MEASURING D-AMINO ACIDS IN THE CENTRAL NERVOUS SYSTEM USING CAPILLARY ELECTROPHORESIS

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DISSEPTION

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Abstract

Understanding what molecules are used by neurons and glia for cell-to-cell signaling is important as this knowledge provides insights into neuronal network function within the brain. Even after decades of research, novel signaling molecules remain to be uncovered in the nervous system. Since their discovery in the central nervous system, D-amino acids (D-AAs) have drawn intense research interest because several of them are involved in signal transmission processes and brain development. However, a lack of appropriate analytical tools has prevented more refined studies to elucidate the roles of D-AAs in intercellular signaling. Here new and improved analytical platforms are described with the aim of sampling the complex neuronal microenvironment, accomplishing chiral separations and quantifying low abundant D-AAs reliably.

Among D-AAs found across the metazoan, two D-AAs are studied extensively, namely D-serine (D-Ser) and D-aspartate (D-Asp). D-Ser can be synthesized endogenously by serine racemase which converts L-Ser to D-Ser. D-Ser can affect the activity of N-methyl-D-aspartate (NMDA) receptor, which is an important receptor involved in learning and memory formation. D-Asp levels correlate with embryonic development stages and D-Asp concentrations drop to much lower levels in adult animals. Through the past ten years of studying D-Asp within Sweedler group, we have determined its neuronal localization, biosynthesis, transport, and demonstrated several of the physiological responses it elicits within Aplysia californica (A. californica).

Capillary electrophoresis (CE) is an ideal method to characterize D-AAs in the central nervous system due to the compatibility of CE with small size sample and the low detection limits it provides when coupled with laser-induced fluorescence (LIF) as a
detection method. Through a variety of CE measurements, two main questions are answered in this thesis. First, is D-Asp released from A. californica neurons and is it released in a stimulation-dependent manner? Second, is D-Ser released from glia via a vesicular release pathway? The first question was answered by selectively extracting and quantitatively measuring the extremely diluted D-Asp from the releasates of A. californica ganglia. Our results indicate a stimulation-dependent release of endogenous D-Asp, which is the last criterion needed to be satisfied to show that D-Asp meets the requirements of a traditional neurotransmitter within A. californica. The second question was addressed by quantifying the amino acid contents in intact and leaked glial vesicles. Various CE methods were incorporated to validate the quantitation results. These analytical measurements clearly show the enrichment of D-Ser, along with another signaling molecule L-glutamate (L-Glu), inside glial vesicles. Together with the results of biochemical assays from our collaborator, our data demonstrate D-Ser being stored in glial vesicles and released from glia via vesicular release pathway.
To my parents and my fiancé
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Chapter 1
Introduction

1.1 Research summary

The research in Sweedler group is conducted at the interface of bioanalytical chemistry and neuroscience. On one hand, we develop analytical platforms enabling new measurements that drive scientific advances. On the other hand, animal model systems are carefully chosen to fit the specific questions that we are trying to answer under the umbrella of neuroscience. One of the long-term projects within the group is to elucidate unusual neurochemical pathways, and more specifically novel cell-to-cell signaling molecules. A prime example of such an overlooked class of putative signaling molecules is the D-amino acids. The lack of appropriate analytical approaches has hindered the investigation of the role of chirality in signal transmission. Previous group members in this project had set the background for these experiments [1-5] and created the solid foundation on which I started my efforts. In my research under the guidance of Professor Jonathan Sweedler at the University of Illinois, I studied the role that D-Asp and D-Ser plays in the central nervous system. This research, like much of the research in the group, required the development of new analytical methods in order to better access these D-AAs from the brain, and then measure them to answer several critical questions such as their presence in vesicles and their release in a chemical-stimulation dependent manner.

In this chapter, I give background on the foundations of this research. First, neurochemistry is discussed with an emphasis on D-AAs as putative signaling molecules in the central nervous system. Secondly, capillary electrophoresis as the main technique
used throughout this research is described, focusing on its usage in neuroscience studies. Finally, this chapter ends up with an overview of each chapter in this thesis.

1.2 Neurochemistry and signaling molecules

Cells within nervous system communicate in two ways, both chemically and electrochemically. The cell-cell chemical signaling uses chemical compounds released from one cell and produces information flow in the following cell(s) by activating specific receptors. Chemical communication is oftentimes categorized as either classical neurotransmission (using classical neurotransmitters) and neuromodulation (via molecules such as neuropeptides).[6] Classical neurotransmitters are released by the nerve ending of a neuron and cause strong and fast responses in adjacent neurons by direct gating of ion channels. Neuromodulators are not the same. They can have long-term effects that are slower to appear; they can trigger responses that involve second messengers; they can alter the sensitivity of postsynaptic neurons instead of directly acting on them and they are not necessarily released from nerve terminals.[7] Traditional neurotransmitters include small molecules such as L-glutamate (L-Glu), γ-aminobutyric acid (GABA), glycine (Gly), and acetylcholine. In order to define a compound as a classical neurotransmitter, it has to satisfy five criteria.[8] Firstly, the compound is present in the presynaptic neuron. Secondly, it is biosynthesized within that presynaptic neuron. Thirdly, the compound is released under chemical and/or electrical stimulation. Fourthly, it causes polarization of the postsynaptic neuron. Lastly, there is a specific reuptake mechanism for its recovery. It is important to realize that many molecules no longer are categorized so neatly, such as nitric oxide, ATP, adenosine. Regardless, the formalism of the classical transmitter still is useful in many cases.
Recently, several free D-AAs have been found in the brain and endocrine tissues of rats, mice, human beings and many invertebrate nervous tissues.[9] Among them, D-serine (D-Ser) and D-aspartate (D-Asp) are the best studied and understood. The biosynthesis of D-Ser is catalyzed by serine racemase, which converts of L-Ser to D-Ser.[10] Moreover, studies reveal that D-Ser is a co-agonist of the N-methyl-D-aspartate (NMDA) receptor,[11] which is an essential receptor required for long-term memory formation. D-Asp levels correlate with embryonic development stages and its concentrations drop to much lower levels in adult animals.[12] Although several lines of evidence suggest that both D-AAs are involved in signal transmission, many questions remain, and whether the link is direct or indirect is not well established.

1.3 Capillary electrophoresis

The research outlined in the following chapters uses CE as the major approach to measure small-volume samples for D-AAs. What is CE? While a short description is given here, several recent reviews provide much greater detail.[13-16] The simplest mode of CE, capillary zone electrophoresis (CZE), separates compounds based on their differences of size and charge. Of course, racemic pairs have the same charge and size to each other, so other separation modes are employed such as cyclodextrin modified micellar electrokinetic chromatography (CD-MEKC), which is used the most in analyzing biological samples.[17] Differences of amino acid (AA) enantiomers partitioning into cyclodextrins (CDs) play an important role in separating them.

CE has found significant applications in neuroscience.[18-26] A number of features of CE make this approach very suitable for neuroscience investigations. The
small sample requirements of CE, along with the ability to manipulate small volumes, allow single cell or even subcellular analyses to be performed on CE. Of course, a single cell does not have much material in it and so detection becomes a challenge. Luckily, CE can be coupled with a number of detection methods, such as laser-induced fluorescence (LIF), which then creates a system with excellent mass detection limits for a wide range of signaling molecules. When mass spectrometry (MS) is coupled with CE, separated compounds can be identified with confidence and information of unknown peaks can be obtained. A detailed discussion on measuring endogenous D-AAs using CE is presented in section 2.2.1 in chapter 2.

1.4 Overview of thesis

The content of subsequent chapters in the dissertation is summarized below. Chapter 2 reviews the techniques and highlights the field of measuring endogenous D-AAs in the brain, which were reported from 2005 to 2011. Although the main focus is on the brain, other studies that investigate functional roles of D-AAs in invertebrate nervous system are included. Measurements conducted using cell cultures with significance in neuroscience are also included. Significant effort is made to highlight: (1) newly developed or newly implemented approaches and (2) measurement results that lead to a better understanding of the functional roles of D-AAs.

Chapter 3 presents research to determine whether D-Asp is released in a stimulation-dependent manner in Aplysia californica (A. californica). This was critical information missing from our prior studies and is needed in order to understand if D-Asp can be a neurotransmitter. We employ two different sample collection and sample
preparation methods. One is by directly collecting extracellular environment. We
developed a protocol using liquid-liquid extraction method to specifically extract amino
acids out while leave salt behind. Another uses solid phase extraction (SPE)–based bead
sampling technology as used in previous peptide studies by our group,[27] which allows
more defined analyses in terms of spatial information on the release locations. Our
findings show that D-Asp is released from neurons in a stimulation-dependent manner.
With our prior work, we now have demonstrated that D-Asp fulfills all criteria of
neurotransmission within the A. californica central nervous system.

Chapter 4 describes a fundamental study on D-Ser, representing a large
international collaboration project. The goals of this work were to show the storage and
uptake of D-Ser into astroglial vesicles. Whether D-Ser is released from glia via
vesicular pathway is a controversial topic within D-Ser community. Here we address the
question by quantifying the amino acid contents in immunoisolated glial vesicles, both
leaked and intact. Our analysis shows the existence of a population of GVs which stores
D-Ser and L-Glu, with estimated intravesicular concentrations of ~20 mM. This level is
within the acceptable range of the concentration of other signaling molecules. Because
this collaboration is meant to address this question in a definitive manner, we verified the
quantitation results using three different CE methods, on two CE platforms, CE-LIF and
CE-MS. When our analysis results are combined with the imaging, molecular,
physiological and biochemical data from our collaborators, we conclude that vesicles in
astroglia do exist, and they store and release D-serine, L-glutamate, and most likely other
neuro- and gliamodulators.
Chapter 5 is more preliminary in nature and describes several ongoing method development projects on CE-LIF aiming to provide sensitive, reliable measurement of D-glutamate, D-alanine and D-proline. They D-AAs are poorly studied and little is known about their functions except that they occur in the central nervous and endocrine systems.[28-32] Although most analytical measurements of these three D-AAs employed high-performance liquid chromatography (HPLC), my goal was to adapt CE, useful because of the reasons given above (e.g., the ability to analyze d-AAs in single cells.) The newly developed analytical methods, in combination with appropriately designed experiments, will help to uncover the roles of these D-AAs play in cell to cell signaling in nervous and endocrine system.
1.5 References


Chapter 2

Measuring Endogenous D-Amino Acids in Brains

2.1 Introduction

Chirality is a common feature in most compounds found in nature. The physiological environment within a living system is chiral and the biological activities of molecules of different chirality differ dramatically.\[1\] Among the twenty essential amino acids which commonly occur in all organisms, only glycine lacks a chiral center. It was long believed that only L-amino acids (L-AAs) exist in higher animals and their D counterparts, are present solely in lower species like microorganisms and bacteria.

The first report of significant endogenous D-amino acids (D-AAs) in the animal kingdom was free D-alanine (D-Ala) from the blood of the milkweed bug isolated by paper chromatography and detected using D-amino acid oxidase-linked (DAAO-linked) colorimetric assay. This was followed by finding of several D-AAs in mammals, such as D-aspartate (D-Asp) in humans,\[2, 3\] neonatal rat brain,\[4\] as well as D-serine (D-Ser) in human and rat brain.\[3\] Hundreds of studies now document the widespread occurrence of D-AAs across the Metazoan.

The presence of D-AAs in the brain or central nervous system (CNS) has implied their importance and further studies have confirmed their roles. For example, D-Ser accumulates in astrocytes located nearby neurons with a high expression of N-methyl-D-aspartate (NMDA) receptor,\[5\] which is an important player in learning and memory formation. Intriguingly, D-Ser binds to the glycine binding site of the NMDA receptor
while potentiates its activity.\[6\] D-Asp is the second most studied endogenous D-AA in animals. So far, it has been found in all examined animals. Interestingly, D-Asp levels are relatively high during embryonic development, but its concentration decreases substantially in adult animals.\[3\] These D-AA studies describe specific biological functions, with D-Ser playing an important role in neurotransmission and D-Asp in development. Moreover, altered levels of D-AA are related to the developmental stages of many nervous system diseases, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD), although their involvements in these processes have yet to be confirmed.\[7\]

Endogenous D-AAs in animal CNS have been characterized using a variety of separation and measurement techniques. Techniques can be categorized into separation based and non-separation based. Separation based approaches comprise of capillary electrophoresis (CE), high performance liquid chromatography (HPLC) and gas chromatography (GC). These approaches can be coupled with different detection methods such as laser-induced fluorescence (LIF), mass spectrometry (MS) or ultraviolet (UV). These instrumental platforms usually provide high separation efficiency, good sensitivity as well as great repeatability and allow high-throughput measurements of D-AAs along with other analytes of interest in one single run.\[8\] On the other hand, non-separation based techniques, including immunostaining and enzyme-linked assays, could have advantages over separation based approaches, especially when specific analytes are being assayed. For example, immunostaining provides a straightforward image that relates analytes with morphological structures and enzyme-linked assays can deliver close to real-time responses, although both require pre-selected D-AAs.
This review primarily focuses on the measurements of endogenous D-AAs in the brain using the techniques stated above that were reported from 2005 to 2011. The main focus is on the brain, including mammals and invertebrate nervous system. Measurements conducted using cell cultures with significance in neuroscience are also included. We balance our discussion between techniques themselves and the scientific significance of those measurements. Measurements in clinical research and disease diagnosis are discussed separately. Significant effort is made to highlight: (1) newly developed or newly implemented approaches and (2) measurement results that lead to a better understanding of the functional roles of D-AAs.

2.2 Separation based techniques

2.2.1 CE

2.2.1.1 Techniques

2.2.1.1.1 Separation

Cyclodextrin-mediated enantioseparation is the most widely used separation technique used in measuring D-AAs in biological matrix using CE.[9] It can be categorized into three different modes: CD-capillary zone electrophoresis (CD-CZE), in which neutral CDs are added into background electrolyte (BGE); CD-electrokinetic chromatography (CD-EKC), in which charged CDs are added; CD-micellar electrokinetic chromatography, in which surfactants and CDs are both added. The mechanism of these modes has been discussed in detail in a recent review.[10] A variety of CDs (native CDs and CD derivatives) and several surfactant (chiral and achiral) have been employed in bioanalytical CE approaches, which have been recently reviewed.[9, 11-14] Besides the
types and concentrations of CDs and surfactants, the chiral separation efficiency can also be affected by pH, BGE and other additives, such as saccharides [15] and organic modifier.[11] It is important to note that chromatographic mechanism plays a more significant role by distributing chiral amino acid at different extents between bulk solution and chiral selectors.[16]

One of the most attractive advantages of EKC is that one can alter the separation efficiency towards various analytes by changing the types and concentrations of components in the separation buffer, without modifying a traditional CE system. Moreover, it is cost-effective because usually low concentration of chiral selectors and small volume of media are required in the buffer condition.[17]

2.2.1.1.2 Detection

Besides the separation, it is important to be able to detect the analytes. Laser-induced fluorescence (LIF) allows the measurement of low abundant endogenous D-AAs as it provides the lowest reported limit of detection among detection methods coupled with CE.[18] CE-LIF instruments use a laser to excite the fluorescent analytes, and the resulting fluorescence emission is focused by optics and subsequently detected.[19] The ability to use CE-LIF for detecting D-AAs in single cell and subcellular regions has facilitated our understanding of D-AAs as signaling molecules. In addition, LIF is the most common detection system coupled with microdialysis either on-line or off-line.[18]

Light-emitting diode-induced fluorescence (LEDIF), used less often, is an alternative to lasers as a source for fluorescence detection with lower cost and usually,
with poorer detection limits. However, it can provide limits of detection (LOD) that allow measurements of D-AAs in biological matrices. Li et al. described within-column LEDIF detection with a LOD at $2.6 \times 10^{-8}$ M for D-Ser. To avoid the possible light loss from light-emitting diode (LED)’s limited power, the upper end of optical fiber was inserted parallel into the separation capillary with excitation light transmitted directly to reach the detection window.

