Chiral Measurement of Aspartate and Glutamate in Single Neurons by Large-volume Sample Stacking Capillary Electrophoresis

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Supporting Information

Additional Experimental Details

D-Aspartate Oxidase Cloning and Purification. Mouse kidney D-aspartate oxidase (D-AspO) was cloned by a reverse transcription polymerase chain reaction (RT-PCR) technique and produced as His-tagged protein in *Escherichia coli*. The cloning primers were based on a published cDNA sequence (Katane, M.; Furuchi, T.; Sekine, M.; Homma, H. *Amino Acids,* **2006**, 32, 69.) and synthesized by Integrated DNA Technologies (Coralville, IA). Mouse kidney cortex was dissected and homogenized in 2 mL of TRIzol (Invitrogen, Carlsbad, CA) and the RNA extracted from the homogenate. First strand cDNA was prepared from total RNA with Superscript III reverse transcriptase (Invitrogen). The first strand cDNA was then PCR-amplified using platinum pfx DAN polymerase (Invitrogen, Carlsbad, CA) and the following primers: 5'-GGCATATGGACACAGTGTGTATTGCGGT-3' (forward, NdeI site underlined) and 5'-CCGGATCCCTACAGCTTCGACAAGGAAGC -3' (reverse, Bam HI site underlined). For PCR amplification, the following temperature program was used: 94 °C for 5 min (1 cycle); 94 °C for 15 sec, 55 °C for 30 sec, and 68 °C for 80 sec (29 cycles); 68 °C for 10 min. Next, the target PCR fragment (~1000 bp) was isolated from an agarose gel with a QIAGEN kit, ligated to pCR2.1 ®-TOPO® vector (Invitrogen) and transformed into TOP10 chemically competent cells (Invitrogen). Ten colonies were picked from the transformation plate, grown up, and their plasmids purified and sequenced. A sequence-verified clone was then grown up, and the plasmid was isolated and digested with NdeI and Bam HI restriction enzymes (New England Biolabs, Ipswich, MA). The digested plasmid was then ligated to bacterial protein expression vector pET15b (EMD Millipore, Billerica, MA) and transformed into TOP10 cells.

Following sequence confirmation, the clone plasmid was purified from TOP10 and transformed into BL21(DE3) cells (EMD Millipore). The D-AspO was then expressed by growing a 3 mL bacterial overnight culture at 30 °C in 250 mL Luria broth medium until OD600 reached 0.5. Next, 0.01 mM isopropyl β-D-1-thiogalactopyranoside was added and incubated at 30 °C for 20 h with continuous shaking. The bacteria culture was pelleted by centrifugation at 10,000 *g* for 10 min at 4 °C. The resulting pellet was lysed with 10 mL of BugBuster Mix (EMD Millipore) supplemented with 2 mM dithiothreitol (DTT) and a protease inhibitor cocktail. The lysate was then centrifuged at 16,000 *g* for 20 min at 4 °C. The supernatant was mixed with 2 mL NiNTA resin in 10 mL NiNTA bind buffer (EMD Millipore) and incubated with rocking for 1 hr at 4 °C. A 24-mL Econo column (Bio-Rad Laboratories, Hercules, CA) was then gravity-packed with the resin. The column was washed with 40 mL NiNTA wash buffer and then protein was eluted in 7 mL of NiNTA elution buffer. The eluted protein was concentrated to 2 ml with an Amicon Ultra Centrifugal filter (Thermo Fisher Scientific, Waltham, MA) and then subjected to gel filtration chromatography using 50 mM Tris-HCl buffer, pH 8.0, with 2 mM DTT at 1 mL/min flow rate with a HiPrep Sepharcryl 200 HR 16/60 column (GE Healthcare Life Sciences, Pittsburgh, PA). The enzyme-containing fractions were buffer exchanged by Amicon into enzyme storage buffer containing 50 mM $NaPO_4$, 150 mM NaCl, pH 8.0, 20% glycerol, and 1 mM DTT, and stored at –20 °C until use.

