Electrochemical Microphysiometry Detects Cellular Glutamate Uptake

Dusty R. Miller,1,∗ Ethan S. McClain,1,✉ and David E. Cliffel 1,2,✉,∗

1Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235-1822, USA
2Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt University, Nashville, Tennessee 37235-1809, USA

Glutamate is one of the 20 canonical amino acids that together provide the structural and enzymatic foundation of proteins. Alone, glutamate plays a very different role and is itself an excitatory signaling molecule widely distributed throughout the central nervous system. In fact, glutamate is the most prevalent neurotransmitter and its function is essential for proper neurocognition, learning, and memory. Over-activation of glutamate receptors causes excitotoxicity, a pathological process whereby neurons are damaged or killed, which may result in neurodegeneration.1,2 Because of its central role in metabolic and cognitive processes, a strong effort has been put toward developing methods to detect glutamate.3–5

Accurate detection of glutamate can be accomplished using many techniques, including spectrometry, spectroscopy, and electrochemistry. The benefits of spectrometry and spectroscopy include sensitivity and selectivity. However, mass spectrometry requires chromatographic separation or vacuum preparation, which decreases the temporal resolution of the system.3 Although spectroscopic techniques, such as the iGluSNFR,2 offer impressive temporal resolution, they require optical transparency thereby limiting the scope of samples that can be analyzed. In contrast, electrochemical sensors require almost no sample manipulation and can be placed directly in the area of interest, allowing, for example, detection of glutamate and dopamine signaling in the brain.6,8 Electrochemical sensors are also versatile: They can be made on the nanoscale,1 have been 3D-printed,9 and can be inexpensive.10,11 The highly translatable nature of electrochemical techniques has already been demonstrated with the advent of Smart Bands12 and iSTAT meters (Abbott Point of Care Inc.).13,14 Detecting glutamate has many advantages: It advances our knowledge of the effects of pesticide exposure, explains some of the erratic behaviors seen after traumatic brain injuries, informs our dietary considerations (MSG), and, perhaps most importantly, functions as a window to cellular bioenergetics in real-time.17 In addition, microfluidics allow for low sample volumes (26 μL sample chamber), enhancing the ability to detect small metabolic changes.5,23 This versatile platform can easily integrate cells or other enzyme sensors to monitor cellular bioenergetics in real-time.17

In addition to increasing the analytical power, microfluidic systems also help recapitulate physiological conditions.23 Microfluidic systems can add flow, and, as a result, mechanical forces. This feature alone has spawned an entire area of research into organotypic cultures. An organotypic culture is a 3D-culture of heterotypic cells and mechanical features25 that aims to mimic an organ or organ system.26 These platforms are more representative of an in vivo system than traditional cell cultures and are used for drug development, predictive toxicology, and basic research. A number of noteworthy examples exist including a liver bioreactor that models detoxification,26,27 a fetal membrane model used to study preterm birth,28 and a neurovascular

*These authors contributed equally to this work.
✉Electrochemical Society Member.
✉E-mail: d.cliffel@vanderbilt.edu

© The Author(s) 2018. Published by ECS. This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 License (CC BY, http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse of the work in any medium, provided the original work is properly cited. [DOI: 10.1149/2.020181jes]

Manuscript submitted June 1, 2018; revised manuscript received July 26, 2018. Published August 9, 2018. This paper is part of the JES Focus Issue on the Brain and Electrochemistry Honoring R. Mark Wightman and Christian Amatore.
unit that recreates transit across the blood-brain barrier.\textsuperscript{29} The ideal platform for studying glutamate would be sensitive and selective, recapitulating physiological conditions, and allow for quantification.

In this work, a sensitive, stable, and selective sensor was developed and incorporated into the \( \mu \)CA (Figure 1) to detect glutamate metabolism of model neuronal cells. This system automated sensor calibration and integrated cell culture through the use of microfluidics, allowing for quantitative, real-time measurements of cellular bioenergetics.

Materials and Methods

Material procurement.—All cells used in these experiments were PC12 cells purchased from American Type Culture Collection (Manassas, VA). For cell culture and electrode modification, penicillin, streptomycin, T25 flasks, collagen type I, trypsin-EDTA, glutamate oxidase, bovine serum albumin, and glutaraldehyde were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco’s modified eagle’s medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) 1640 were purchased for cell culture from ThermoFisher Scientific (Waltham, MA). Fetal bovine serum (FBS) was purchased from ATCC, (RPMI) 1640 was purchased for cell culture from ThermoFisher Scientific (Waltham, MA). Fetal bovine serum (FBS) was purchased from ATCC, (RPMI) 1640 was purchased for cell culture from ThermoFisher Scientific (Waltham, MA).