Radionuclide detection coupled with CE is an excellent choice for studies on conversions from L-AA to D-AA, such as D-AA biosynthesis, transport and reuptake process.[20] Those investigations can be facilitated by carefully designed experiments using radiolabeled precursors of D-AAs. Newly synthesized D-AAs can be differentiated from both the precursor (i.e. radiolabeled L-AAs) via CE separation and the endogenous pool via radionuclide detection. As a result, the percentage of newly synthesized D-AAs can be measured and calculated.

2.2.1.3 Sampling

Bulk tissues and body fluids are still the most common type of samples being analyzed by CE. With tissue punches and slices, a degree of spatial resolution and relatively consistent sample size can be obtained. D-AAs have been measured in a variety of brain samples, such as, *A.californica* ganglia,[15, 21, 22] arctic ground squirrel brain tissue,[23] rat brain tissues [15, 24] and human CSF.[25]

Sampling smaller tissue such as single cells can often provide valuable information because of the heterogeneity in chemical composition and function of
individual or even adjacent neurons.[26] These measurements are made feasible because of the compatibility of CE with small sized sample and its high sensitivity.

Besides dissection, microdialysis has become a useful tool to retrieve information from brains in vivo. The small volumes of CE work well with the small sampling probes used in microdialysis. Temporal resolution is an important consideration and is often limited by both derivatization reaction time and separation time.[27] Various approaches have been developed to improve on this point by either exquisitely designing the microdialysis-CE interface [28] or collecting samples off-line at short intervals.[29] For example, O’Brien et al. established a high-throughput on-line microdialysis-capillary electrophoresis (MD-CE) system [28] using a customized flow-gated injection interface (Figure 2.3a) which switched between injection and separation. This MD-CE system greatly improved sample throughput by eliminating several manual steps including sample pretreatment, derivatization and by shortening the migration time of D-Ser to only 22s. An alternative way to improve temporal resolution is by collecting samples off-line at short intervals. Kirschner et al. designed a new brain slice microperfusion device coupled offline to CE-LIF with a 2 min temporal resolution by manually collected dropwise off-line every 2 min and separated by a CE-LIF system.[29]

2.2.1.2 Applications

The suitability of CE for the determination of D-AAs in biological matrix has encouraged its use in various studies, most aiming to elucidate the functional roles of D-AAs in the CNS. There are a number of references that detail the CE methods used to measure endogenous D-AAs.[17, 30] In the past 6 years, CE measurements have further documented the occurrence of D-AAs in the brains of many animals. These include D-

Moreover, a number of insightful results have been obtained towards understanding the roles of these D-AAs in the CNS.

Single cell and subcellular analysis has proven to be fruitful in studying heterogeneity of D-AA levels not only from cell to cell, but also from different subcellular domains of a cell. For example, our group found that the percentage of the Asp in the D-form considerably varied in different identified neurons of *A. californica*. The ratio of D-/L-Asp was reproducible in the same regions (cell clusters or single cells) and varied region to region with small variance from animal to animal (Figure 2.1).[32]

In addition, the analysis of subcellular domains revealed the presence of D-Asp in both cell soma and cell processes, suggesting it undergoes transport to distant release sites.[31]

With the question that whether D-Asp acts as a traditional neurotransmitter in mind, various CE-LIF measurements were performed in *A. californica* to further investigate the mechanisms including synthesis, transport, and secretion of this putative signaling molecule. A pharmacological study suggested both D-Asp and L-Asp were transported in a major nerve in a colchicine-dependent manner, where colchicine can serve as a vesicle transport blocker. These results pointed to an active transport of D-Asp via vesicles in *A. californica.[31]*

In a more recent study, we detected the activity-dependent release of endogenous D-/L-Asp from ganglia of *A. californica* upon KCl and calcium ionophore stimulation. When calcium ionophore was used as stimuli, the release of D-Asp was even much more pronounced compare to that of L-Asp. These results support the concept of Ca$^{2+}$-dependent D-Asp release from neurons, in line with vesicular
release mechanism.[21] A CE-LIF system assembled with off-line fraction collection [20] and radionuclide detection has been implemented by our group to examine the biosynthesis of D-Asp from L-Asp in *A. californica*. (Figure 2.2) An average of 36% \[^{14}\text{C}]\text{D-Asp}\) radioactive signal provided clear evidence of active synthesis of D-Asp from L-Asp in the *A. californica* CNS.[32] All these data support the fact that D-Asp satisfies the criteria of a traditional neurotransmitter within *A. californica*.

The dynamic nature of D/L-Asp was investigated by S.L. Zhao *et al.* by quantitatively measuring them in rat brains at different development stages. D-Asp levels appeared to decrease rapidly after birth while L-Asp acted oppositely.[35] This corresponds to the pioneer work of D-Asp analysis by Hashimoto using HPLC,[3] and can be advantageous over HPLC when dealing with small volume samples, such as single cells.

Some insights as to the neuromodulator action of D-Ser as well as its fluctuation in nervous system development were also provided by CE measurements. With a CE-LEDIF method, Li *et al.* measured D-Ser contents in the midbrain of mice induced with PD symptoms, showing first increase then steep drop of D-Ser level. The results suggested that the biosynthesis and the transportation of D-Ser might be involved in PD pathogenesis.[24] By analyzing the off-line collected samples from push-pull perfusion, Kirschner *et al.* found that D-Ser and L-Glu efflux from acute slices had similar timing of release. This is critical considering that both agonists are required for NMDA receptor excitotoxicity.[29] In a set of pharmacological-assisted measurements using MD-CE, it was suggested that extracellular concentration of D-Ser was regulated by the involvement of Ala-Ser-Cys neutral amino acid transporters.[39] The advances of MD-CE technique
also helped to demonstrate that the release of D-Ser upon depolarization was a physiological pathway in vivo.[36] Due to the improved temporal resolution (1.25 min) compared to traditional microdialysis (20 min), Rosenberg et al. were able to observe the significant increase in extracellular D-Ser concentration elicited by (Figure 2.3b) veratridine,[36] which was not observed previously.[40] This is an excellent example demonstrating the benefit of faster MD-CE.

2.2.2 HPLC

2.2.2.1 Technique

In chromatographic separations, chiral separations can be categorized as indirect or direct. In indirect approach, the resolution of enantiomers is achieved by forming diastereomers with chiral reagent, followed by their separation using achiral chromatographic modalities. The indirect approaches [41, 42] developed decades ago are still used in analyzing D-AAs in biological matrix nowadays. Direct approach requires no chemical derivatization with chiral reagent prior to separation, but involves using chiral selectors either in mobile phase or on stationary phase known as chiral stationary phase (CSP). There have been several newly developed direct approaches reported in the past six years.[43, 44] Although not required in direct approaches, derivatization usually is to be performed to attach a fluorescent moiety onto D-AAs to assure good detection.[16]

2.2.2.1.1 Methods based on indirect separation mechanisms

Several derivatizing systems were developed to form diastereomers with D-AAs and enabled enantioseparation of D-AAs in biological samples on common C18 columns.
The chiral derivatizing agents used in HPLC separation of AA enantiomers have been recently reviewed.[45] One of the first methods identifying endogenous D-AAs was developed by Hashimoto et al. almost two decades ago,[42] and is still widely used for the analysis of endogenous D-Ser, D-Asp and D-Ala.[46-48] Chiral resolution is achieved by derivatization using OPA and a chiral thiol N-tert-butyloxy-carbonyl-L-cysteine (BOC-L-cys) in combination with gradient elution. Another indirect HPLC method being successfully implemented in analyzing D/L-Asp in biological matrix is an OPA and N-acetyl-L-cysteine (NAC) derivatization system introduced by Aswad.[41]

Marfey’s reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, is also widely used in the analysis of chiral amino acids. The resulting derivatives of amino acids can be detected by both, simple UV as well as more selective mass spectrometry.[49-51] A rapid and sensitive gradient elution liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-/MS/MS) approach was developed recently utilizing C-18 column for separation and a Q-trap in negative ion mode for detection. Quantitation was carried out by MS/MS in selective reaction monitoring (SRM) mode.[50]

**2.2.2.1.2 Methods based on direct separation modes**

Besides forming diastereomers followed by non-chiral columns, methods have also been reported utilizing achiral fluorescent derivatizing reagents in conjunction with chiral HPLC columns to separate AA enantiomers. For example, the achiral NBD-F labeling reagent offers superior stability compared to OPA while requires elevated reaction temperature and longer reaction time.[52] The increased hydrophobicity after NBD-F derivatization also facilitates sample pre-concentration. Song et al. reported a novel sensitive chiral capillary high performance liquid chromatography-tandem mass
spectrometry (HPLC-MS/MS) method. This system employed a C18 column to pre-concentrate samples and a chiral column packed with teicoplanin aglycon to separate NBD-F tagged amino acid enantiomers. The detection using tandem MS with SRM led to excellent sensitivity and selectivity. Thanks to this high selectivity, a stable isotope-labeled analyte analogue (i.e., L-Asp-2,3,3,-d3) could be introduced as an internal standard to improve the reproducibility and reliability of quantitative analyses.[43, 44]

Many separations of NBD-F tagged racemic AAs have been performed on a very neat automate column-switching 2D-HPLC system (Figure 2.4a). This technique particularly allows highly sensitive and selective analysis of AA enantiomers in complicated biological system because in the first dimension, the racemic mixtures can be preselected from complicated biological matrix on a reversed-phase column. The first stage separation greatly reduced the complexity of fractions to be separated on the second stage by a chiral column.[53] This system had an limit of detection (LOD) of 500 amol/injection for NBD-D-Ser and NBD-D-Ala as well as a total 30 min separation time, which were both advantageous over the similar system reported earlier.[54] In addition, the quantitation values of AA enantiomers could also be confirmed using an opposite enantioselective column with reversed elution order, which greatly increased the reliability of the results. Separation of AA pairs could be achieved by optimizing parameters of individualize protocols, including mobile phase composition, column, etc. High cost is a major limitation of this system owing to the two fluorescent detectors and multiple LC columns.[52]

2.2.2.2 Applications
In the past 6 years, various LC methods have been used to further characterized D-AAs in different biological matrix, including neuronal culture,[46] subcellular organelles of *Aplysia limacine* (*A. limacine*),[55] *A. californica* CNS,[43, 44] rat brain tissues,[44, 48, 56-60] mouse brain tissues,[53, 61] microdialysates from rat brain,[47, 50] and human CSF.[51]

LC measurements have provided many insightful results increasing our understanding of the biosynthesis of D-Ser in the brain. The synthesis of neuronal D-Ser was demonstrated in neuronal cultures containing minimal amounts of glia. Interestingly, in pure neuronal culture, synthesis of D-Ser in neurons required the uptake of extracellular L-Ser,[46] which may be supplied by glial cells under normal conditions.[62] Yang *et al.* performed a 2D-HPLC analysis to elucidate the role of brain L-Ser synthesis in modulating D-Ser level by measuring their concentration in the brain of conditional Phgdh mutant mice. Phgdh catalyzes the first step in L-Ser synthesis. A markedly decrease of D/L-Ser in the cerebral cortex and hippocampus of knock-out mice provided proof that endogenous L-Ser synthesis via phosphorylated pathway can play a pivotal role in controlling the level of endogenous D-Ser.[61]

Moreover, several proteins possibly regulating SR have been investigated on their effects on D-Ser synthesis, including protein interacting with C-kinase 1 (PICK1), aminooxyacetic acid (AOAC). Levels of D-Ser were decreased in the forebrains of neonatal mice with PICK1 knockout, indicating the regulation of brain D-Ser levels by PICK1 in a spatially and temporally specific manner.[63] Protein kinase C (PKC) activation reduced D-Ser levels *in vitro* while its inhibition increased cellular D-Ser level. Manipulating PKC activity *in vivo* also regulated D-Ser level in rat frontal cortex.[48]
AOAC acts as an inhibitor of pyridoxal phosphate-dependent enzymes, which SR belongs to. The perfusion of AOAC can induce a significant decline in the extracellular concentration of D-Ser in the striatum.[64]

DAAO has been long known to have D-Ser degradation activity.[65] Endogenous D-Ser and D-Ala in the tissues and physiological fluids of mice with various DAAO activities were measured with a 2D-HPLC method. The amounts of both DAAs increased drastically with a lowered DAAO activity except for the cases of D-Ser in the frontal brain regions (Figure 2.4b and 2.4c).[53] Besides DAAO, SR has been proposed to be another moiety regulating endogenous D-Ser levels through an α,β-elimination activity, especially in brain areas where DAAO is not present. This was supported by the results that levels of D-Ser synthesized with impaired α,β-elimination activity were several-fold higher than wildtype both in vitro and in vivo.[66]

In addition and biosynthesis and degradation, new measurements of endogenous release have also been accomplished by LC. Kanematsu et al. observed a reduced cortical extracellular content of D-Ser during the intra-cortical infusion of a glia toxin. These findings suggested that glial cells participated in controlling extracellular D-Ser contents in the rat medial prefrontal cortex.[47] Kakegawa measured D-Ser secreted from glia in rat cerebellar slices and showed that endogenous D-Ser was released mainly from Bergmann glia by parallel fiber burst stimulation in immature, but not mature cerebellar slices.[58] Spinelli et al. detected high levels of D-Asp in isolated synaptosomes and synaptic vesicles in *Aplysia limacine*. Moreover, D-Asp release was observed from synaptosomes by both K⁺ and ionomycin stimulation, indicating a possible role for D-Asp in neurotransmission.[55]
Topo et al. investigated the role of D-Asp in learning and memory processes of the rat. They found a good and significant correlation between endogenous D-Asp concentration in rat hippocampus and the amount of time to complete a task, indicating the involvement of D-Asp in learning and memory formation.[56] The 2D-HPLC measurements of D-Ala in rats demonstrated an intriguing circadian rhythm of this molecule. Its amount in rat plasma, pancreas and anterior pituitary gland was high during the sleeping period and low during the active period in rats with both diurnal and nocturnal habits. Interestingly, L-Ala and other D-amino acids including D-Asp and D-Ser didn’t show an obvious circadian rhythm.[57]

