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Rapid analysis of glutamate, glutamine and GABA in mice frontal cortex microdialysis samples using HPLC coupled to electrospray tandem mass spectrometry

Celine Defaix[a], Audrey Solgadi[b], Thu Ha Pham[a], Alain M. Gardier[a], Pierre Chaminade[b,c], Laurent Tritschler[a,*]

[a] CESP/UMR-S 1178, Univ. Paris-Sud, Fac. Pharmacie, INSERM, Université Paris-Saclay, Chatenay Malabry, 92290, France
[b] SAMM, Institut Paris-Saclay d'Innovation Thérapeutique, UMS IPSIT UPSud – US 31 Inserm – UMS 3679 CNRS, Fac. Pharmacie, Université Paris-Saclay, Chatenay Malabry, 92290, France
[c] Lip(Sys)^2 – EA7357, Chimie Analytique Pharmaceutique, Université Paris-Sud, Université Paris-Saclay, Chatenay Malabry, 92290, France

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ABSTRACT

In vivo measurement of multiple neurotransmitters is highly interesting but remains challenging in the field of neuroscience. GABA and L-glutamic acid are the major inhibitory and excitatory neurotransmitters, respectively, in the central nervous system, and their changes are related to a variety of diseases such as anxiety and major depressive disorder. This study described a simple method allowing the simultaneous LC–MS/MS quantification of L-glutamic acid, glutamine and GABA. Analytes were acquired from samples of the prefrontal cortex by microdialysis technique in freely moving mice. The chromatographic separation was performed by hydrophilic interaction liquid chromatography (HILIC) with a core-shell ammonium-sulfonic acid modified silica column using a gradient elution with mobile phases consisting of a 25 mM pH 3.5 ammonium formate buffer and acetonitrile. The detection of L-glutamic acid, glutamine and GABA, as well as the internal standards [d6]-GABA and [d5]-glutamate was performed on a triple quadrupole mass spectrometer in positive electrospray ionization and multiple reaction monitoring mode. The limit of quantification was 0.63 ng/ml for GABA, 1.25 ng/ml for L-glutamic acid and 3.15 ng/ml for glutamine, and the intra-day and inter-day accuracy and precision have been assessed for the three analytes. Therefore, the physiological relevance of the method was successfully applied for the determination of basal extracellular levels and potassium-evoked release of these neuroactive substances in the prefrontal cortex in adult awake C57BL/6 mice.

1. Introduction

In mammals, L-glutamic acid (glutamate, Glu) and γ-aminobutyric acid (GABA) are the main amino acid excitatory and inhibitory neurotransmitters in the brain, respectively. Both are involved in many aspects of normal brain functioning including behavior as well as the physiological homeostasis of the whole organism. Both neurotransmitters create an opposite excitatory/inhibitory balance in the brain. Therefore, the physiological equilibrium between Glu and GABA has a great impact on the brain function, in healthy conditions, but also in cerebral pathologies: epilepsy is probably the best example in which the disequilibrium between excitatory and inhibitory neurotransmission leads to induce seizures [1]. At the cellular level, more particularly at the synapse, the Glu and GABA are mainly uptaken by astrocytes. Neuron-astrocyte signaling is a classical example of cell–cell communication *via* the Glu/GABA and glutamine (Gln) cycle. Astrocytes support neuronal metabolism and prevent extracellular accumulation of neurotransmitters and excitotoxicity, especially for Glu. The activity of astrocytes is reflected at least in part, by the Gln synthesis. This amino acid is the metabolic link between astrocytes and neurons in the "tripartite synapse" functional organization encompassing the presynaptic neuron, the postsynaptic neuron and the astrocytes [2]. It is then informative to have the quantification of these three analytes in the same time, in order to have the whole picture of the tripartite synapse. Thus, the ratios of extracellular Gln/Glu levels and extracellular Glu/GABA

* Corresponding author.
E-mail address: laurent.tritschler@u-psud.fr (L. Tritschler).

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levels reflect the astrocyte/neuronal cycling and the balance excitation/inhibition, respectively, in a particular brain region [3].

To obtain samples containing these amino acid neurotransmitters with the minimal effects on the brain function, the microdialysis technique was chosen. This technique is based on the implantation of a probe with semi-permeable membrane into a specific brain area. The implanted probe is then perfused with an artificial cerebrospinal fluid (aCSF) with the same osmolarity as the interstitial space avoiding water flux. The pores of the membrane allow the crossing of small molecules (cut-off 20,000), such as amino acid neurotransmitters. It permits continuous collection of samples with small molecular weight molecules [4]. Interestingly, microdialysis is performed in awake, freely moving rodents reflecting as near as possible their physiological state. Although this technique was developed decades ago, it is still one of the gold standards for the *in vivo* evaluation of the brain neurotransmissions (release, reuptake), with a special interest in mouse, knockout of this species being widely used for genetic manipulations as animal models of psychiatric diseases [5]. Furthermore, we can draw a correlation between responses to behavioral tests and changes in extracellular levels of neurotransmitters when both parameters are measured in the same mouse [6].