Sensor stability during use and storage.—To determine the stability of the sensor over experimentally relevant time periods, the current generated by 300 \( \mu \)M glutamic acid in buffer (2 mM PBS, 120 mM KCl, pH 7) was monitored (4 min) every hour for eleven hours and compared to the original signal. In between glutamate measurements, the baseline was set by measuring the current with the electrode in buffer (56 min). Storage capacity was determined by comparing the signal of the electrode before and after storage. For these experiments, three different working electrodes were monitored for four minutes in glutamic acid solution and four minutes in buffer for ten cycles before and after being stored in buffer at 4\( ^\circ \)C for a week. Electrodes were tested once a week for a month.

Effect of nafion on interference.—To determine the utility of Nafion to exclude interference, the effect of ascorbic acid on glutamate detection was investigated. For these experiments, electrodes were prepared as above by drop-casting the glutamate oxidase solution (1.0 \( \mu \)L) onto the working electrodes. However, before applying the Nafion film, the current generated by 300 \( \mu \)M glutamic acid in buffer (2 mM PBS, 120 mM KCl, pH 7) was monitored (4 min) on three different working electrodes with increasing amounts of ascorbic acid (0, 1, 10, 100 1000 \( \mu \)M). A Nafion film was then applied (1.0 \( \mu \)L, 5\% v/v) to the same electrodes and, after drying (45 min), the effect of ascorbic acid on the signal was investigated at the same conditions.

Microclinical analyzer fabrication.—The \( \mu \)CA bioreactor was designed in-house and fabricated by Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE) at Vanderbilt.\textsuperscript{17} The screws, magnets, O-rings, membranes, nuts, and ferrules can be purchased commercially. The screen-printed electrode was designed in-house and fabricated by Pine Research. The \( \mu \)CA bioreactor opens to insert or remove the electrode and cells from the internal chamber (\( A = 113\ mm^2 \), \( V = 26\ mL \)) and interfaces the pump, valve (with calibrants, treatments and media), analytics (electrode), and biological system of interest (PC12 cells immobilized on a membrane) with Tygon tubing (internal \( r = 0.25\ mm \)). The VIIBRE five-port rotary valve automated treatments with up to five different solutions and the VIIBRE RPPM accommodated flow speeds from low \( \mu \)L/min to mL/min.

Cell culture.—PC12 cells cryopreserved in liquid nitrogen were thawed, added to warmed DMEM culture media, and spun down (180 \( \times \) g, 7 min). The supernatant was discarded and the pellet was triturated with 1 mL of DMEM with 5\% FBS, 0.1 mg/mL penicillin, and 100 units/mL streptomycin. The resulting suspension was brought to \( \sim \)1 million cells/mL and transferred to two collagen-coated T25 flasks. All cell culture flasks and transwells were coated in collagen type I overnight at room temperature before adding cells. The cells were grown to confluence (\( \sim \)10 days) at 37 \( ^\circ \)C, 5\% CO\textsubscript{2}, trypsinized, (0.25\% wt/v trypsin-EDTA) and plated onto two T25 flasks and a
six-well PET-track etched 3.0 μm transwell. These cells were grown to confluence (10–14 days) before use. All cellular experiments and cell culture were conducted at 37 °C and 5% CO2 within an incubator.

**Potassium chloride and glutamate treatment.**—Confluent PC12 cells in a six-well transwell were maintained in glucose-free RPMI media for twelve hours before being transferred into the μCA bioreactor. Before addition of the cells, the electrodes were calibrated as above with modifications. Calibrants (RPMI, 20–1000 μM glutamate, 100 mM KCl, pH 7) were passed over the electrodes (20 μL/min, 37 °C) and were electrochemically monitored until a steady state was reached (~10 min). After calibration, a transwell membrane with immobilized PC12 cells was removed from its ridged plastic support with a paring knife and transferred to the μCA bioreactor. To transfer the membrane, the housing was opened and a membrane was placed on top of the electrode with the cells facing up. A second 0.3 μm membrane was placed on top to secure the cells in place. The two membranes were aligned with the electrode and the O-ring by the magnets on either side of the housing and compressed with the screws to seal the cell chamber. During treatment, the bioreactor chamber was amperometrically monitored by three different glutamate-sensitive electrodes along the cell-containing membrane at 0.6 V vs. Ag/AgCl. First the cells were allowed to equilibrate in RPMI for 30 min. RPMI with 100 mM KCl was then passed over the cells for 30 min. Finally, RPMI with 1.0 mM glutamate and 100 mM KCl was passed over the cells for 30 min before the treatment cycle repeated. After these treatments, the cells were allowed to recover for 30 min under flow in RPMI. Flow was stopped for two seconds in between treatments to prevent pressure backup during the valve change, otherwise a flow of 20 μL/min was maintained. After both treatment cycles, the membranes were removed and the electrodes were calibrated again. All treatments were done at 37 °C and 5% CO2 in an incubator.