2.2.3 GC

While gas chromatography is a fast and efficient way to separate amino acid enantiomers in bulk tissue, it is less used in measuring endogenous amino acid enantiomers in small biological samples due to the difficulty in sample preparation.[52] Among the GC methods for chiral amino acids separation, one of the most successful approaches to analyze biological samples is achiral derivatization with pentafluoropropionic anhydride (PFPA) followed by chiral separation on a Chirasil L-valine column.[67] Chirasil L-valine column is a chiral column in which the stationary phase of L-Val was coupled to a fused silica glass. Separation is dependent on formation of multiple hydrogen bonds of analytes with the stationary phase.[68] This GC approach coupled with mass spectrometry in the selective ion detection mode was described to quantify D-AAAs in the brains of a wide range of higher vertebrates. D-Ser has been detected in almost all surveyed animals except birds, indicating the ubiquity of D-Ser in vertebrate brains.[69]
2.2.4 Enzymatic methods to verify D-AA identity in separation results

In the complex biological system, a clean discrimination of chiral enantiomers cannot always be easily achieved even when standards can be well resolved. This is more common when the differences in concentrations between the D-AA and the L-AA are great and D-AAs only exist at trace level, or when small changes of D-AAs needs to be detected. Several approaches have been developed to overcome these challenges. If the expected D-AA is masked by a known peak, specific enzyme can be applied to remove the masking peak. For instance, Rosenberg et al. employed glutaminase to remove glutamine, which masked the small D-Ser peak in their HPLC analysis. It greatly improved the chromatographic separation of D-Ser and allowed endogenous D-Ser release from cultured neurons to be measured.[36] Even when there is no known peak co-migrating with the targeted D-AA peak, more than one technique or addition confirmation using enzyme reaction is preferred to validate the identity of D-AA peak in complicated biological matrix. DAA peak can be selectively removed by enzyme and antibody incubation, including DAAO, D-Ser deaminase,[70] d-aspartic acid oxidase (D-ASPO) [55, 56, 71] and D-Asp antibody.[72]

2.3 Non-separation based techniques

2.3.1 Enzyme-linked assays

Although analytical separation methods discussed above offer highly sensitive, greatly selective and very quantitative measurements of endogenous D-AAs, they are typically not suitable for on-line analysis except when coupled on-line with microdialysis. Even microdialysis sampling could be limited by their large sized probes as well as the
slow diffusion of molecules through the dialysis membrane. In addition, purchasing, building and maintaining a complicated analytical instrument can be a huge investment. An alternative way is to use enzyme-based techniques, which offer simplicity of design, high sensitivity and more importantly the potential to quantify D-AA variations in vivo in real time.

2.3.1.1 Microsensor

Pernot et al. have developed a microbiosensor by covering a cylindrical platinum microelectrode with a membrane of poly-m-phenylenediamine (PPD) and a layer of immobilized D-amino acid oxidase from the yeast Rhodotorula gracilis (Figure 2.5).[73] The diameter of the tip was about 25 µm. This sensor functioned by detecting the hydrogen peroxide produced from enzymatic degradation of D-Ser and had an in vivo detection limit of 16 nM. Rhodotorula gracilis D-amino acid oxidase (RgDAAO) provided a more active and more selective detection over the previously used porcine kidney D-amino acid oxidase (pkDAAO). In addition, the used of a highly selective PPD layer blocked 97% of the nonspecific oxidation of endogenous oxidizable molecules. Although several D-amino acids could be potential substrates for RgDAAO, D-Ser was the only endogenous substrate sufficient to be detected by the sensor in the central nervous system. This device has been tested on measuring D-Ser in rat cortex after an intraperitoneal injection.[73]

2.3.1.2 Chemiluminescent detection

Besides detecting the resulting H₂O₂ oxidation current induced by DAAO enzyme reaction, chemiluminescent provides an alternative detection option. This detection, although can also provide real-time responses with simplicity, does not offer as good
spatial resolution as microbiosensor, because the detection is usually performed in a solution instead of a single spot. The mechanism is based on measuring the light of a luminescent compound produced by the reaction of hydrogen peroxide with peroxidase and luminol.[74] Assays using this method have been performed to measure D-Ser during vestibular nuclei (VN) postnatal development, showing a strong variation depending on the postnatal developmental stages. The level of D-Ser peaked at one week and dropped gradually afterwards.[75] Mothet et al. applied this enzyme-linked method to monitor D-Ser release from cultured astrocytes in a neuropharmacological study, aiming to learn the mechanism of D-Ser release from glia. One of the most intriguing observation was that receptor-mediated D-Ser release was calcium-dependent, a feature shared by other gliotransmitters such as glutamate and ATP (Figure 2.6). They also demonstrated that agonist-evoked release of D-Ser was sensitive to tetanus neurotoxin, which proteolyzed soluble NSF attachment protein receptors (SNAREs) on the vesicle membranes. Their data together supported Ca$^{2+}$-dependent vesicular release of D-Ser from glial cells.[76] The same chemiluminescent assay was applied to determine the D-Ser content in hippocampus, revealing its amount in young rats decreased to only about 1/6 in aged rats.[77]

### 2.3.1.3 Colorimetric detection

D-AAs can also be determined by colorimetrically measuring the α-ketoacids produced by oxidation of D-AAs with DAspO or by DAAO. Spinelli et al. have used DAspO to measure the sum of D-Asp+NMDA+D-Glu and DAAO to measure the total other DAAs in the nervous tissues of *A. limacina*.[55]

### 2.3.2 Immunostaining

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Immunostaining is an excellent method to locate D-AAs of interest in brain tissues. Antibodies have been raised specifically to the targeted analytes including D-Ser,[46, 75, 78-81] D-Asp,[82] D-Ala,[83, 84] and D-Glu.[85] Cross-reactivity is a challenge that needs to be addressed before immunostaining is performed on biological samples. Specificity of antibodies can be tested using dot-blotting or western blotting to make sure the purified antibodies do not or negligibly conjugate with molecules other than targeted analytes. In addition, standard control experiments need to be performed including the use of pre-immune sera, pre-absorption of diluted antibodies with the target peptide or amino acid that they are raised against.[86]

2.3.2.1 D-Ser

D-Ser has been intensively studied by immunostaining to determine its distribution in tissues and subcellular localization inside both neurons and glia. One of the controversial topics in D-Ser community is the distribution of D-Ser. Is D-Ser is localized exclusively in neurons or glia, or both? Is this distribution changes along with development? Numerous immunostaining studies have unveiled the answers. Immunostaining has been performed extensively to investigate the localization of D-Ser in both brain tissues and cell cultures. Previous research has demonstrated that D-Ser was found mainly in astrocytes, but not neurons. Kartvelishvily et al. improved the staining by employing a different antibody raised against D-Ser-glutaraldehyde conjugate and incubating thinner sections for longer time. These advances enhanced immunoreactivity and resulted in lower background staining, which was crucial for the observation of neuronal staining. They also quenched glutaraldehyde autofluorescence. All these improvements enabled a clear staining for D-Ser in neuronal cell bodies and processes in
all layers of the cerebral cortices of rats. However, in other brain areas, glial staining was predominant (Figure 2.7).[46] Another interesting study stained D-Ser in rat retina to investigate the developmental change of D-Ser concentration level in neuronal ganglion cells of the retina. Sections were fixed for 10 min in 4% p-formaldehyde prepared in phosphate-buffer saline (PBS). The level of D-Ser was quite high in ganglion cells of neonatal retinas while decreased rapidly postnatally.[81] Glutaraldehyde-fixation can result in the difficulty of co-localization of D-Ser antibody with glutaraldehyde-fixation-sensitive antigens. To overcome this challenge, Williams et al. raised a new D-Ser antibody optimized for formaldehyde-fixation instead of glutaraldehyde fixation. With this method, they were able to characterize novel anatomical compartmentalization of D-Ser in glia. Instead of uniformly distributed in cytoplasm, D-Ser was found to be enriched into vesicle-like compartments in astrocytes and radial glial cells. Moreover, in aged animals, immunolabeling for D-Ser was absent in patches of cortex and hippocampus, suggesting an impaired glial modulation of forebrain glutamatergic signaling might occur.[78]

Double-staining can deliver a more comprehensive and more convincing results about cellular distribution of D-AAs by supplying information of the co-localization of two targets. More importantly, by staining subcellular organelles and D-AAs simultaneously, double-staining can implicate the subcellular localization of D-AAs. Magalie et al. performed an elegant study to address the question whether D-Ser was distributed in vesicles of the regulated secretory pathway using cultured cortical astrocytes. eGFP-synaptobrevin/VAMP2 (eGFP-Sb2) and eGFP-cellubrevin/VAMP3 (eGFP-Cb) were used to mark synaptic-like vesicles and their co-localizations with D-Ser
were quantified. Their results indicated that near one-fourth of D-Ser is present in partial but not all vesicles of the regulated secretory pathway (Figure 2.8).[87] A single and double immunolabelings also revealed high concentration of D-Ser and its biosynthetic enzyme, serine racemase, in the supraoptic nuclei (SON). By double immunolabeling, it was demonstrated that both were localized in all SON astrocytes but D-Ser was not observed from neuronal elements.[79] In neurons, D-Ser was revealed in a subset of glutamatergic neurons with dual immunofluorescence labeling for glutamate and D-Ser.[78] Puyal et al. double-immunolabeled D-Ser with neurons and glial cells respectively in VN sections of rat brains. B2-Tubulin was used to label neurons while GLAST, GFAP and S100β to verify D-Ser in glial cells. Interestingly enough, the cellular localization was found to be associated with postnatal development stages. For example, during the first three weeks, it was mainly localized in glial but switched from glia to neurons afterwards, indicating that D-Ser may have distinct functional roles depending on the maturation stage of the vestibular networks.[75] In neurons, D-Ser was revealed in a subset of glutamatergic neurons with dual immunofluorescence labeling for glutamate and D-Ser.[78]

### 2.3.2.2 D-Asp

As D-AAs like D-Asp are usually thought to be synthesized endogenously, their co-localization with various enzymes has been well studied. Immunohistochemistry revealed that D-Asp and D-Asp racemase (DR) have similar localizations in the rat brain including paraventricular nuclei (PVN) and supraoptic nuclei (SON) as well as other tissues, indicating that DR plays an important role to generate endogenous D-Asp.[82]

### 2.3.2.3 D-Ala

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Using mouse monoclonal antibody against D-Ala,[83] Etoh et al. visualized the cellular localization of D-Ala for the first time in the rat pituitary gland, which is the tissue containing the highest amount of D-Ala in the rat brain. In particular, they double stained D-Ala with five types of cells secreting specific hormones to identify the type of D-Ala immunopositive cells. The double-immunostaining results display a similar distribution of D-Ala with adrenocorticotropic hormone (ACTH) cells. Along with their previous results of D-Ala co-localization with insulin-secreting beta-cells in rat pancreas [83], the data shows an intriguing colocalization between important hormones, ACTH and insulin, that regulate blood glucose levels, and D-Asp.[84] More research is needed to resolve these questions.

2.3.2.4 D-Glu

Given the importance of L-Glu transmission in the brain of all animals studied, it is surprising how little data exists on D-Glu. Mangas et al. showed a clear visualization of a sparse localization of D-Glu-immunoreactive cell bodies for the first time in the rat brain by taking advantage of the high affinity and specificity of the antiserum directed against conjugated D-Glu. The immunoreactivity of D-Glu was only observed in mesencephalon and in the thalamus and confined to neuronal structures.[85]

2.4 Clinical and pharmaceutical applications

D-AAs have drawn intense interest from pharmaceutical industry because of their possible correlation with several mental diseases, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD). Measurements have been performed on samples obtained from diseased vertebrate animal models or even human beings. Given its excellent
quantitation ability, high reproducibility and automation, HPLC methods are used almost exclusively in clinical studies.

The method of reversed-phase HPLC following Boc-L-Cys-OPA derivatization,[42] was employed to investigate the difference of D-Ser level in schizophrenia patients vs. healthy control subjects. A significant 25% decrease in CSF D-Ser levels suggests D-Ser disposition maybe altered in the disease.[70] Yoshikawa et al. applied the same method to monitor D-Ser level after chronic morphine treatment. D-Ser was shown to augment slightly but significantly in the rat forebrain areas: striatum, hippocampus, and cortex while no significant effect were noticed in the diencephalon, midbrain, pons-medulla, and cerebellum.[88]

With a column-switching HPLC system, Hashimoto et al. studied whether D-Ser to total serine ratio in CSF was altered in first episode and drug-naïve schizophrenic patients. It was found this ratio was significantly lower in 25 male first episode and drug-naïve schizophrenic patients than that of 17 age-matched male healthy subjects, suggesting the abnormal synthetic or metabolic pathways of D-Ser in the brain of drug-naïve patients.[89] In a more recent study, plasma levels of D-Ser were compared between patients with schizophrenia and healthy controls as well as during the clinical course of schizophrenia. It was found that plasma D-Ser levels of schizophrenia patients were higher than those of healthy controls. In addition, the increase of D-Ser levels seemed to be related with improvements of clinical symptoms.[90]

Using Marfey’s reagent derivatization followed by C18 column separation, Fuchs et al. measured D-Ser concentrations in cerebrospinal fluid of healthy children and those
with a defective L-Serine biosynthesis. This study elucidated possible roles of D-Ser in human central nervous system development. Healthy children demonstrated high D-Ser level immediately after birth while the levels were non-detectable in children with deficiency. L-Ser therapy restored D-Ser concentration and interestingly, when L-Ser was supplied prenatally, a nearly normal D-Ser concentration was observed at birth of patient and the clinical phenotype was normal (Table 2.1).[91]

2.5 Conclusions

The remarkable increase in our understanding of D-AA distribution, function in the brain as well as their clinical significance is, in many ways, a result of combining existing and newly-developed bioanalytical approaches with novel experimental strategies.

Choosing the appropriate techniques for these measurements is important. CE-LIF is well suited for single cell analysis due to their compatibility with minute sample size and excellent sensitivity provided by LIF detection. Single cell measurements can aid neuroscience studies because of the heterogeneity in function and chemical composition of even adjacent neurons. In addition, the significance of immunostaining in relating morphological structures to D-AA distribution and subcellular localization is widely recognized. Thus while older than other approaches, it is not likely to be replaced by other techniques. Of course, immunostaining may not be most appropriate for quantifying multiple D-AAs within a defined brain tissue. Therefore, a thorough understanding of the questions to be answered via D-AA measurements is necessary to
ensure that the most appropriate bioanalytical tool is selected from the extensive tool box packed by the analytical community.

