One-second time resolution brain microdialysis in fully awake rats Protocol for the collection, separation and sorting of nanoliter dialysate volumes

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Abstract

Capillary zone electrophoresis is capable of analyzing nanoliter volumes, reducing the challenge posed by brain microdialysis time resolution improvement to the management of nanoliter dialysate volumes. This fact has not been overlooked and 12- and 6-s time resolution microdialysis have been reported in anesthetized rats. However, behavioral experiments require fully awake and freely moving animals. To achieve high temporal resolution brain microdialysis in awake unrestrained rats, we have developed an online device that mixes the outflowing dialysate with fluorescein isothiocyanate and buffer within a 26-nl reactor. The mixture was continuously accumulated in a 99-μm-bore capillary tube. After the experiment the tube was cut into 4-mm pieces and the content of each piece (30 nl, equivalent to 1 s dialysate) was transferred to a test tube. After allowing 18 h for derivatization, the samples were diluted with water and injected into a capillary electrophoresis laser-induced fluorescence detection instrument. This protocol was tested first in an in vitro assay and proved to be capable of detecting glutamate concentration changes in only 1 s. For the in vivo assays, a probe was inserted into the primary somatosensory cortex of eight rats divided in two groups. One group was stimulated by gently moving its whiskers for 10 s. The other group had no whisker manipulation. Moving the whiskers released glutamate in the experimental group. The first and only change was observed at the 12th s. This method allows 1-s time resolution brain microdialysis in freely moving rats and multiple amino acid analysis every second during sensory perception or motor actions in behavioral experiments.

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1. Introduction

Glutamate is the most abundant and ubiquitous excitatory neurotransmitter in the brain [1]. It is released by specialized neurons, uptaken by glial cells and participates in fast neuronal actions such as movements, sensations, learning and memory [2]. As glutamate neurotransmission is a fast event, the need of a method capable of measuring glutamate and other fast acting neurotransmitters with high specificity and temporal resolution becomes evident.

Several techniques have been developed to tackle this task. The most successful seem to be biosensors and microdialysis. Biosensors permit continuous

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monitoring of substances with high temporal resolution, but are limited by their low sensitivity, low specificity and by the fact that they can monitor only one substance at a time [2]. Brain microdialysis (BM) on the other hand, is capable of collecting virtually any substance in the brain with a limited amount of tissue trauma [3,4], and when coupled to offline or online analytical techniques these substances can be measured with high specificity and sensitivity. The major limitation of microdialysis lies in its low temporal resolution, typically ranging between 10 and 35 min.

BM temporal resolution depends on the analytical method. Traditionally high-performance liquid chromatography (HPLC) coupled to electrochemical or fluorometric detection, has been used. This technique requires microliter volumes and picomole to femtomole amounts for detecting neurotransmitters in brain dialysates. Alternatively, capillary zone electrophoresis (CZE) with laser-induced fluorescence detection is a technique capable of analyzing nanoliter volumes and zeptomole amounts.

With CZE available, the microdialysis temporal resolution problem is reduced to the management of very small dialysate volumes. This fact has not been overlooked. In 1997, Lada et al. [5] measured glutamate and aspartate using microdialysis online with CZE with a 12-s time resolution in anesthetized rats. In the same year, Tucci et al. [6] achieved a 6-s time resolution also in anesthetized rats. However, behavioral experiments require awake and freely moving animals. Currently, temporal resolution ranges from 30 to 60 s (when CZE is used).

Improving microdialysis temporal resolution in freely moving rats poses two major challenges: (1) reduction of collection times is accompanied by lower sample volume, (2) reduction of time intervals is difficult for sample collection and sorting.

The first challenge seems to be solvable by increasing the perfusion flow-rate. However, this alternative is of little help because perfusion rates above 2 μl/min deplete the neurotransmitter in the tissue creating non-physiological conditions around the probe [7,8] and high flow-rates may cause tissue damage [3].

Both of these difficulties might be overcome by designing an appropriate protocol for the collection, separation and sorting of very small dialysate volumes. We designed an online device (Fig. 1) that mixes the dialysate with an FITC derivatizing solution within a 30-nl mixing reactor. The outflowing mixture is continuously accumulated in a 99 μm I.D. fused-silica capillary tube for its posterior separation and sorting. To estimate if molecular diffusion was slow enough to allow a posteriori sample separation we used the Einstein–Smoluchowski diffusion equation [9]:

$$(\Delta X)^2 = 2Dt$$

in which ΔX represents the net displacement in the X direction, D is the diffusion coefficient and t is time. When a glutamate diffusion coefficient of 7.6×10^{-6} cm^2/s is used [10], the equation predicts that in 10 min the net displacement in the X direction will be 0.95 mm for 68% of the molecules (one standard deviation) and 2.85 mm for 99.7% of the molecules (three standard deviations). If a 1 μl/min rate is used to perfuse the probe and the FITC-containing solution is pumped at the same rate, 33 nl will enter the collecting tube every second, this volume will fill 4.3 mm of the 99 μm I.D. capillary tube. If the tube is sectioned in 4-mm pieces, each sample will contain 30 nl corresponding to 1 s dialysate. Obviously, tube cutting has to be done in the shortest possible time (it usually takes less than 10 min) to minimize that factor in the diffusion equation.