The separation and quantification of the analytes in microdialysates need a sensitive and selective analytical method. To date, several methods have been used for the quantification of amino acid neurotransmitters in tissues or biological fluids using a wide range of reagents, additives, derivatization procedures, equipments and detectors. Thus, high performance liquid chromatography (HPLC) can be combine with various detection systems such as ultra-violet detection (LC-UV), fluorescent detection (LC-FD) [7], electrochemical detection (LC-EC) [8] or mass spectrometry (LC–MS) (see [9] for a review). Among these methods, HPLC coupled to tandem mass spectrometry (LC–MS/MS) is commonly used for multi-analyte detection; an analyte can be identified by its retention time, molecular weight and characteristic fragmentation ions. Thus, it has proven to be a reliable method to detect a vast variety of analytes in different biological matrices. Plasma, serum, urine and native CSF are substrates, obtained in clinic, where LC–MS/MS was used to perform multiple amino acids assay, including Glu and Gln. In such studies, the samples needed to be processed (deproteination, precipitation, desiccation, suspension) [10–12]. By contrast, in the present study, the use of a dialysis membrane allows avoiding any pretreatment of dialysate samples to measure extracellular brain neurotransmitters levels in rodents.

Few microdialysis studies previously reported the simultaneous determination of amino acid neurotransmitters levels by LC–MS/MS or LC-FD in various rat brain areas (Table 1). Thus, Buck et al. [13] investigated changes in extracellular Glu and GABA concentrations in the globus pallidus by using LC–MS/MS method. Otherwise, another study described the simultaneous LC-FD quantification of multiple D- and L-amino acids, among them extracellular levels of GABA ($GABA_{ext}$) and Glu (Glu_{ext}) in frontocortical dialysates in freely moving rats ([7]; see Table 1). In the same way, multi-analyte approach for the measurement of neuromediators was develop from rat hippocampus microdialysates [14] or cerebrospinal fluid [15]. However, most of the studies quantified amino acid neurotransmitters in rat [16–18] or in post-mortem homogenates of mouse brain tissues ([19–21]; see Table 2). More recently, the UHPLC–MS/MS method was set up for simultaneous determination of dopamine, serotonin and their metabolites, as well as Glu and GABA in rodent brain tissue and extracellular fluid [22]. To validate this method, the authors measured extracellular levels of these neurotransmitters in rat nucleus accumbens and ventral tegmental area (VTA).

Such studies being rare in freely moving mice, the present study describes a simple method using sensitive method HPLC coupled to a triple quadrupole tandem mass spectrometry dedicated to quantify simultaneously Glu_{ext}, Gln_{ext} and $GABA_{ext}$ in mice dialysate samples. This method was applied in the medial prefrontal cortex (mPFC) to study the excitatory neurotransmitter Glu, the inhibitory one, GABA as well as the intermediate metabolite Gln. The small volume of dialysate analyzed (5 μl) permits a high temporal resolution, which is compatible with pharmacological studies. As a proof of concept, we chose as mood relevant brain area, the mPFC, which is mainly involved in the antidepressant-like activity of selective serotonin reuptake inhibitors (SSRI). Moreover, neuronal depolarization with a high potassium concentration (120 mM) was performed to induce neurotransmitters' release [23]. KCl-evoked neurotransmitter release is one of the tests used to verify the neuronal origin of extracellular neurotransmitter levels measured in dialysate samples. The time course of modifications of Glu_{ext}, Gln_{ext} and $GABA_{ext}$ in dialysates was then successfully measured with the method developed here.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice were purchased from Taconic Farms (Lille Skensved, Denmark). All mice were 7–8 weeks old, weighed 23–25 g at the beginning of the experiment, and were maintained on a 12 l:12 D schedule (lights on at 06:00 h). They were housed in groups of five. Food and water were provided *ad libitum*. All the experiments in animals were performed on compliance with the European Ethical Guidelines (86/609/EEC), as well as with the French National laws (project approval of the Ethical Committee, number APAFIS#5489-2016052717037691 v2).

2.2. In vivo *microdialysis procedure*

Each mouse (n = 17) was anesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and implanted with two microdialysis probes (CMA7 model, Carnegie Medicine, Stockholm, Sweden) located in the right and left mPFC. Stereotaxic coordinates in mm from bregma: A = +2.2, L = ±0.5, V = −3.4 (A, anterior; L, lateral; and V, ventral) [6]. On the next day, the probes were continuously perfused with an artificial cerebrospinal fluid (aCSF, composition in mM: NaCl 147, KCl 3.5, $CaCl_2$ 1.26, $MgCl_2$ 1, NaH_2PO_4 1.0, $NaHCO_3$ 25, pH 7.4 ± 0.02) at a flow rate of 1.0 μl/min through the mPFC using CMA/100 pump (Carnegie Medicine, Stockholm, Sweden), while animals were awake and freely moving in their cage. Dialysate samples were collected every 15 min for 120 min. Basal Glu_{ext}, Gln_{ext} and $GABA_{ext}$ were determined one hour after the onset of aCSF perfusion, corresponding to a stabilization period. These basal levels, determined from fractions 1–3 (15–45 min), were used to establish the reference values B0 defined as 100% for each compound. To activate the neurotransmission, a high concentration of potassium (KCl 120 mM) was added to the aCSF during fraction 4 (45–60 min). Samples were then collected up to the fraction 8, with the same conditions described for the fractions 1–3. The correct location of the probes was controlled macroscopically at the end of the dialysis experiment. Only mice correctly implanted with the probes were included in the data analysis.

2.3. Analytical standards and reagents

γ-aminobutyric acid (GABA), L-glutamic acid (Glu), glutamine (Gln), DL-glutamic acid-2,3,3,4,4-d5 (Glu-d5), 4-aminobutyric acid-2,2,3,3,4,4-d6 (GABA-d6), acetonitrile, ammonium formate, formic acid and all compound used to aCSF were purchased from Sigma-Aldrich (L'isle d'Abeau Chesnes, France).