The growth in knowledge of D-AA functions in the brain has prompted a new range of questions that require novel techniques with better sensitivity, selectivity, reliability as well as enhanced temporal and spatial resolution. The development of 2D-HPLC system overcame the challenge of peak co-migration, which frequently occurs due to the complexity of brain samples. As a result, targeted D-AA can now be detected without ambiguity and quantified reliably. Moreover, the newly designed enzyme-linked microbiosensor is miniaturized, obtains a 2 s temporal response and 16 nM in vivo detection limit.[73] There is no question that our understanding of D-AAs in the brain is being driven by such technology advances, and that as more detailed questions are asked, scientists continue to push the analytical tools.
2.6 References


22. Zhao, S.L. and Y.M. Liu, Quantification of D/L-aspartic acids in Aplysia californica central nervous system by beta-cyclodextrin modified micellar


chromatographic system combining reversed-phase and enantioselective columns.


2.7 Tables and figures

Figure 2.1. Individual *Aplysia californica* neurons have different levels of D-Asp. (a) Electropherograms of single neurons from neuronal clusters in cerebral ganglia; some peaks are truncated. (b) D-Asp percentage to total Asp in different neurons. F = F-cluster top-layer neuron; C = C-cluster top-layer neuron; G = G-cluster neuron; MCC = metacerebral cell; OGT = optical ganglion top-layer neuron. (Adapted from ref. [32] with permission.)
Figure 2.2. Diagram of the CE system highlighting the nanovial sampling system, the postcolumn fraction collector, and the complementary detection modes of LIF and radionuclide detection. (Reprinted with permission from (Page, J.S., S.S. Rubakhin, and J.V. Sweedler, Single-neuron analysis using CE combined with MALDI MS and radionuclide detection. Analytical Chemistry, 2002. 74(3): p. 497-503). Copyright (2002) American Chemical Society.)
Figure 2.3.  A) Diagram of the MD-CE interface.  B) Effect of veratridine on extracellular D- and L-Ser concentrations in rat striatum. Online MD-CE was used to monitor D-Ser (left) and L-Ser (right) concentrations in rat striatum. CE analyses were performed every 22 s. Black bar denotes the 3-min period (corrected for time to travel to the detector) where artificial CSF (aCSF) perfusing the probe was changed to aCSF spiked with 100 µM veratridine. Smaller peak (indicated by v) is an artifact that occurs when the valve is switched to introduce the drug-containing aCSF into the line. Larger peak (indicated by asterisk) corresponds to veratridine-induced d-or l-serine release. Dashed lines mark se of 3 replicate measurements. (Adapted from ref. [28, 36] with permission.)
Figure 2.4. A) Flow diagram of the 2D-HPLC system for the determination of Ser and Ala enantiomers. M, Mobile phase; DG, degasser; P, pump; AS, auto sampler; CO, column oven; D, fluorescence detector; CS, column-switching valve; W, waste. B) Reversed-phase separation and the continuously interlinked enantiomer separation of NBD-Ser (A) and NBD-Ala (B) using Sumichiral OA-2500S in the serum of dY/DAO+/- mice. The fractions indicated by gray bars (60 µL, 120 s) were online collected to a loop and transferred to the enantioselective column. (Adapted from ref. [53] with permission.)
Figure 2.5. Design of the biosensor. (A) Photomicrograph of the tip of an RgDAAO microbiosensor. The enzymatic layer appears as a translucent yellow membrane. (B) Schematic RgDAAO microbiosensor. A platinum wire is covered with a layer of PPD and an enzymatic membrane of RgDAAO. (C) Enzymatic reaction allowing D-serine detection at the microbiosensor: D-serine is oxidized into hydroxypyruvate by RgDAAO with equimolar production of $\text{H}_2\text{O}_2$ that diffuses through the PPD layer and is oxidized by the platinum wire. $\text{H}_2\text{O}_2$ oxidation gives rise to two electrons detected by the patch-clamp amplifier. (Reprinted with permission from (Pernot, P., J.P. Mothet, O. Schuvalio, A. Soldatkin, L. Pollegioni, M. Pilone, M.T. Adeline, R. Cespuglio, and S. Marinesco, Characterization of a yeast D-amino acid oxidase microbiosensor for D-serine detection in the central nervous system. Analytical Chemistry, 2008. 80(5): p. 1589-1597.) Copyright (2008) American Chemical Society.)
Figure 2.6. Ca\(^{2+}\)-dependent release of D-serine from astrocytes measured by DAAO/HRP/luminol assay. (A) Luminol-derived chemiluminescence (LDCL) trace shows D-serine release in response to (S)-\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (black arrow) in control conditions (normal calcium), in extracellular calcium-free solution (ECFS) (0 Ca\(^{2+}\)/2 mM EGTA), in high Ca\(^{2+}\)-containing medium (5 mM), or after intracellular Ca\(^{2+}\) chelation with 1,2-bis(2-aminophenoxy)ethane-\(N,N,N',N'\)-tetraacetateacetoxymethyl ester (BAPTA-AM) (5 µM; 1 h). Application of the ionophore A23187 (open arrowhead; 10 µM) induced a slow and long increase of chemiluminescence. (Scales: 9 fmol per 5 and 20 s.) (B) Statistical histogram of the different pharmacological manipulations during AMPA stimulation. (C and D) Effect of thapsigargin (10 µM; 20 min) on the response induced by t-ACPD (100 µM). Data are mean ± SEM of \(n \geq 3\) independent experiments. **, \(P < 0.01\); ***, \(P < 0.001\), one-way ANOVA plus Scheffé test. (Adapted from ref. [76] with permission.)
Figure 2.7. D-Serine localizes to neurons and astrocytes in the brain. (A–C) Staining for D-serine in neurons of several layers of the cerebral cortex (Ctx) of a P9 rat. CC = corpus callosum. D) Glial staining for D-Ser in the corpus callosum. (Adapted from ref. [46] with permission.)
Figure 2.8. Small synaptic-like vesicles contain D-serine. Primary astrocytes were transfected with eGFP-Sb2 (A) and GFP-Cb (B). Then, cells were double labelled with antibodies against D-Ser (red) and GFP (green). The merged images show in yellow the colocalization of D-Ser with eGFP-Sb2 (A) and eGFP-Cb (B) throughout the cell cytoplasm. The zoom insets are representative of the distribution of labeling observed in the cells analyzed. Arrows indicate double-labeled structures, arrowheads indicate membrane or juxtamembrane eGFP-Sb2/Cb. n, nucleus. Scale bars: 10 µm. (Adapted from ref. [87] with permission.)
Table 2.1. D-Ser, L-Ser, and Gly concentrations and percentage D-Ser of total Ser, percentage Gly of the sum of Gly and L-Ser, and ratio of D-Ser to Gly in CSF of patients with 3-phosphoglycerate dehydrogenase deficiency before and after L-Ser therapy. Patients 1, 2, and 4 received oral postnatal L-Ser supplementation on diagnosis. Due to adverse effects, L-Ser dosage was lowered in patient 4 at the age of 1 year. Unfortunately, no pretreatment sample of patient 1 was available. Patient 3 received prenatal therapy by maternal supplementation with oral L-serine. The first measurement was performed on her day of birth.

<table>
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<th>Patient</th>
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<th>After therapy</th>
<th>N\textsuperscript{a}</th>
<th>Age (days)</th>
<th>D-Serine (μM)</th>
<th>L-Serine (μM)</th>
<th>Glycine (μM)</th>
<th>D-Serine/Total Serine (%)</th>
<th>Glycine/Glycine + L-Serine (%)</th>
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<td>2,522 – 4,089</td>
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<td>0.4 (0.4 – 0.5)</td>
<td>12.0 (11.2 – 12.9)</td>
<td>3.5 (3.3 – 4.3)</td>
<td>3.5 (3.1 – 3.9)</td>
<td>23.9 (22.9 – 24.9)</td>
<td>0.1 (0.1 – 0.1)</td>
</tr>
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\textsuperscript{a} Number of CSF samples available for analysis. (Adapted from ref. [91] with permission.)
Chapter 3

Investigating D-Aspartate Release in the Aplysia Californica Central Nervous System

Notes and Acknowledgements


This project was initiated by Cory Scanlan, and I specifically developed the method for measuring D-Aspartate (D-Asp) release from Aplysia californica. I designed and performed the experiments of measuring D-Asp release stimulated by elevated extracellular potassium. Experiments from Stanislav Rubakhin and C.S. measuring D-Asp uptake from the published study are not reported; release results using another stimuli and different methods (solid phased extraction (SPE) bead technique) are reported here with the purpose of providing an informative context of the whole project. S.R. provided training on dissection and always offered helpful discussions whenever needed. The manuscript was written by all coauthors. The project described was supported by Award No. CHE-05-26692 from the National Science Foundation (NSF), Award No. P30 DA018310 from the National Institute On Drug Abuse (NIDA) and by Award No. 5RO1NS031609 from the National Institute of Neurological Disorders and Stroke (NINDS).
3.1 Introduction

It had been long presumed that amino acids, so vital to organismal function, predominantly occurred in the L-form, especially in higher animals. This view persisted for decades, even though a variety of D-amino acids (DAAs) were reported since the 1930s in mammalian tissues,[2] intriguing many when they were observed in human tumors.[3, 4] Hundreds of studies have now documented the widespread occurrence of DAAs in animals. Nevertheless, DAA function in higher organisms remained enigmatic until fairly recently, when the role of D-serine as a co-agonist of the NMDA receptor,[5, 6] and its participation in gliotransmission [7] were established. These observations, combined with additional available experimental evidence, support the likelihood that other endogenous DAAs also have physiological role, specifically, D-Asp,[8, 9] D-alanine,[10, 11] D-glutamate,[12, 13] and D-proline.[14]

Of these, D-Asp is one of the most studied endogenous DAAs in Metazoan.[6, 8, 9, 13-17] It has been detected in a wide variety of species and is present in the nervous system of both vertebrates and invertebrates. For example, within mollusks, free D-Asp has been found in the nervous tissue of the Cephalopoda species Octopus vulgaris, Loligo vulgaris, and Sepia officinalis;[18] in the buccal ganglia of the opistobranch mollusk Aplysia fasciata;[19] in several ganglia of Aplysia californica;[8, 20, 21] and Aplysia limacine,[22] as well as in the reproductive glands of O. vulgaris.[18, 23]

Interestingly, D-Asp appears at relatively high levels during embryonic development throughout Metazoan, but the concentration decreases to much lower levels in adult animals. In vertebrates, high D-Asp concentrations occur in the developing
retina and CNS of chickens,[24] rats,[25, 26] lizards,[27] frogs,[16] and humans.[28] D-Asp drops to trace levels in most animal tissues after maturation, exceptions being specific endocrine and exocrine systems. In adult rats, for example, D-Asp has been measured in significant quantities in the testes and in the adrenal and pineal glands,[29, 30] with concentrations in these tissues reaching up to 40% of the free Asp.

Despite these reported observations, the functions of D-Asp are not established; however, progress is being made. Indirect evidence demonstrates that changes in D-Asp levels are correlated with disease state [31] and similar to D-Ser, it is found in mammalian neurons during development.[28, 31] In an exciting recent report, Kim et al. showed that D-Asp is involved in adult mouse neurogenesis, playing a role as a trophic factor influencing dendritic development. Clearly, more studies are required to understand its role in the adult animal.[32]

We and others have hypothesized that D-Asp may function as a neurotransmitter or hormone.[8, 22, 26] However, confirmation of a physiological role for D-Asp as a signaling molecule requires that several crucial characteristics be demonstrated: presence in a pre-synaptic neuron, activity-dependent release, generation of an effect in the post-synaptic cell via specific cellular mechanisms, and inactivation of the molecular signal by uptake or catabolism. To examine the function of D-Asp in morphologically documented neuronal networks, we have selected the well-characterized neurobiological model, A. californica. For many years, this marine slug has been used in studies linking neurochemical pathways to function, and thus, it is an excellent choice for our current investigation. In our prior analyses of the A. californica CNS, we observed significant quantities of D-Asp in the C-, F-, and G-clusters of the cerebral ganglia, with over 85%
of the free Asp present in the insulin-producing neurons of the F-cluster being in the D-form.[8] D-Asp is actively transported along the pleural-abdominal connective in a colchicine-dependent manner and induces electrophysiological responses in several neurons. These observations, along with the results of a single-cell study,[21] demonstrate that D-Asp is present in A. californica neurons and may play roles in cell-to-cell signaling.

Here, we continue these earlier investigations by studying the stimulated release of D-Asp. Briefly, we employ two different sample collection and sample preparation methods. One is by directly collecting extracellular environment without bias. We then develop a protocol using liquid-liquid extraction method to specifically extract amino acids out while leave salt behind. Another is using SPE bead sampling technology used in previous peptide studies by our group, which allows more defined analyses in terms of spatial resolution. Our findings showed that the stimulation-dependent release, along with other data collected on D-Asp biosynthesis and uptake (not presented here) prepared D-Asp being a signaling molecule involved in neurotransmission and/or neuromodulation within the A. californica nervous system.

3.2 Experimental procedures

3.2.1 Reagents and solutions

We prepared 500 mL of 50 mM borate buffer (pH 9.4) by dissolving 2.38 g of sodium borate (Na2B4O7·10H2O) (Sigma–Aldrich, St. Louis, MO, USA) in 456 mL of ultrapure de-ionized (DI) water (Milli-Q Ultrapure Water Filtration Systems; Millipore, Bedford, MA, USA) and mixing with ~44 mL of 0.2 M NaOH; this solution was used in
sample preparations and as a sheath flow buffer, unless otherwise noted. The separation buffer solution consisted of 20 mM of β-cyclodextrin (β-CD) and 50 mM of sodium dodecyl sulfate (SDS) in 50 mM of borate buffer (pH 9.4) and 15% methanol (V/V). Naphthalene-2, 3-dicarboxaldehyde (NDA) was from Molecular Probes (Eugene, OR, USA). As indicated in several cases, a citric acid sheath buffer (25 mM, pH 2.5) was used that was made by dissolving 5.25 g of citric acid monohydrate (C6H8O7·H2O; Sigma–Aldrich) in 1.0 L of ultrapure DI water (ELGA Purelab Ultra water system; USFilter, Lowell, MA, USA), and titrating to pH 2.5 using 0.10 M NaOH. Other reagents were obtained from Sigma–Aldrich at the highest purity available. Solutions were filtered through 0.45 μm Acrodisc syringe filters (Gelman Laboratory, Ann Arbor, MI, USA) before use. Solid-phase extraction beads (~40 μm diameter) were obtained from Waters (Waters Oasis HLB, Milford, MA, USA).

### 3.2.2 Capillary electrophoresis instrument

There were two CE systems used in this project. The analyses of samples prepared by SPE technique were conducted on the first system. The second system was employed on the measurement of samples prepared by directly collecting extracellular medium followed by solvent extraction.

