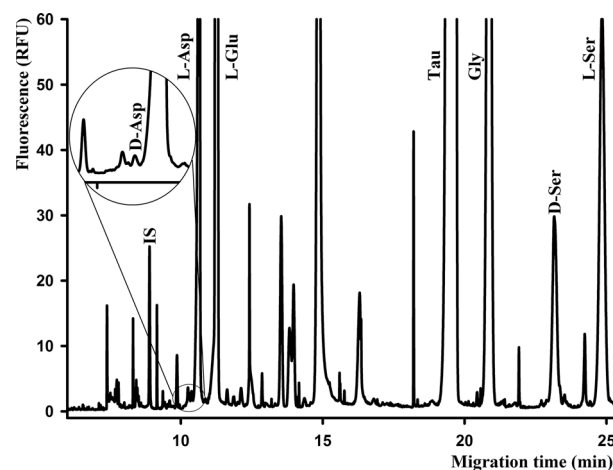


Figure 3. An electropherogram of a prefrontal cortex sample of an adult mouse. BGE: 50 mM pH 7 HEPES buffer with 6 mM HPA- β -CD; 50/60 cm $\times$ 75 μ m id coated fused-silica capillary, –24 kV. Sample introduced by 6894 Pa pressure for 20 s.

dala is suggested to be involved in implicit memory, while hippocampus and prefrontal cortex in explicit memory formation [3, 29–31].

4 Concluding remarks

NMDA receptor activation has been justified to be important in learning processes and memory formation. The precise function of D-serine and D-aspartate is not well established,

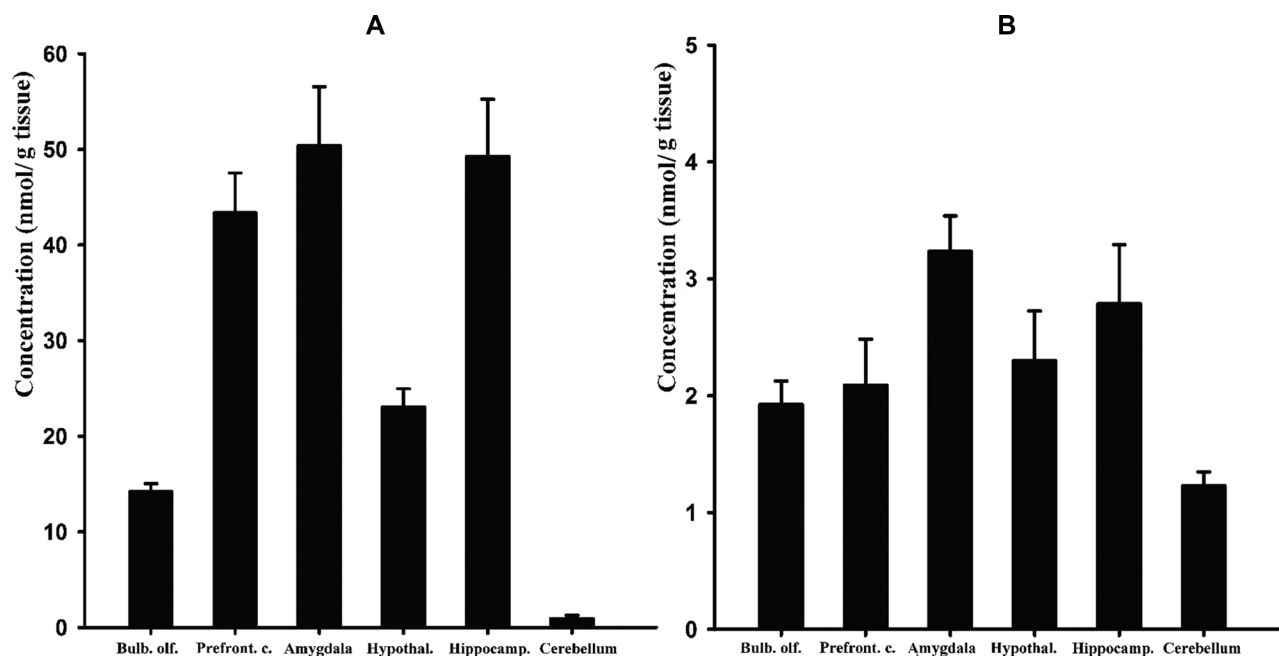


Figure 4. Diagrams show the amount of D-serine (A) and D-aspartate (B) in different brain regions.

although their modulator function on NMDA receptors has been known. To simultaneously determine these amino acids a validated, sensitive chiral CE method with LIF detection has been developed. The optimized method using 6 mM HPA- β -CD as chiral selector in 50 mM HEPES buffer of pH 7 was appropriate for separation and quantification of D-aspartate and D-serine along with L-aspartate and L-glutamate. D-Amino acids have been determined with an LOQ of 0.05 μ M concentration. The method was used to measure neuromodulator amino acids in different regions of murine brain. According to our preliminary results areas involved in memory formation, for example, amygdala, hippocampus and prefrontal cortex showed higher level of D-serine and D-aspartate compared to other brain regions. These results indicate that the elaborated method is capable of determination of these D-amino acids in various biological samples.

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The authors have declared no conflict of interest.

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