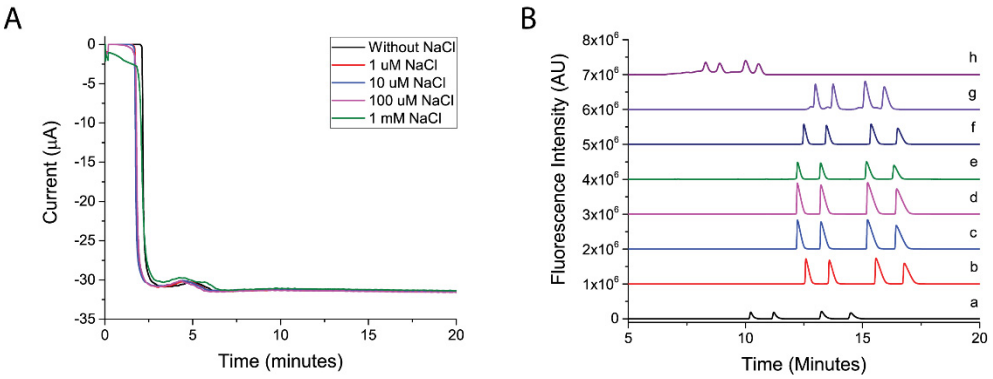

Figure S1. Influences of inorganic ions on the separation and detection performance of CZE-LIF and LVSS-CE-LIF. (A) Current profiles for LVSS-CE analyte separations with 1, 10, and 100 uM or 1 mM NaCl, as well as without NaCl, in the injected sample plug. (B) CE-LIF electropherograms from the analysis of D- and L-Asp, and D- and L-Glu, with various levels of NaCl in the injected sample plug. a) CZE of 1 uM AAs without NaCl; b) LVSS-CE of 10 nM AAs without NaCl; c) LVSS-CE of 10 nM AAs in 1 uM NaCl; d) LVSS-CE of 10 nM AAs in 10 uM NaCl; e) LVSS-CE of 10 nM AAs in 100 uM NaCl; f) LVSS-CE of 10 nM AAs in 1 mM NaCl; g) LVSS-CE of 10 nM AAs in 10 mM NaCl; h) LVSS-CE of 10 nM AAs in 100 mM NaCl. Peaks: From left to right, L-Asp, D-Asp, D-Glu and L-Glu.

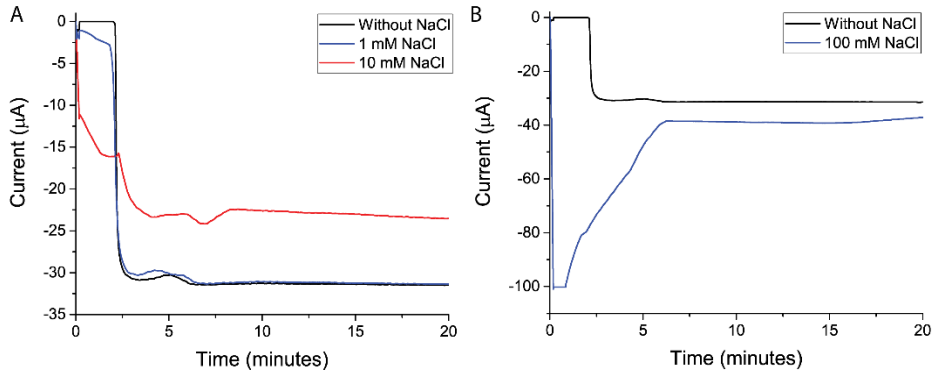

Figure S2. (A) Current profiles for LVSS-CE separations without, and with, 1 and 10 mM NaCl in the injected sample. (B) Current profiles for analysis of samples without NaCl and with 100 mM NaCl.