The first system was a lab-assembled CE-LIF system described previously,[33] converted to a 457.9 nm excitation wavelength by adapting the Argon Ion laser (Melles Griot, Carlsbad, CA), focusing lens and optics. Analytes were separated in a 65–75 cm long, 50 μm ID/360 μm OD fused-silica capillary (Polymicro Technologies, Phoenix, AZ).
The second system was a previously described, laboratory-built CE-LIF system,[34] converted to a 457.9-nm excitation wavelength by adapting the Argon Krypton mixed laser (Coherent, Santa Clara, CA, USA), focusing lens and optics. The schematic is shown in Figure 3.1. Analytes are separated in an 86 cm long, 50 µm ID/360 µm OD fused-silica capillary, with an effective length of 50 cm. The separation voltage is set at 20 kV. The separation buffer solution consists of 20 mM of β-cyclodextrin (β-CD) and 50 mM of sodium dodecyl sulfate (SDS) in 50 mM of borate buffer (pH 9.4).

3.2.3 Buccal, cerebral and pleural ganglia isolation

*A. californica* (200–300 g) were obtained from Charles Hollahan (Santa Barbara, CA, USA) and kept in an aquarium containing continuously circulating, aerated and filtered sea water (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) at 14–15°C until used. Animals were anesthetized by injection of isotonic MgCl₂ (~30 to ~50% of body weight) into the body cavity. The cerebral and buccal ganglia were dissected and placed in artificial sea water (ASW) containing (in mM): 460 NaCl, 10 KCl, 10 CaCl₂, 22 MgCl₂, 6 MgSO₄, and 10 HEPES (pH 7.8), or in ASW-antibiotic solution: ASW supplemented with 100 units/mL penicillin G, 100 µg/mL streptomycin, and 100 µg/mL gentamicin (pH 7.8).

To improve isolation of cellular clusters and individual neurons, the ganglion sheaths were digested enzymatically by incubating the ganglia in 1% protease (Type IX: Bacterial; Sigma–Aldrich) ASW-antibiotic solution at 34 °C for 1–2 h depending on animal size and season. Next, the ganglia were washed in fresh ASW. For several
measurements, the total protein has been determined to correct for animal to animal variations in the ganglia.

3.2.4 Capillary electrophoresis (CE)-laser induced fluorescence (LIF) cerebral ganglion KCl-stimulated release analysis

The cerebral ganglia were pinned down in a small ~450 µL well. The ganglia were allowed to rest in ASW for > 1.5 h, and then stimulated by increasing the extracellular potassium concentration to 53 mM for 20 min. Aliquots (200 µL) of extraganglionic media were collected before, during and after the KCl stimulation. Three washes of the ganglia were made after the KCl stimulation and before collection of the last sample. Each 200 µL was divided into five 40 µL aliquots in PCR vials and stored frozen until the CE-LIF investigation. The details of sample collection procedure are described in Figure 3.2.

The 40 µL aliquots were dried in the oven overnight and reconstituted with 5 µL water or 5 µL internal standards containing 1 µM D-Asp. The addition of internal standard allowed accurate determination of extracellular D-Asp and L-Asp levels and reduces methodological uncertainties. KCN and naphthalene-2, 3-dicarboxaldehyde (NDA) solutions were added to the reconstituted samples and the reactions were allowed to continue for 20 min in the dark to form N-substituted 1-cyanobenz[f]isoindole amino acid (CBI-AA). The large amount of inorganic salts in these samples created issues with CE when the solutions were concentrated; thus, the salts were removed via extraction. Specifically, HCl solution was added to the reaction mixture to adjust the pH and the resulting neutrally charged CBI-AA was extracted into CH₂Cl₂. The lower layer was
placed into a PCR tube and the extraction process was repeated three times. The combined collected organic layer was dried and reconstituted with buffer. The samples were then assayed via CE as described.

3.2.5 *F-cluster ionomycin-induced release analysis by CE-LIF*

A microvial containing SPE beads and releasate was placed in the injection port of the multichannel CE-LIF instrument. Two microliters of 8 mM NDA and 2 μL of 20 mM KCN in 50 mM borate buffer (pH ~9.2) were added to the microvial to elute and derivatize the collected releasate analytes. Derivatization was allowed to continue for 30 min at which time nearly all the solution in the microvial had evaporated. Once dry, 0.5 μL of 50 mM borate buffer was added to the vial, and the sample was then hydrodynamically injected into the system by lowering the outlet end of the capillary 15 cm below the inlet end for 30 s (injecting ~7.6 nL). Each sample was analyzed by CE-LIF at 14 kV with an average current of 44 μA. Running buffer was 50 mM borate (pH 9.4), with 20 mM β-CD and 50 mM SDS.

3.3 Results

3.3.1 *Liquid-liquid extraction method optimization*

*A. californica* is a fantastic model in terms of neuroscience investigations due to its well-characterized nervous system and large accessible neurons. However, as it is a sea creature, experiments such as release measurements have to be conducted in sea water to maintain the cell viability. This renders difficulty in sample preparation step as most analytical separation techniques are not compatible with this high salt concentration.
Therefore, the liquid-liquid extraction method was developed and optimized to extract and concentrate amino acids while removing most of the inorganic salts.

A mechanism of pH-mediated solvent extraction of CBI-Asp was developed as follows. First, the AAs in the samples were allowed to react with NDA at pH 9.4. The PKa values of the COOH group, amino group and COOH as R group are 1.88, 9.6, 3.65 respectively and PI equals 2.77 (Figure 3.3). At these conditions, the –COOH groups in both moieties were deprotonated, so that the targeted analyte partitions into the aqueous media. Subsequently, the pH value was modified to near the PI of Asp, therefore the two –COOH groups were partially charged while the amino group was protonated and positively charged. At this point, the CBI-Asp partitions into organic phase while most inorganic salts are left in aqueous phase.

The detailed procedure of CBI-Asp extraction for biological samples with organic solvent is illustrated by Figure 3.4. Optimization of both the choices of organic solvent and final sample solution pH employed exactly the same procedure as that of biological sample preparation. Choosing an appropriate organic solvent for solvent extraction was the very first step as this solvent must satisfy several criteria. The first criterion is that it has to be immiscible with water because the purpose is to leave the salts behind in the water. The second is to make the evaporation of the phase faster. Although CBI-AAs are relative stable, they are light sensitive. The less the time spent on drying the sample, the better the preservation of the CBI-AAs. Last but not least, the polarity of organic solvent needs to match that of CBI-Asp in order to achieve high extraction efficiency of CBI-Asp but no other derivatized AAs or peptides.
The organic solvent being tested included ethyl acetate, methylene chloride, hexane, butyl acetate, chloroform and dichloromethane. As the derivatized product of Asp, CBI-Asp, is a green fluorescence color; the extraction efficiency of different organic solvents can be compared by observing the strength of color partitioned into the organic layer. Dichloromethane was finally chosen as the solvent to be used in liquid-liquid extraction procedure due to its high extraction efficiency and ease to evaporate.

The desired pH was chosen based on the CBI-Asp peak intensity while the pH of the standard solutions were modified from around 1-4 by adding different volume of HCl. Due to the small size of sample (~40 µL), it was rather hard to accurately measure the pH after adding HCl. So although the exact final pH was not known, the amount of HCl added in could be easily quantified and controlled. The optimal extraction efficiency was achieved when 0.3 µl 5 M HCl solution was added to the reaction mixture. So it was used for all the sample preparation process. Through this solvent extraction process, the peak intensity of Asp could be increased up to 20 fold. Moreover, the peak distortion caused by high salt was also eliminated (Figure 3.5).

3.3.2 Liquid-liquid extraction method validation

When measuring D-Asp release from whole ganglia, it is desired that the amount of D-Asp collected can be compared from one experiment to another. Or it would be difficult to know whether D-Asp is truly secreted during stimulation and how much is released during one event. However, because the solvent extraction protocol presented earlier is not a quantitative method due to the difference of extraction efficiency, a quantitative method needs to be developed and validated.
One way to make the method quantitative was to induce D-Asp itself as an internal standard. A prerequisite was that one needed to make sure that the ratio of D-Asp versus L-Asp after liquid-liquid extraction process represented the ratio of them in the original sample. Different ratios of authentic D/L-Asp standards were mixed and the samples underwent exactly the same sample preparation process as real samples. And the results presented in Figure 3.6 showed that the solvent extraction procedure didn’t affect the ratio of D/L-Asp in the solution. We further validated the method by preparing blank control (ASW only) using exactly the same procedure to make sure that there were no contamination (Figure 3.7).

Appropriate amount of D-Asp standard was added into samples as internal standard (IS) at the very beginning after the releasates were collected. The samples were run twice, with IS or without and electropherograms of the two were aligned based on peak height/peak area of L-Asp, whose levels were the same between two samples. This step could give a peak height/peak area of the IS. The electropherograms of before and after stimulation samples were then aligned again based on the peak height/peak of IS, which was kept constant from one sample to another. As a result, a quantitative measurement of D-Asp can be achieved by calculating the relative peak height of D-Asp total peak to D-Asp internal standard.

3.3.3 *Secretion of D-Asp from whole ganglia or F-cluster cells*

Establishing activity-dependent release from neurons is an important criterion for determining if a compound functions as a neurotransmitter and/or neuromodulator. Increase in the extracellular concentration of both endogenous D-Asp and L-Asp was observed upon stimulation of intact cerebral ganglia by elevated potassium ion
concentrations in the media surrounding the ganglia (Figure 3.8). Interestingly, while Asp-levels increased, the ratio of the enantiomer differed from experiment to experiment (Figure 3.9), with instances when D-Asp (experiment #2) or L-Asp (experiment #3) contributed most to this increase. This variability differs from the range of levels of D- and L-Asp present in cells and tissues and, therefore, may indicate intrinsic regulation of D-Asp release.

To further refine the information on D-Asp release, we studied the secretion of D-Asp from two cerebral ganglion neurohemal regions—the anterior tentacular and upper labial nerves—using bead sampling technology [35, 36] (Figure 3.10) with CE-LIF measurements of the collected releasates. Our previous experiments showed that cerebral F-cluster cells have high levels of D-Asp present in their cell body.[8] Many of these neurons send their processes to the neurohemal areas located at the bases of the anterior tentacular and upper labial nerves, as well as the cerebral commissure.[37, 38] Larger Aplysia insulin-producing neuroendocrine cells have their terminals located in the first two areas.[37] We detected a small amount of both D- and L-Asp in prestimulation samples, suggesting a small, constitutive secretion rate (Figure 3.11). Following stimulation with 10 μM of the calcium ionophore, ionomycin, a marked increase in D-Asp release is detected, suggesting that secretion of the enantiomer into the hemolymph does in fact occur and is, moreover, induced by calcium entry. In one set of experiments, >160 pmol of D-Asp was collected after stimulation with ionomycin from the F-cluster cells. These data support the concept of Ca^{2+}-dependent release of D-Asp from neurons, consistent with vesicular exocytosis of both enantiomers.
3.4 Discussion

3.4.1 D-Asp release

Several groups described D-Asp release from brain tissue upon chemical or electrical stimulation in mammalian endocrine tissues using radioactive labeling [39-41] and HPLC.[17, 42] Both KCl and acetylcholine-induced D-Asp release were more pronounced than that of L-Asp in adrenal slices, and only D-Asp (but not L-Asp or L-Glu) was shown to be released from rat adrenal gland when stimulated with nicotine.[42] In the rat brain, electrically evoked [3H]-D-Asp release from hippocampal slices mimics the release of L-Glu.[41] Similarly, electrical field-induced D-Asp release has been demonstrated in cerebral cultures and is not only Ca2+-dependent but also sensitive to synaptic vesicle toxins (including the relatively specific V-type H+-ATPase inhibitor), suggesting that D-Asp can accumulate in synaptic vesicles.[43] Studies on different animal models have also shown that D-Asp is stored in secretory granules,[17, 44] actively transported in nerves in a colchicine-dependent manner,[8] present in synaptosomes [44] and released in a Ca2+-dependent manner, similar to that of dopamine.[17] However, these experiments are not often at the level of a single cell, but rather, are made using large populations of cells containing multiple cell types (e.g., neurons, glia, etc.), thereby making it difficult to differentiate the neuronal contribution to D-Asp release.

Our findings show that F-cluster cells are capable of releasing a relatively high quantity of D-Asp at *A. californica* neurohemal areas. Released D-Asp may influence nearby neuronal terminals, sheath and vasculature cells at these locations, as well as distant
targets via a hormonal role. It is particularly intriguing that different chemical stimuli induce Asp release where the ratio of D-Asp to L-Asp varies. For example, ionomycin stimulation results in the predominant release of D-Asp, and was also found to be a more potent stimulus compared to an elevated potassium concentration for D-Asp release from A. limacina synaptosomes.[44] Thus, it appears that D-Asp and L-Asp release from the same physical location can be independently varied depending on a number of factors.

3.5 Conclusions

In this chapter, we report an off-line coupling of pH-mediated concentration and extraction of amino acids with CE to overcome the challenge of measuring extremely diluted D-Asp molecules from samples containing high levels of salts. With this method, the separation of amino acids from other biological compounds, as well as removal of salts in the same matrix was realized.

This new method along with the bead sample technology we have developed earlier provided a good toolset to study D-Asp release from whole Aplysia ganglia as well as specific locations using both KCl and ionomycin as stimuli.