To test this protocol, in vitro and in vivo assays were performed. The in vitro assay consisted in rapidly changing a microdialysis probe from a Ringer solution to a 10 mM glutamate solution, both solutions were thermostated at 37 °C. This assay demonstrated the feasibility of detecting glutamate concentration changes within 1 s.

For the in vivo assays the primary somatosensory cortex of the rat, specifically the posteromedial barrel subfield (PMBSF), was used as anatomical target. The event observed was the glutamate release in this area in response to a mechanical stimulation of the contralateral rostral vibrissae.

Unlike most cortical regions in other mammals, the layer IV neurons of the somatosensory cortex and their thalamic input in mice and rats are distributed as uniform aggregates that are easily visible in routine histological preparations [11]. In Nissl preparations [12] of sections made perpendicular to the
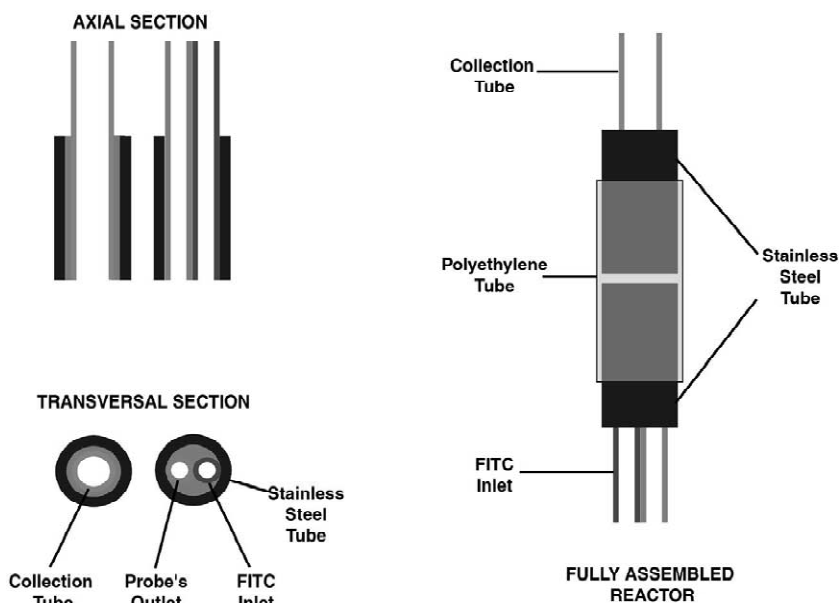
Fig. 1. The mixing reactor couples two inlets (FITC and probe lines) to a 26-nl mixing chamber with a collection tube (16 cm long, 365 μm O.D.×99 μm I.D. fused-silica capillary tube) at the other end. Both inlets and the outlet were included within a 1-cm-long 22 gauge stainless steel tube and epoxied. These included ends were coupled together by a No. 50 polyethylene tube leaving 0.1 mm between them.

surface of the pia mater (pial surface) the arrangement appears to be one of ''cellular columns'' oriented perpendicularly to the pial surface and being as tall as the layer IV itself. These aggregates were termed barrels by Woolsey and Van der Loos who demonstrated that their shape, size, organization and number are particularly clear and constant. They suggested, based on morphological and physiological evidence, that barrels in the PMBSF are the cortical correlates of mystacial vibrissae, and that one barrel represents one vibrissa [13]. A classical experiment performed by Van der Loos and Woolsey demonstrated that damaging a row of whisker follicles on the face of a new born mouse alters the distribution of layer IV neurons in such a fashion that the cortical representation of that row appears as a single fused band rather than as a series of discrete barrels [14]. Further, Killackey demonstrated that such abnormal organization is not confined to the somatosensory cortex but is also seen in the brainstem and the thalamic trigeminal nuclei [15]. These experiments give additional support to the barrels in the posteromedial barrel subfield as the cortical representation of the mystacial vibrissae. Because of its histological, physiological and anatomical characteristics as well as the easiness for manipulation, we chose the vibrissae tactile organ as the biological subject for testing our protocol.