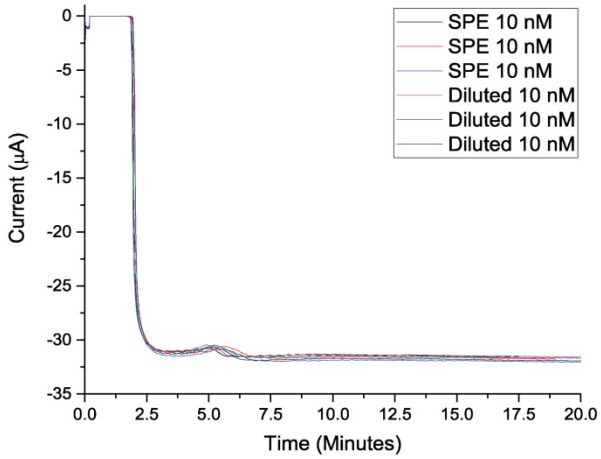

Figure S3. Overlay of current profiles from the analysis of 10 nM D- and L-AA standards following desalting by solid phase extraction (SPE), as well as dilution, where dilution was used to minimize the concentration of ionic species generated from derivatization.

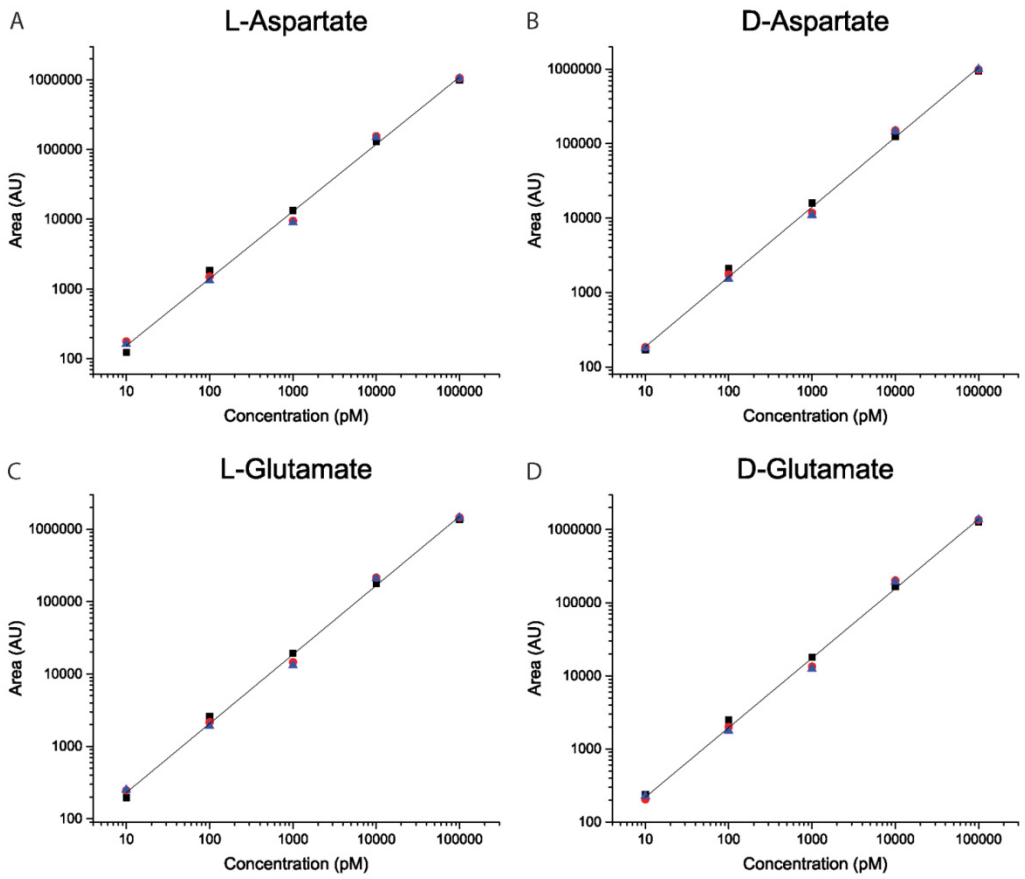

Figure S4. Calibration curves for L-Asp, D-Asp, L-Glu and D-Glu. Three technical replicates were performed for each analyte concentration. Data produced by the LVSS-CE-LIF method demonstrate linear dependence on analyte concentration over the concentration range of interest (10 pM–100 nM). The R^2 values for all plots were greater than 0.996, and the limits of detection for each amino acid were 30 fM or better.