The advantage of combining A. californica CNS studies with small volume analytical methods is the ability to study D-Asp dynamics at the neuronal cluster or even single neuron level. Information on the formation, uptake/accumulation (results not presented here) and release, of D-Asp in the A. californica nervous system indicates it plays a likely role in cell-cell signaling and justifies follow-up physiological studies on D-Asp function in neuronal systems. Additional work is now aimed at determining the specific enzymes involved in D-Asp synthesis.
3.6 References


3.7 Tables and figures

Figure 3.1. The schematic of the CE-LIF detection system. Analytes are separated in an 86 cm long, 50 µm ID/360 µm OD fused-silica capillary, with an effective length to the detector of 50 cm. The separation voltage is set at 20 kV. The separation buffer solution consists of 20 mM of β-cyclodextrin (β-CD) and 50 mM of sodium dodecyl sulfate (SDS) in 50 mM of borate buffer (pH 9.4). Reprint with permission from (Floyd, P.D., L.L. Moroz, R. Gillette, and J.V. Sweedler, *Capillary electrophoresis analysis of nitric oxide synthase related metabolites in single identified neurons*. Anal. Chem., 1998. 70(11): p. 2243-7.) Copyright (1998) American Chemical Society.)
Figure 3.2. Schematic representation of collecting extraganglionic media after KCl stimulation. Ganglia were allowed to rest from at least one hour before pre-stimulation samples were collected. Stimulated releasates were collected after 20 min stimulation of 40 mM extracellular concentration.
Figure 3.3. Reaction of NDA derivatizing primary amines. The bottom figure shows that the pH of CBI-Asp can be manipulated close to the pI of Asp, so the molecules of CBI-Asp can mostly have a neutral charge.
Figure 3.4. Schematic view of the solvent extraction procedure. The purpose of performing this step is to concentrate the extremely diluted D-Asp while leave the salt behind. The lower layer is organic phase containing CBI-Asp and the upper layer is aqueous layer containing salts.
Figure 3.5. Representative electropherograms of stimulated samples with or without undergoing solvent extraction procedure. The top trace shows clear D/L-Asp peaks with a Gaussian shape, while the bottom trace shows no recognizable peaks.
Figure 3.6. Method validation of solvent extraction protocol. a) Calibration curve showing the measured D/ (D+L)-Asp peak ratio of samples undergoing solvent extraction process versus the ratio of amount in original sample. The linearity and slope of one suggests that D/L-Asp is extracted into the organic phase to the same extent at all levels. b) Representative peak profiles plotted in the calibration curve.
Figure 3.7. Representative electropherograms of blank control versus biological samples. The comparison of control (middle trace) and sample (bottom trace) demonstrates the negligible amount of contaminations from blank, which would not affect the measurement of D/L-Asp from biological samples.
Figure 3.8. Elevated extracellular concentrations of potassium ions potentiate release of D/L-Asp from the cerebral ganglia. Representative electropherograms show cerebral ganglion releasate before KCl stimulation (left) and after KCl stimulation (right). In both cases, the black trace shows the original sample and the red trace shows the sample spiked with 1 µM D-Asp, used to calibrate the signal levels. Arrows indicate the increase in D-Asp signal because of the addition of standard and the change because of potassium ion stimulation (labeled standard and stimulation, respectively). Adapted from “Synthesis, accumulation, and release of D-aspartate in the Aplysia californica CNS” by Scanlan, C., T. Shi, N.G. Hatcher, S.S. Rubakhin, and J.V. Sweedler that was published in Journal of Neurochemistry, 2010. 115(5): p. 1234-1244.[1] Adapted with permission.
Figure 3.9. Stacked histograms summarizing results from five experiments, with statistical data placed in the sixth column. For each experiment, the extracellular potassium concentration was increased to 53 mM during the 20 min stimulation. Adapted from “Synthesis, accumulation, and release of D-aspartate in the Aplysia californica CNS” by Scanlan, C., T. Shi, N.G. Hatcher, S.S. Rubakhin, and J.V. Sweedler that was published in Journal of Neurochemistry, 2010. 115(5): p. 1234-1244.[1] Adapted with permission.
Figure 3.10. Microphotograph of solid phase extraction (SPE) collection beads placed upon the anterior tentacular and upper labial nerves of a cerebral ganglion from *Aplysia californica* to collect releasate from neurohemal areas containing terminals of the F-cluster neurons. (Inset) Close-up of the SPE beads on the nerves. Scale bar = 1mm. Adapted from “Synthesis, accumulation, and release of D-aspartate in the *Aplysia californica* CNS” by Scanlan, C., T. Shi, N.G. Hatcher, S.S. Rubakhin, and J.V. Sweedler that was published in *Journal of Neurochemistry*, 2010. 115(5): p. 1234-1244.[1] Adapted with permission.
Figure 3.11. Capillary electrophoresis with laser-induced fluorescence electropherograms of F-cluster releasate collected by solid phase extraction beads. (a) Releasate from F-cluster cells before stimulation. (b) Releasate from F-cluster cells after chemical stimulation with 10 µM ionomycin. (c) Accuracy of L- and D-Asp signal assignments is determined via standard addition using both enantiomers. Adapted from “Synthesis, accumulation, and release of D-aspartate in the Aplysia californica CNS” by Scanlan, C., T. Shi, N.G. Hatcher, S.S. Rubakhin, and J.V. Sweedler that was published in Journal of Neurochemistry, 2010. 115(5): p. 1234-1244.[1] Adapted with permission.
Chapter 4

Measuring D-Serine in Astrocytic Synaptic-Like Vesicles Using Capillary Electrophoresis

Notes and acknowledgements

This is a collaborative work between the Mothet group (Université de Bordeaux and now located at the Université Aix-Marseille) and the Sweedler group, as well as several other groups including those at the Universität Münster, Université de Lausanne, Université Paris, and the Max-Planck-Institut für Biophysikalische Chemie with the work being prepared for publication with the title and authors as follows (“Storage and uptake of D-serine into astroglial vesicles specify gliotransmission” by Martineau, M., T. Shi, J. Puyal, A.M. Knolhoff, J. Dulong, B. Gasnier, J. Klingauf, J.V. Sweedler, R. Jahn, J.P. Mothet, in preparation). This international collaboration has as its goal to answer to several outstanding questions related to gliotransmission. I was responsible for much of the analytical chemistry measurements, which includes sample pretreatment, CE analyses (with Ann Knolhoff’s help on CE-MS analyses) and related data interpretation. This chapter presents the work I was involved in, and does not include the detailed information regarding biochemistry and in situ hybridization which were performed mainly by Magalie Martineau and Jean-Pierre Mothet. However, some of the results from these other measurements are included here in this chapter, in order to provide a complete picture of this collaborative work.
I would like to acknowledge the following scientists for their enormous contributions: Magalie Martineau, Julien Puyal, Ann M. Knolhoff, Jérôme Dulong, Bruno Gasnier, Jürgen Klingauf and Jonathan Sweedler. Some of the people I didn’t even have a chance to meet in person, but this didn’t affect the always exciting and productive environment of this collaboration. M.M. implemented cell cultures, established the protocols for vesicle purifications at the beginning of this project, designed and conducted biochemical experiments on vesicles and transport assays with acridine orange (although not detailed here in this chapter). It is no exaggeration to say that M.M. set up the foundation of this project. J.P. conducted electron microscopy studies on brain slices. A.M.K conducted the CE-MS analysis. J.D. and J.P.M. conducted cell cultures, purification of vesicles at the later stage of the project. J.P.M also performed the immunofluorescence staining B.G. and J.P.M. performed the transport assays with radiolabelled amino acids (not presented here due to limit of space). J.K. contributed to final experiments and analysis. J.P.M., the advisor of M.M., conceived and directed the study. We acknowledge grant supports from CNRS (JPM), INSERM (JPM), Conseil Régional d’Aquitaine (JPM), the National Research Agency (JPM), the U.S.A. NSF [CHE-1111705 and CHE0526692] and NIH [P30DA018310] (JVS).

4.1 Introduction

Traditionally, synaptic activity only refers to the signal transmission of information from one neuron to another. It occurs when neurotransmitters, which are neuron-derived molecules, are released upon depolarization of the nerve terminal of the presynaptic neuron and bind to specific receptors on the postsynaptic neuron. However, neurons are not the only type of cells making up the central nervous system (CNS). The
CNS is composed of both neurons and glia, with glia being by far more numerous. The numeric preponderance of glia over neurons can be a 4:1 ratio in humans.[1] The traditional model of chemical synapse which only involves neurons needs to be revised due to recent findings describing the role of glial cells as a third party of the chemical synapse.[2-4] Anatomically, glia are well positioned to exchange chemicals with neurons at synapses, where chemical communication takes place via their fine processes which are in close proximity to synapses.[5] Despite glia’s intimate structural relation with neurons, the role of glial cells in chemical synapses was long ignored by neurophysiologists who considered them no more than housekeeping cells and only supplying nutrients for neurons. Now there is an emerging concept of the tripartite synapse considering glial cells, particularly astrocytes, as dynamic partners of neurons at synapses, controlling synaptogenesis [6] and synaptic transmission.[7]

A possible detailed mechanism of gliotransmission can be described as follows. In this model, astroglial cells first sense the level of synaptic activity through the interaction of various chemicals with a range of ion channels, transporters or receptors on the cell surface.[8, 9] This event triggers intracellular calcium elevation of glia,[10, 11] which induces the release of diverse signaling molecules termed gliotransmitters. Those gliotransmitters in turn regulate synaptic events and neuronal excitability by affecting pre- and postsynaptic neuronal elements.[12] Among those identified gliotransmitters are glutamate,[13, 14] adenosine triphosphate (ATP) [15, 16] and taurine.[17]

Another major glial-derived neuromodulator, D-Ser has been unveiled by the studies over the last decade.[18, 19] The discovery of D-Ser in the CNS revolutionized our thinking and forced us to reconsider the long believed fact that only L-isomers of
amino acids exist in mammalian tissues and body fluids. It is known today that higher organisms produce D-amino acids and in particular D-Ser.[20, 21] More importantly, works have shown that D-Ser is mainly present at astrocytes.[22] although very low levels can be detected in some neurons.[23] Another intriguing feature of this molecule is that it fulfills all criteria to be the major ligand for the strychnine-insensitive glycine-binding site of the N-methyl-D-aspartate receptors (NMDARs),[19] a key receptor for excitatory transmission and cognitive functions. Activation of NMDARs required glutamate and a co-agonist, which used to be thought as glycine.[24] The evidence of high levels of D-Ser present in regions enriched in NMDARs suggests the possibility of D-Ser to be an alternative co-agonist for NMDAR activation.

The biosynthesis of endogenous D-Ser was later reported by Snyder, showing the localization serine racemase (SR), an enzyme that synthesizes D-ser from L-Ser, was mainly present at astrocytes.[25, 26] Subsequently, it was demonstrated that selective degradation of D-Ser, but not of glycine, by D-amino acid oxidase (DAAO) significantly reduced NMDAR-dependent neurotransmission in the hippocampus.[27] This unambiguously indicated that glial-derived D-Ser is an endogenous ligand of NMDARs and glial cells can contribute actively to NMDAR-dependent processes in the mammalian brain.

However, the mechanism of D-Ser release from glia is still not clear. Do astrocytes release signaling molecules, such as D-Ser? Is the mechanism exocytosis, just like how most small molecule neurotransmitters are released from neurons? And is exocytosis of conventional small vesicles? These questions need to be addressed. It has been proposed that astroglia release D-Ser through a Ca2+- and synaptobrevin 2 (Sb2)-
dependent mechanism.[28-30] However, whether the released gliotransmitter originates from a cytosolic pool or from secretory organelles (the glia exocytosis hypothesis) remains controversial.[31] An issue with the general acceptance for astroglia exocytosis of neuromodulators, like D-Ser or L-glutamate (L-Glu), is that the demonstration of the enrichment of these gliotransmitters and their uptake in glia vesicles (GVs) has yet to be established. Understanding the molecular mechanisms of the release and uptake of gliotransmitters and notably D-Ser is an important step towards explaining the role of glia in the neuron-glia synapse. Currently, there is a gap in our understanding of glia transmitters.

Here we address the question of whether D-Ser and other gliotransmitters are packaged into vesicles and released via exocytosis similar to most neuron-based signaling molecules. Vesicles have been purified from astrocytes using magnetic beads coupled to vesicle specific antibodies. Immunoisolated Sb2-containing GVs display morphological and biochemical features similar to synaptic vesicles (SVs). In order to measure low levels of amino acids within those purified GVs, we use a custom capillary electrophoresis system with laser-induced fluorescence detection that has been optimized for characterizing chiral amino acid pairs from chemically-complex, small-volume samples. Our analyses results show the existence of a population of GVs which stores D-Ser and L-Glu, with estimated intravesicular concentrations of ~20 mM. This level is within the acceptable range of the concentration of signaling molecules. While not reported here, other results studied the localization, uptake and transport, producing a complete and convincing story. More specifically, when these results (not completely
presented here), we conclude that vesicles in astroglia do exist with the function to store and release D-serine, L-glutamate, and most likely other neuro- and gliamodulators.

4.2 Experimental

4.2.1 Reagents

We prepared 500 mL of 50 mM borate buffer (pH 9.4) by dissolving 2.38 g of sodium borate (Na2B4O7·10H2O) (Sigma–Aldrich, St. Louis, MO, USA) in 456 mL of ultrapure de-ionized (DI) water (Milli-Q Ultrapure Water Filtration Systems; Millipore, Bedford, MA, USA) and mixing with ~44 mL of 0.2 M NaOH; this solution was used in sample preparations buffer, unless otherwise noted. The separation buffer solution consisted of 10 mM of γ-cyclodextrin (γ-CD) and 30 mM sodium dodecyl sulfate (SDS) in 75 mM of borate buffer (pH 10.5). A CE-LIF non-chiral separation condition (50 mM borate buffer at pH 8.42) as well as CE-MS was used to verify the quantitation results. Naphathalene-2, 3-dicarboxaldehyde (NDA) was from Molecular Probes (Eugene, OR, USA). Methanol was purchased from Fisher Scientific (Fair Lawn, NJ) and formic acid was from Thermo Scientific (Rockford, IL). Other reagents were obtained from Sigma–Aldrich at the highest purity available. Solutions were filtered through 0.45 µm Acrodisc syringe filters (Gelman Laboratory, Ann Arbor, MI, USA) before use.

4.2.2 Capillary electrophoresis instrument

CE-LIF system
The system was a previously described, laboratory-built CE-LIF system,[32] converted to a 457.9-nm excitation wavelength by adapting the Argon Krypton mixed laser (Coherent, Santa Clara, CA, USA), focusing lens and optics. Analytes are separated in an 86 cm long, 50 µm ID/360 µm OD fused-silica capillary, with an effective length of 50 cm. The separation voltage is set at 20 kV. The chiral separation buffer solution consists of 10 mM of γ-cyclodextrin (γ-CD) and 30 mM sodium dodecyl sulfate (SDS) in 75 mM of borate buffer (pH 10.5). The non-chiral separation buffer solution consists of 50 mM borate buffer at pH 8.42.