**Discussion**

Glutamate, the principal excitatory amino acid, plays a key role in neurocognition, yet it is excitotoxic in high quantities. Developing methods to detect glutamate is essential for understanding its biochemistry. In this work, a sensitive and stable glutamate sensor was developed and incorporated into the microclinical analyzer (μCA). This easy-to-use and versatile format (Figure 1) was then used for glutamate microphysiometry by adding model neuronal cells to the analytical chamber and measuring their response to the amino acid.

As evidence of the μCA’s biological relevance, the glutamate sensor spanned the physiological to pathophysiological temporal range. The biologically relevant temporal range of glutamate parallels its scope of function. In traumatic brain injuries, extracellular excitotoxic glutamate builds up over the course of hours and can take days or weeks to return to normal levels. In synaptic signaling, glutamate is released from and taken up by neurons in less than a second. Therefore, a sensor that can respond on the sub-second time scale but is also stable for weeks is required to monitor this wide temporal range. Here, the sensor had a sub-second response time, making it suitable for monitoring rapid synaptic signaling. After 11 hours of use, the sensor retained 91 ± 1% of its original signal, demonstrating optimal stability over experimentally relevant time periods. During this time, the enzymatic sensor had a consistent (R² = 0.96) and therefore predictable signal loss of 0.8 ± 0.1% per hour, which may be due to decreasing enzyme activity. Overall, the sensor showed the fast response time and long-term stability necessary for monitoring both glutamate signaling and trauma-induced excitotoxicity.

As further evidence of the sensor’s biological relevance, the sensitivity of the electrode spanned the physiological to pathophysiological concentration range. Within the brain, glutamate concentration is thought to range from low micromolar under normal conditions up to hundreds of micromolar in conditions of stress, such as stroke. The sensitivity of the disk electrode (19 ± 1 μA·mM⁻¹·cm⁻²) enabled detection down to 8 ± 1 μM and quantitation down to 11 ± 1 μM (Figure 2). The upper limit of quantitation, beyond which the electrode began to saturate and was no longer able to linearly quantitate glutamate, was 1 mM. Between these limits (11–1000 μM), the sensor response was linearly quantifiable, making our sensor suitable for measuring physiological to pathophysiological concentrations.

Even at high concentrations, small changes in glutamate could be observed. At the upper limits of linearity (1 mM), this system could detect a 1.5% (15 μM) difference in glutamate concentration with 95% confidence. Together with the low limit of quantitation (11 μM), the high-resolution of this system makes it a powerful analytical tool for measuring glutamate dynamics.

The sensor was designed to decrease interference by addition of Nafion. To examine the ability of Nafion to exclude interferents, the effect of ascorbic acid on glutamate detection was investigated both with and without a Nafion film. Ascorbic acid, a common interferent linked to glutamate physiology, introduced a large change in current (1.04 ± 0.05 nA/μM, n = 3) without the use of Nafion. With Nafion, this slope decreased (0.29 ± 0.04 nA/μM of ascorbic acid, n = 3),
This sensor also demonstrated optimal stability during storage. After one month in storage, the sensor retained 123 ± 11% of the signal. This may be due to a combination of the storage method (wet storage), the use of Nafion (antifouling), and the enzyme entrapment technique (crosslinking), which all stabilize enzyme sensors.6,17 The dynamic and durable nature of this sensor make it well suited for integration into the μCA to measure cellular microphysiometry.