Our experiment consisted in mechanically stimulating rat vibrissae and measuring glutamate release in the somatosensory cortex. We were able to measure a 1-s glutamate concentration change.

2. Experimental

2.1. Microdialysis probes

The microdialysis probe was made of concentric fused-silica polyimide-covered capillary tubing (150 μm O.D.×75 μm I.D.) into a 26 gauge stainless steel tube. A cellulose hollow fiber was plugged with epoxy at one end and attached inside the 26 gauge tube. This 2-mm cellulose tube has a 13,000 molecular mass cut-off and its permeability data have been reported elsewhere [16].

2.2. Mixing reactor

The reactor described here (see Fig. 1) was designed for mixing two different fluids in a 26-nl chamber before entering a capillary tube. We used this online reactor to mix the dialysate from a microdialysis probe with a 1:1 FITC–buffer solution (2.57 mM FITC in acetone–20 mM carbonate buffer at pH 9.4). Therefore the two inlets were: the microdialysis probe's outlet (3 cm long) and the FITC line (16 cm long). Both were made of 150 μm O.D.×75 μm I.D. fused-silica capillary tubes. The ends of the two inlets were included within a 1-cm-long 22 gauge stainless steel tube and glued with epoxy. After hardening of the epoxy, one end of this conglomerate was ground with a fine file until a smooth flat surface was attained. To avoid obstruction of the capillaries, water was pumped through them while they were filed. In the same way the end of a 160-mm-long, 365 μm O.D.×99 μm I.D. fused-silica capillary tube was included in an identical stainless steel tube. The smoothed ends of both assemblies were coupled together with 6 mm of No. 50 (0.29 mm I.D.) polyethylene tube leaving 0.1 mm between them. The whole reactor was sealed with epoxy. The only outlet was the 160-mm-long, 99 μm I.D. capillary that collected the mixture.

2.3. Microdialysis and derivatization

Ringer solution (136 mM NaCl, 3.7 mM KCl, 1.2 mM CaCl$_2$, 1 mM MgCl$_2$ and 10 mM NaHCO$_3$ at pH 7.4) and FITC–buffer solution were set in two separate 500-μl gas tight Hamilton syringes and pumped at 1 μl/min flow-rate into a double channel swivel [17] the outputs of which were connected to the probe inlet and the FITC line. The syringes were connected to the swivel by 99 μm I.D. fused-silica capillaries, and the same tubing was used to connect the swivel with the probe and FITC line. Unions were made with PE 20 polyethylene tubes except in the case of the probe's inlet which required a PE polyethylene tube (see Fig. 2).

Immediately after the experiment the FITC inlet and the probe's outlet were cut off with a diamond pencil and sealed with epoxy. At once, the collection tube was sectioned with a diamond pencil in 4 mm
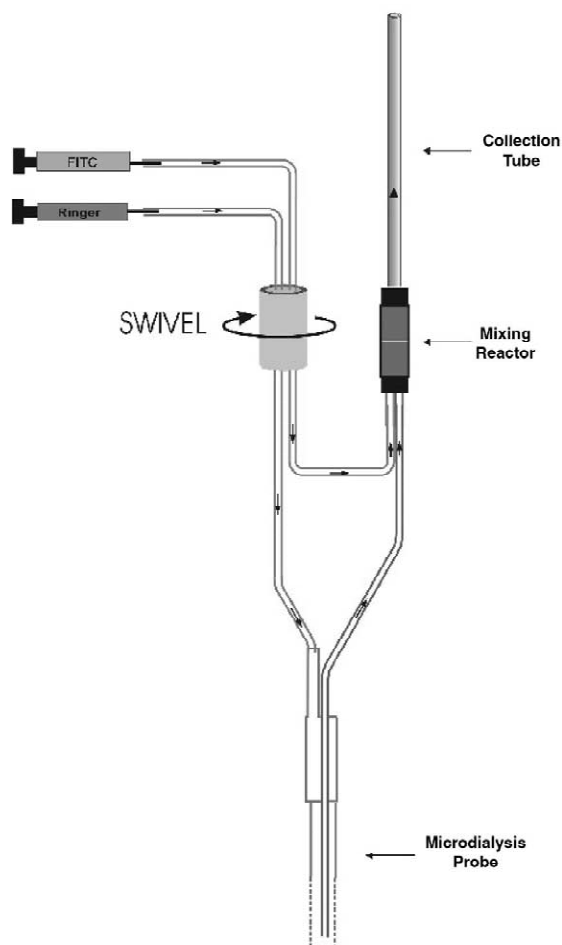