CE-ESI-MS system

Samples were analyzed by a custom-built CE-ESI-MS platform that was partially based on an earlier system.[33] The signal was converted to 20 kV and was directly applied across a 90 cm-long fused silica capillary (40 µm inner diameter (i.d.), 110 µm outer diameter (o.d.), Polymicro Technologies, Phoenix, AZ) for electrophoresis separations. This separation capillary was connected to a custom-built coaxial sheath-flow ESI source. The sheath liquid (50% methanol with 0.1% formic acid) was supplied through the emitter at a 750 nL/min flow rate using a syringe pump. The separation capillary was coaxially fed through the emitter and protruded ~50-80 µm into the Taylor cone for stable and efficient ion generation by microelectrospray source.[34]

4.2.3 Analysis of vesicle contents

Starting material and immunoisolates were treated with 5% trichloroacetic acid to extract the free amino acids as described.[35] Amino acids were determined by a laboratory-assembled CE-LIF system using pre-column derivatization with 10 mM naphthalene-2,3-dicarboxaldehyde (NDA) and 20 mM KCN for 20 min in the dark.
Chiral separation and quantitation (3 or 4 technical replicates) was achieved by employing a separation buffer consisting of 10 mM γ-cyclodextrin, 30 mM sodium dodecyl sulfate (SDS) and 75 mM borate buffer at pH 10.5. Quantification results were accomplished by establishing calibration curves (Figure 4.1) for each molecule of interest within the linear range of the detector. The samples were diluted accordingly to ensure that concentrations of molecules being measured fall in the range of calibration curves. A CE-LIF non-chiral separation condition (50 mM borate buffer at pH 8.42) as well as CE-MS was used to verify the quantitation results. The laboratory-assembled CE-MS instrument and separation conditions were as described previously.[34]

To verify the D-Ser peak assignment, biological samples were incubated with Rhodotorula gracilis D-amino acid oxidase (RgDAAO), which was kindly provided by Dr. Pollegioni’s group at the University of Insubria in Italy. The original concentration of RgDAAO was 10.6 mg/ml, and it was diluted 1000 times in 100 mM phosphate buffer (pH = 8.25), giving a final concentration of ~10 µg/ml. The diluted enzyme solution was stored on ice to keep the activity of enzyme. Samples and enzyme solution were mixed with 1 to 2 volume ratio. In control experiments, enzyme solution was replaced by phosphate buffer. Samples were allowed to incubate with RgDAAO overnight at 30 °C. Derivatization was performed as described in previous paragraph before CE analyses were performed.

4.2.4 Antibodies

Monoclonal antibody clone 69.1 against rat synaptobrevin 2 (Sb2) was previously characterized.[36] The antibodies against the following proteins were used: cathepsin D (Santa Cruz biotechnology), cellubrevin (Covalab), D-serine (GemacBio), EEA1 (clone
14) and GM130 (clone 35) (Transduction Laboratories), Na\(^+\)/K\(^-\)-ATPase and SV2 (Developmental Studies Hybridoma Bank), PDI (clone RL90, Abcam), serine racemase (Santa Cruz Biotechnology and clone 29, BD Biosciences), synaptophysin (clone 7.2), TfR (clone H68.4, Invitrogen), vGlut1 and vGlut 2 (Millipore), Glutamine synthase (clone SH-B1, Sigma), β3-tubulin (clone TU-20, Chemicon) and glial fibrillary acidic protein (GFAP, DAKO). Rabbit polyclonal antibodies against Serine Racemase, SNAP23 and v-ATPase were kind gift of Herman Wolosker (Technion-Israel Institute of Technology, Haifa, Israel), Thierry Galli (INSERM, Paris, France) and Nicolas Morel (CNRS, Gif-sur-Yvette, France), respectively. Mouse monoclonal IgG1 was from Serotec (France). Secondary antibodies conjugated to horseradish peroxidase and Alexa Fluor 488 or 546 were obtained from P.A.R.I.S. and Invitrogen, respectively. Biotinylated donkey anti-rabbit antibody was from Jackson ImmunoResearch and the goat anti-rabbit IgG coupled to 1.4-nm gold particles was from Nanoprobe.

4.2.5 **Cell cultures**

 Cultured cortical astroglia cells were prepared from 0- to 4-day-old postnatal Wistar rats as previously described.[29] Cells were plated onto 150 cm\(^2\) flasks, kept at 37 °C in a 5% CO\(_2\) incubator for 14-20 days. Astroglia cultures purity (>95%) was assessed by immunostaining against the astrocytic marker GFAP.

4.2.6 **Organelle immunoisolation**

 The scheme of immunoisolation procedure is presented in Figure 4.2. All steps were carried out at 4-6 °C for western blot and transport analysis or at 0 °C for amino acid content analysis. For the immunoisolation of GVs, cultured astroglial cells were
washed, harvested by scraping and resuspended in homogenization buffer (4 mM HEPES-KOH pH 7.4, 100 mM K$_2$-tartrate, 2 mM MgCl$_2$, protease inhibitor cocktail (Complete EDTA-free, Roche)). Cells were homogenized using a cell cracker (clearance 10µm, European Molecular Biology Laboratory, Heidelberg, Germany) and the lysate was centrifuged for 10 min at 400 g. Low speed supernatants (LSS) (containing ~1 mg/ml protein) were incubated with monoclonal antibodies against Sb2 for 2 h under rotation and with magnetic beads coupled to protein G (Ademtech) one additional hour. The beads were further washed 3 times with homogenization buffer. Unbound membranes in the supernatant and LSS were pelleted by centrifugation at 175,000 g for 2 h in 80Ti rotor. All membrane or bead pellets were resuspended in SDS-PAGE sample buffer before being processed for gel electrophoresis and immunoblotting with standard methods, or in appropriate buffer for further analysis. Isolations with murine IgG or at 21 °C were used as a control. Vesicular amino acid contents were corrected for nonspecific adsorption on beads determined by isolating vesicles at 21 °C.

Immunoisolation of synaptic vesicles was performed following the same protocol, with the following modifications. The rat cerebral cortex of a male Wistar rat (2 months) was homogenized with a glass/Teflon homogenizer (10 strokes at 2,500 rpm) in homogenization buffer immediately after decapitation. The homogenate was centrifuged for 25 min at 35,000 g. The resulting supernatant (containing ~3 mg/ml protein) was incubated 1 h with anti-Sb2 and 1 h with magnetic beads.
4.2.7 Electron microscopy on isolated vesicles

Immunobeads with bound organelles were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h at 20 – 22 °C. After a rinse in 0.1 M sodium phosphate (pH 7.4), the beads were embedded in 1% agarose in the same buffer. Agarose blocks were then postfixed for 1 h with OsO₄ (1% in phosphate buffer), dehydrated and embedded in Epon. During dehydration, they were stained en bloc with uranyl acetate in 50% ethanol. Thin sections (~60 nm) were counter-stained with lead citrate and examined in a Philips 301 electron microscope.

4.3 Results

To uncover the existence of astroglial secretory organelles that store D-serine, we carried out a detailed analysis of Sb2-containing vesicles from rat cortical astrocytes. Glial organelles were immunoisolated from a low speed supernatant (LSS) of cultured astrocytes using the cytoplasmic epitope of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor) protein Sb2 as a bait.[29, 37, 38] Electron microscopy analysis revealed that purified Sb2-containing organelles (Figure 4.3a) are clear round shape vesicles with a homogenous diameter of 40 ± 16 nm (Figure 4.3b). GVs appear similar in size and morphology to GVs and SVs in situ.[39, 40] Control beads coupled to non-immune IgG were devoid of bound membranes, suggesting that membrane contamination is negligible.

Defining D-serine as an exocytically released gliotransmitter requires characterizing the amino acid content of purified GVs. Capillary electrophoresis analyses, with laser-induced fluorescence as detection method, revealed that vesicle fractions
contained D-serine and L-glutamate in amounts that were largely enriched over control incubations (Figure 4.4).

One challenge we have encountered using CE-LIF to measure D-amino acids in complicated biological samples was that peak co-migration, although rare, still could exist. In this work, several steps have been taken to ensure an unambiguous identification of D-Ser peak and an accurate quantification of all amino acids of interest. First step was to incorporate an D-amino acid oxidase (DAAO) enzyme reaction, which could specifically convert the amino group on D-Ser to a keto group (Figure 4.5). As a result, D-Ser peak should be removed after enzyme incubation. The removal of D-Ser with *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO) [28] verified the identity of D-Ser (Figure 4.6). The second step was to take two complementary non-chiral separation approaches, using either laser-induced fluorescence (Figure 4.7) as detection method or mass spectrometry (Figure 4.8). The two complementary approaches validated our quantitation results of the amino acid content within purified vesicles. The quantitation results of all the amino acids were consistent among different methods, except GABA. The CE-LIF chiral separation method presented a significant GABA peak while both two non-chiral methods detected only a trace level (Figure 4.9). This indicated that there was another unknown molecule co-migrating with GABA under the condition of chiral separation. This was an excellent example to show the need of using more than one measurement protocols in CE-LIF when analyzing complex biological samples. The quantitation results from three different methods on two CE platforms (Table 4.1) agree well, further enhancing the credibility of our quantitation data.
We also characterized immunoisolated SVs using CE-LIF analysis. Isolated SVs contained a significant amount of L-glutamate, glycine and γ-aminobutyric acid (GABA) (Figure 4.10). These vesicular neurotransmitters agreed with previous studies.[41] Although present in the cortex homogenate, D-Ser was not detected in SVs further indicating that neurons may not exocytose D-Ser but release it from a cytosolic pool.[42, 43] Accordingly, neuronal D-Ser immunostaining was confined to the soma of neurons but was excluded from their distal neuritis or boutons (data not presented here). The retrieval of D-Ser only GVs argues for the existence of a glia-specific transport mechanism.

Assuming similar expression of Sb2 copies in GVs and SVs [44] and taking into account the degree of reported colocalisation between the gliotransmitters and Sb2,[29, 45] we estimated the intravesicular concentration of d-serine and L-glutamate to be ~20 mM.

4.4 Conclusions

It has been previously established that release of gliotransmitters such as L-glutamate or D-Ser is dependent on Ca\(^{2+}\)-regulated exocytosis.[28-30, 38, 40, 46] However, the vesicular origin of the release gliotransmitter remained controversial.[31] Here we provided a direct and unequivocal demonstration that two gliotransmitters, namely L-Glu and D-Ser, are stored inside GVs. The existence of different populations of vesicles in glia to store and release one or several gliotransmitters requires further investigation. Our evidence of vesicular release of D-Ser doesn’t exclude the possibility
of non-vesicular release mechanism, such as gap junction hemichannels, reversal of uptake carriers, which can be responsible for L-Glu release from astrocytes.[31]

Our approach, combining immunopurification and various analytical chemistry techniques, reveals the storage of both D-Ser and L-Glu in a group of astrocytic synaptic-like vesicles contain both D-Ser and L-Glu. This evidence, along with other data on D-Ser vesicular transport (not presented here), support the concept that astrocytes possess specific vesicles capable of sequestering and storing D-Ser and Glu as gliotransmitters, which undergo calcium-dependent exocytosis and modulate synaptic transmission.
4.5 References


4.6 Tables and figures

Figure 4.1. Representative calibration curves generated for amino acid quantitation using CE-LIF. The linear range is from 0-2000 nM, with three technical replicates.
Figure 4.2. Scheme of immunoisolation procedure. The same process was performed both at 0 °C and elevated temperature 20 °C, when most amino acid contents leak out of the vesicles[41]. So samples prepared at 20 °C were used as blank controls to correct for nonspecific binding of amino acids onto the beads.
Figure 4.3. Morphological characterization of Sb2-positive GVs. a) Electron micrographs of organelles immunoabsorbed onto beads. Scale bar: 100 nm. Control immunopurification (Ctl) shows no bead-bound vesicle. b) Size distribution of immunoisolated vesicles bound to Sb2 beads. The number of vesicles analyzed (total = 300) is reported within the bars. Black curve represents the Gaussian fit of diameter size distribution.
Figure 4.4. Representative electropherograms of immunoisolated glial vesicles (GVs). Astrogila low-speed supernatant (LSS), Sb2-containing vesicles immunoisolated at 20 °C (leaked GVs) and at 0 °C (Intact GVs) were analyzed by CE-LIF. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrows point to the peak of L-Ser (LS), D-Ser (DS), GABA + unknown substance (γ*), glycine (G) and L-Glu (E).
Figure 4.5. Reaction of DAAO with D-amino acid. DAAO can selectively convert the amino group on D-amino acids, but not L-amino acids to a keto group.
Figure 4.6. Representative electropherograms of intact vesicle samples treated with (top) or without (bottom) DAAO. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrows point to the peak of L-Ser (LS), D-Ser (DS), GABA + unknown substance (γ*), glycine (G) and L-Glu (E).
Figure 4.7. Representative electropherograms of vesicle amino acid content using CE-LIF with a non-chiral separation method. To validate quantitation results via CE-LIF chiral separation as shown in Figure 4.4, astroglial Sb2-containing vesicles immunoisolated at 20 °C (leaked GVs) and at 0 °C (intact GVs) were analyzed using a non-chiral CE-LIF method. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrows point to the peak of Ser (S), GABA (γ), glycine (G) and L-Glu (E).
Figure 4.8. Representative data of vesicle amino acid content using CE-MS with a non-chiral separation method. To validate quantitation results via CE-LIF chiral separation as shown in Figure 4.4, astroglial Sb2-containing vesicles immunoisolated at 20 °C (leaked GVs) and at 0 °C (intact GVs) were analyzed using a non-chiral CE-MS method. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. S = serine, E = glutamate.
Figure 4.9. Electropherograms of one sample run by three different methods with GABA ($\gamma$) peak highlighted out. The CE-LIF chiral separation method (a) presents a significant GABA peak while both two non-chiral methods (b,c) detect only a trace level, suggesting a co-eluting peak with GABA in CE-LIF chiral separation method.
Table 4.1. A table summarizing representative quantitation results. D-Ser quantitation of one sample set using three different separation methods on two CE platforms are compared. 3 technical replicates were performed with CE-LIF while 1 technical replicate was performed with CE-MS. The final quantitation results of each amino acid were calculated from data of all methods, except for GABA.

<table>
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<tr>
<th>Unit=μM</th>
<th>CE-LIF</th>
<th>CE-MS</th>
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<tr>
<td></td>
<td>Chiral separation</td>
<td>Non-chiral separation</td>
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<tr>
<td></td>
<td>D-Ser</td>
<td>L-Ser</td>
</tr>
<tr>
<td>Leaked vesicles</td>
<td>ND</td>
<td>2.2</td>
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<td>Intact vesicles</td>
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Figure 4.10. Cortex homogenate, Sb2-containing synaptic vesicles immunoisolated at 20 °C (leaked GVs) and at 0 °C (intact GVs) were analyzed by CE-LIF. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrowheads point to the position of L-Ser (LS), D-Ser (DS), GABA (γ), glycine (G) and L-glutamate (E).
Chapter 5

Developing Methods to Measure D-Glutamate, D-Alanine and D-Proline in Biological Matrix

Notes and Acknowledgments

The following members of the Sweedler group assisted in the research presented in this chapter. The assistance of Nobutoshi Ota was vital to achieve the baseline separation of D/L-glutamate (D/L-Glu). Stanislav Rubakhin and Xiying Wang helped tremendously with the sample dissection and preparation of rat pituitary and pancreas. S.R. also provided numerous insightful suggestions on experimental design.