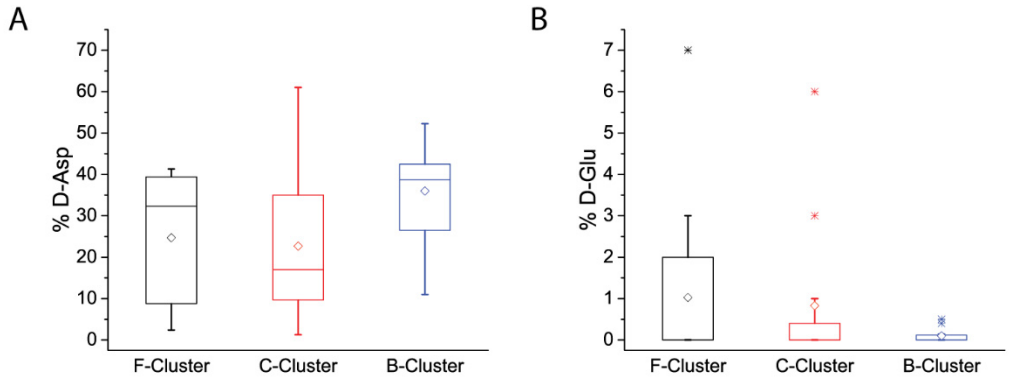

Figure S5. Detection of D-Asp and D-Glu in individual neurons using LVSS-CE-LIF. Box and whisker plots of the relative to total amino-acid content of (A) % D-Asp and (B) % D-Glu in individual neurons from the F-, C-, and B-clusters of the cerebral ganglion. Asterisks indicate outlier data points as determined by inner fences. ◊ = Mean. Lower inner fence = Lower quartile – 1.5*(interquartile range). Upper inner fence = Upper quartile + 1.5*(interquartile range). Lower/middle/upper lines indicate the 1st quartile/median/3rd quartiles, respectively. Number of single neurons analyzed per cluster, sample size (n): F-cluster = 15, C-cluster = 17, B-cluster = 16.

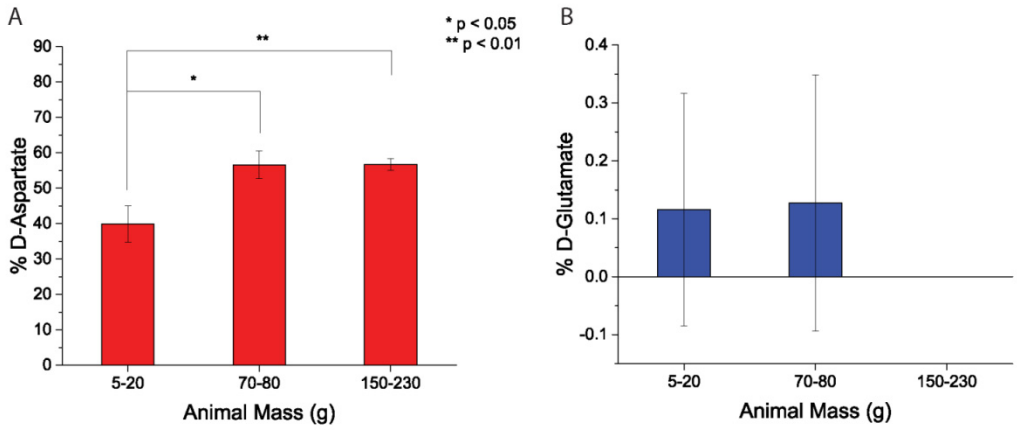

Figure S6. Relative quantification of (A) D-Asp and (B) D-Glu in entire F-clusters from animals of different sizes, where the 5–20 g animals are juveniles, and the 70–80 g and 150–230 g are adult *Aplysia*. The error bars represent standard deviations for data acquired from three biological replicates. Two-tailed t-test, *$p < 0.05$, **$p < 0.01$, $n = 3$ for each group.