The μCA bioreactor and microfluidic system were designed for enhanced analytical power (Figure 1). Although previous studies have also used microfluidics for glutamate detection, the cells were spatially removed from the electrode in these cases.22 Here, the cells and sensor were brought in close proximity within the bioreactor. The low limit of quantitation of the glutamate sensor combined with the small volume of the μCA bioreactor (V = 26 μL) allowed for monitoring small changes in glutamate such as those seen in synaptic signaling. This configuration improved analytical power by increasing signal-to-noise ratio and time resolution.32

In addition to increasing analytical efficiency, the microfluidic system also imposed shear stress on the cells, thereby mimicking physiological conditions. At a flow rate of 20 μL/min, the cells experience a calculated shear force of 32 mN/m² (assuming 25°C, η = 0.89 cP, laminar flow), similar to that in the brain. The μCA can accommodate flowrates from low μL/min to mL/min to monitor cellular response under a range of physiological conditions.

Here, the μCA was used with PC12s, a model neuronal cell line, to track glutamate microphysiometry upon exposure.33 PC12s, derived from rat pheochromocytomas, have been used extensively to study glutamate metabolism, toxicity, and cell signaling.34 Within the μCA bioreactor, glucose-starved PC12 cells took up 210 ± 8 μmoles of the provided glutamate (35 ± 16% or 7 ± 3 μmoles/min), which increased to 390 ± 50 μmoles (65 ± 9% or 13 ± 2 μmoles/min) during the second exposure (Figure 3). This uptake was not seen when cells were cultured in glucose-containing media. Extracellular exposure to glutamate triggers uptake via EAAC, GLT-1 and GLAST proteins. Once inside the cell, glutamate may act as a fuel substrate for the Krebs cycle and restore bioenergetic homeostasis following prolonged PC12 depolarization and glucose starvation. That the amount of glutamate taken up by PC12 cells increased during a second treatment suggests that these neurotransmitter uptake systems were functional and may increase to mitigate excitotoxicity. Other cell types and enzyme sensors can be used with this system to monitor cellular microphysiometry in response to a range of drugs and environmental toxins.

Figure 3. Representative amperometric i-t curve during a typical four-hour experiment (Left), and average uptake of glutamate by cells during glutamate exposure (Right). (Left) During a typical four-hour experiment PC12 cells were perfused with two cycles of media (glucose-free RPMI, white pillars), 100 mM KCl (light blue pillars), and 100 mM KCl with 1.0 mM glutamate (dark blue pillars) followed by media for 30 min each. The dashed red line indicates the current generated by the addition of 1.0 mM glutamate with no cells present. (Right) On average, cells took up 210 ± 100 μmoles (35 ± 16%) of the glutamate, which increased to 390 ± 50 μmoles (65 ± 9%) during the second exposure. All experiments were performed at 37°C, 5% CO₂, and 20 μL/min in media (glucose-free RPMI) and are represented as the mean (n = 8) and standard error of the measurements, control vs. 1st exposure: p ≤ 0.0062; control vs. 2nd exposure: p ≤ 0.0002; 1st exposure vs. 2nd exposure: p ≤ 0.068.
resolution and sample throughput. Electrodes can be inexpensive (e.g. screen printed) and, when enzymatically modified, are sensitive and selective. The µCA is an easy-to-use and versatile sensor format that can monitor cellular response under a range of physiological conditions and exhibits desirable signal-to-noise ratio and ample time resolution. Patients and clinicians have already benefited from the highly translational nature of electrochemistry and expanding the repertoire of analytes and analytical power of these systems may facilitate the automation and personalization of medicine.

Conclusions

In this work, a glutamate sensor showed the fast response time (<1s), large linear range (11–1000 μmoles of the provided glutamate, which increased to 390 ± 50 μmoles during the second exposure. Other sensors or cell types could be incorporated into this system to track cellular response to a variety of drugs and environmental toxins.

Acknowledgments

This work was supported in part by IARPA grant number 2017-17081500003, EPA grant number 83573601, NIH training grant number ES007028, and using the resources of the Vanderbilt Microfabrication Core operated by the Vanderbilt Institute for Integrative Biosystems Research and Education. We thank Kazi Tasneem for her modeling of the shear stress, professors John P. Wikswo and BethAnn McLaughlin for their critical review of the manuscript, and Jennifer McKenzie, Ph.D. and David K. Schaffer, M.S. for their contributions to the microclinical analyzer design and fabrication.

ORCID

Ethan S. McClain https://orcid.org/0000-0001-7677-0114
David E. Clifford https://orcid.org/0000-0001-8756-106X

References