5.1 Introduction

Perhaps the most interesting subset of putative active compounds found within the central nervous system (CNS) is the D-amino acids (D-AAs). Their discovery forced us to reconsider the long cherished dogma that only L-amino acids (L-AAs) exist in mammalian tissues and body fluids. The D-AAs found in animal kingdom include D-serine (D-Ser),[1-6] D-aspartate (D-Asp),[7-12] D-glutamate (D-Glu),[13] D-alanine (D-Ala),[14, 15] D-proline (D-Pro),[16, 17] D-leucine (D-Leu),[18] D-methionine,[19] D-Asparagine [20, 21] and D-Arginine.[22] Among them, D-Ser and D-Asp are studied most extensively. As highlighted in Chapter 4, it is now known that, D-Ser plays critical roles in glia-neuron communication through N-methyl-D-aspartate (NMDA) receptor-mediated signaling processes.[23-27] Our group also has devoted efforts to demonstrate the role of D-Asp in cell to cell signaling using Aplysia californica (A. californica) as a
well-characterized neuroscience model system.[11, 12, 28, 29] Besides D-Ser and D-Asp, other D-AAs are also found in brain, but their functional roles in nervous system are much less studied.

D-Ala was measured in the brain [14, 30-32] and can function as an co-agonist at the glycine binding site of the NMDA receptor, similarly to D-Ser. D-Ala has been reported in both rat pituitary and pancreatic islets using immunostaining.[15] In particular, in the double-immunostaining experiments, D-Ala displayed a similar distribution with adrenocorticotropic hormone (ACTH) cells. When endogenous D-Ala was measured in the tissues and physiological fluids of mice with various DAAO activities, its amount increased significantly with a lowered D-amino acid oxidase (DAAO) activity.[31] D-Ala was also reported to show circadian changes where its level was high during the sleeping period and low during the active period in rats with both diurnal and nocturnal habits.[14]

Since L-glutamate (L-Glu) is the predominant excitatory neurotransmitter in the brain, the observation of its D counterpart, D-Glu in the nervous system [33-35] is particularly intriguing. D-Glu was also isolated from the liver and kidneys, where D-Glu existed at substantial levels, even exceeding that of D-Asp in some cases.[35] Highly sensitive immunostaining also revealed a sparse distribution of D-Glu immunoreactive neurons in rat brains.[13] Moreover, electrophysiological experiments indicated that D-Glu could induce responses, although less potent compare to L-Glu.[36] However, differed responses to these enantiomers may imply distinct mechanisms of response. Earlier work also showed that when rat brain slices were incubated with a medium
containing glucose and D-Glu, similar accumulation of L-Glu and D-Glu occurred. But associated accumulation of potassium ion was accelerated less by D-Glu.[37]

One of the least studied D-AAs, D-Pro was found at relatively high levels in the pituitary gland, cerebellum and pineal gland of mice, as well as the pancreas.[16, 17]

The information obtained so far on D-Glu, D-Ala and D-Pro by various measurement techniques is limited. We hope to develop a suite of approaches to advance our knowledge of the presence and activity-dependent release of those putative cell to cell signaling molecules. Depending on the specific questions to be answered, measurement can range from entire tissue regions to single cells. Although so far most analytical measurements of these three D-AAs employed high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) can be particularly beneficial in analyzing small sized samples, such as single cells. Developing methods on CE platform will be our main focus presented here.

Rat pituitary and pancreatic islets can be good models to study activity-dependent release because of their isolated distributed within the tissue. In addition, since D-Ala has been reported to follow a circadian rhythm,[14] suprachiasmatic nucleus (SCN) would be an appropriate model to study its relation with biological clock. For D-Glu, the immunostaining of its location in sparse networks can be used as a map to ensure that the appropriate neurons are dissected and analyzed. Single cell assays are highly expected to understand the heterogeneity of D-Glu distribution in those sensory neurons which are often thought to be homogenous.
This chapter describes the ongoing method development on CE platform aiming at providing sensitive, reliable measurements of D-Glu, D-Ala and D-Pro. The newly developed analytical methods, in combination with appropriately designed experiments, will help to uncover the roles of these D-AAs play in cell to cell signaling in nervous and endocrine system.

5.2 Experimental procedures

5.2.1 Reagents and solutions

The D/L-Glu separation buffer solution consists of 80 mM of α-cyclodextrin (α-CD) in 200 mM of borate buffer (pH 9.5). An alternative separation buffer for D-Glu contains 60 mM α-CD, 20 mM β-CD and 40 mM sodium deoxycholate (SDC) in 100 mM Borate buffer (pH 9.3). Naphathalene-2, 3-dicarboxaldehyde (NDA) was from Molecular Probes (Eugene, OR, USA). NDA solution is made in methonal while KCN solution is made in 50 mM borate buffer at pH = 9.5. NDA and KCN solutions are mixed with standards or biological samples and the reaction mixture is allowed to react for 20 min in dark. The D/L-Ala and D/L-Pro chiral separation was achieved using the following conditions: 20 mM β-CD, 30 mM SDS in 100 mM borate buffer at pH = 10. An alternative separation consists of 10 mM γ-CD, 30 mM SDS in 100 mM borate buffer at pH = 9.5. Fluorescein isothiocyanate (FITC) solution is made in acetone. 1 μL sample or standard is mixed with 1 μL 1 mM FITC and 8 μL sample buffer (20 mM borate buffer at pH = 10.0). The derivatization reaction is allowed in dark overnight. Other reagents were obtained from Sigma–Aldrich at the highest purity available. Solutions
were filtered through 0.45 μm Acrodisc syringe filters (Gelman Laboratory, Ann Arbor, MI, USA) before use.

5.2.2 Capillary electrophoresis instrument

The system is a previously described, laboratory-built CE-LIF system [38], converted to a 457.9-nm excitation wavelength by adapting the Argon Krypton mixed laser (Coherent, Santa Clara, CA, USA), focusing lens and optics. Analytes are separated in an 86 cm long, 50 μm ID/360 μm OD fused-silica capillary, with an effective length of 50 cm. The separation voltage is set at 20 kV. The chiral separation is performed under conditions with different buffer components as described in 5.2.1.

5.2.3 Cerebral ganglia and pituitary isolation

_A. californica_ (200–300 g) were obtained from Charles Hollahan (Santa Barbara, CA, USA) and kept in an aquarium containing continuously circulating, aerated and filtered sea water (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) at 14–15°C until used. Animals were anesthetized by injection of isotonic MgCl₂ (~30 to ~50% of body weight) into the body cavity. The cerebral ganglia were dissected and placed in artificial sea water (ASW) containing (in mM): 460 NaCl, 10 KCl, 10 CaCl₂, 22 MgCl₂, 6 MgSO₄, and 10 HEPES (pH 7.8), or in ASW-antibiotic solution: ASW supplemented with 100 units/mL penicillin G, 100 μg/mL streptomycin, and 100 μg/mL gentamicin (pH 7.8).

The rats were anesthetized with diethyl ether and euthanized by decapitation. Anterior pituitary, posterior pituitary were quickly excised and stored at -80 °C before...
use. These tissues were homogenized for 2 min in 200× vol of MeOH. The homogenates were centrifuged at 4500×g for 5 min to obtain the supernatants. Filtration was performed through microcon® filter (3000 MW) to remove proteins as well as other large molecules and the resulting filtrate was evaporated to dryness. To the residue, appropriate amount of buffer was added and the solution underwent CE analysis for the determination of the D- and L-amino acids.

5.2.4 Solvent extraction procedure

The 40 µL aliquots were dried in the oven overnight and reconstituted with 5 µL water or 5 µL internal standards containing 1 µM aminoadipic acid. The addition of internal standard (IS) allowed accurate determination of extracellular D-Glu levels and reduces methodological uncertainties. KCN and NDA solutions were added to the reconstituted samples and the reactions were allowed to continue for 20 min in the dark to form N-substituted 1-cyanobenz[f]isoindo[le amino acid (CBI-AA). The large amount of inorganic salts in these samples created issues with CE when the solutions were concentrated; thus, the salts were removed via extraction. Specifically, HCl solution was added to the reaction mixture to adjust the pH and the resulting neutrally charged CBI-AA was extracted into CH₂Cl₂. The lower layer was placed into a PCR tube and the extraction process was repeated three times. The combined collected organic layer was dried and reconstituted with buffer. The samples were then assayed via CE as described.

5.3 Results
5.3.1 Method developed to measure D-Glu release from Aplysia cerebral ganglia

5.3.1.1 Liquid-liquid extraction method optimization

The liquid-liquid extraction procedure was performed similarly as described in Chapter 3, section 3.3.2 with the modification of using another molecule as an IS instead of D-Glu itself. Because D-Glu peaks in releasate samples were often small even after sample concentration, adding D-Glu as IS was not very practical to ensure precise quantitation.

Several candidates, including β-glutamic acid, threo-β-methylaspartic acid and L-2-aminoadipic acid, were screened to evaluate their potential of being used as IS (Figure 5.1). They have to meet several criteria: 1) having similar structural features and pKa profile as Glu; 2) not co-migrating with D/L-Glu peak; 3) not interfering with peaks in biological samples. Among all the candidates, only L-2-aminoadipic acid fulfilled all requirements. It was used in all sample preparation procedure of measuring D-Glu release.

5.3.1.2 Liquid-liquid extraction method validation

The liquid-liquid extraction method validation was performed similarly as described in chapter 3, section 3.3.2, except that the ratio of L-2-aminoadipic acid to D-Glu was used instead of the ratio of D-Glu to D-Glu+L-Glu (Figure 5.2).

5.3.1.3 Secretion of D-Glu from whole ganglia

D/L-Glu enantiomers could be almost baseline separated under the current condition. In Figure 5.3, a clear increase of D-Glu peak could be observed after the
whole ganglia was stimulated by an elevated extracellular potassium concentration. This indicated the feasibility of this method in measuring the low abundant D-Glu released from *A. californica* ganglia. However, among the releasate samples collected (N>5), the increase of D-Glu peak was only found once. This can be attributed to the low concentration of D-Glu in the ganglia being tested. To overcome this challenge, future efforts need to be made on locating the regions or particular cell populations which contain higher levels of D-Glu.

**5.3.2 Method developed to measure D-Ala and D-Pro in biological samples**

**5.3.2.1 Optimization of separation conditions**

Compared to other chiral amino acid enantiomers, D/L-Ala are especially challenging to separate, perhaps due to its smallest size. When attached with a relatively large fluorescent reagent, this small chirality difference is further reduced, resulting in minor differences between derivatized D/L-Ala. Separation of NDA derivatized D/L-Ala was tried with all separation conditions available in our lab, which employed a variety of chiral selectors such as α-CD, β-CD, γ-CD, S-β-CD. However, under those conditions, CBI-Ala enantiomers were not separated at all, even without a split at top of the peak of their mixtures. In addition to D-Ala, another D-AA of interest, D-Pro is not a primary amine, so it cannot be derivatized with NDA.

Alternative derivatizing reagents with the ability to derivatize secondary amines were tested, including 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and fluorescein isothiocyanate (FITC). Surprisingly, standards of both pairs could be easily baseline
separated on a number of conditions when FITC was used in derivatization. This may be attributed to the relative size of CD’s cavity and the derivatized analytes. For example, γ-CD possesses a truncated cone structure, with a roughly 8 Å inner diameter at the bigger end. Since the naphthalene end of CBI-AA is much smaller than the size of CD cavity, CBI-AA is easier to fit that end into the cavity instead of the end with chiral moiety. As a result, the structural difference of chiral moieties contributes less to the difference of enantiomers partitioning into the chiral selectors. The same situation applies to NBD-AAs. In contrast, FITC has a much larger 3D structure, arranged perpendicular to the functional group that can react with AAs. With the width of three rings to be more than 10 Å, the FITC derivatized molecules are more likely to interact with the CD cavity from the AA end (Figure 5.4). If this is the case, small chirality differences interact more with CD and as a result, improved chiral resolution may be obtained.

Baseline separation was achieved for both Pro and Ala enantiomer standards in one single run (Figure 5.5c). This method was then validated by measuring D-Ala in anterior pituitary, which was reported to contain D-Ala.[14] Although D/L-Ala were well separated with each other and matched their migration times (Figure 5.5a and 5.5b), the putative D-Ala peak showed no difference before and after DAAO treatment (data not shown). DAAO can specific convert the amino group on D-AAs to a keto group. As a result, if nothing co-migrates with the D-AA peak, it should be removed after reaction. The almost unnoticeable change of the putative D-Ala peak suggests: 1) comigration of unknown molecules with D-Ala occurred under the current separation condition; 2) D-Ala, if present, was at negligible level in the tested samples. Previous results also show only a few percent of D-Ala in the total Ala pool. To overcome this challenge of co-
migration, efforts need to be made on further optimizing the separation condition, especially using biological samples instead of pure standards in the optimization process. DAAO enzyme reaction provides a good way of distinguishing the authentic D-Ala from other compounds co-migrating with it.

5.4 Conclusions

In this chapter, methods to quantitatively measure D-Glu release from A. californica ganglia was established and validated in biological samples. Among all releasate samples being analyzed, only one showed a low level of D-Glu released after simulation event. Future work can be directed to locate the regions or particular cell populations which contain higher levels of D-Glu to facilitate the measurements.

In addition, efforts have been made to optimize separation conditions for both D-Pro and D-Ala and examine the reliability of those methods in analyzing biological samples. Both pairs of Pro and Ala enantiomers could be very well separated as standards using FITC as a derivatizing reagent. But when applied in biological matrix, this method suffered from poor selectivity, which might be overcome by further fine tuning each buffer component.
5.5 References


5.6 Tables and figures

Figure 5.1 Results of testing three candidates to be used as IS in measuring of D-Glu after a solvent extraction procedure. L-2-aminoadipic acid was well separated from both D- and L-Glu as well as other peaks in biological samples, thus was finally chosen as IS.
Figure 5.2 Method validation of using L-2-amino adipic acid as IS in quantifying D-Glu after a solvent extraction procedure. The ratio of peak area of L-2-amino adipic acid to that of D-Glu was plotted against the ratio of their concentration in original samples. This equation generated by linear regression curve was used for quantitation purpose.
Figure 5.3 An increase of D-Glu peak was shown after chemical stimulation by elevated potassium concentration.
Figure 5.4 Relative size of CD cavity and FITC.
Figure 5.5 Representative electropherograms of pituitary samples (A and B) as well as standards (C). LP = L-Pro, DP = D-Pro, LA = L-Ala, DA = D-Ala
Appendix A

Abbreviations

2D-HPLC Two dimensional-high performance liquid chromatography

A. californica Aplysia californica

A. limacine Aplysia limacine

AAs amino acids

aCSF artificial cerebrospinal fluid

ACTH adrenocorticotropic hormone

AD Alzheimer’s disease

AMPA (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APOC (+/-)-1-(9-anthryl)-2-propyl chloroformate

Asp aspartate

BAPTA-AM 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetateacetoxymethyl ester

BGE backgournd electrolyte

BOC-L-cys N-tert-butyloxy-carbonyl-L-cysteine
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<td>CDs</td>
<td>cyclodextrins</td>
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<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
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<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
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<tr>
<td>CE-LIF</td>
<td>capillary electrophoresis with laser-induced fluorescence</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CSP</td>
<td>chiral stationary phase</td>
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<td>PPD</td>
<td>poly-m-phenylenediamine</td>
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