Fig. 2. The mixing reactor coupled to the microdialysis probe (28 mm long), to the swivel and both syringes is depicted here. Two lines are distinguished: the FITC line conducts FITC–buffer solution from the FITC syringe through the swivel and into the mixing reactor. The dialysis line conducts Ringer solution from the Ringer syringe through the swivel and into the probe. Within the probe, neurotransmitters are collected by diffusion and the dialysate flows from the probe to the mixing reactor through the probe's outlet. All tubing connecting the elements were 99 μm I.D. fused-silica capillary tubes.

pieces and their content was transferred to test tubes by centrifugation. Each sample contained 30 nl of the mixture equivalent to 1-s dialysate. After 18 h of derivatization time the samples were diluted by adding 1 μl of water. Since in this procedure the
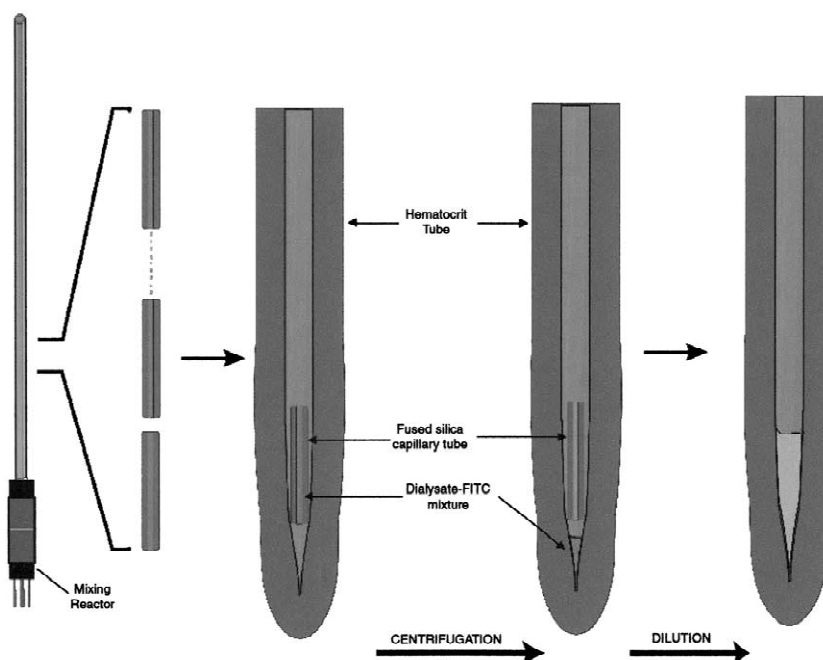
Fig. 3. Separation and sorting of nanoliter volumes is achieved when the collection tube is sectioned in 4-mm pieces. The content of each piece is transferred to a test tube by centrifugation. Each sample contains 30 nl corresponding to 1-s dialysate. After 18 h of derivatization time, samples were diluted by adding water.

assembly is destroyed, a new one must be made for each experiment (see Fig. 3).

2.4. Sample analysis

The diluted samples were injected into a model R2D2-1 CZE-LIFD instrument (Meridialysis, Mérida, Venezuela). Separation of analytes was carried out in a 27 μm I.D and 360 μm O.D. fused-silica capillary column filled with 20 mM carbonate buffer at pH 9.4. The two ends of the column were immersed in buffer reservoirs with Pt–Ir electrodes. A high voltage (20 kV) was applied for 10 min. The analyte was excited by the 20 mW, 488 nm line of an argon ion laser (National Laser Instrument, USA), and the fluorescence was collected through an objective and focused on a photomultiplier tube (PMT). The output current of the PMT was acquired and processed by means of ONICE® software (Dial-

demo, Mérida, Venezuela) in a PC. Glutamate was identified by migration time and spiking.

2.5. In vitro assay

Two beakers, one containing a 10 mM glutamate solution (dissolved in Ringer) and the other with pure Ringer, were placed in a 37 °C thermostatic bath. Once thermal equilibrium was attained, a probe was placed in the Ringer solution for 5 min, then it was placed 10 s in the glutamate solution, immediately after it was returned to the Ringer and remained there for a further 26 s after which the collection tube was cut off and processed. The derivatizing procedure in these assays was slightly modified, introducing the use of fluorescein as an internal standard. The new FITC–buffer solution contained 2.57 mM FITC and 75.3 μM fluorescein in acetone mixed 1:1 with 20 mM carbonate buffer at pH 9.4. Fluorescein was used because it is unable to deriva-

tize glutamate, its peak height is not modified by addition of high concentrations of glutamate and does not coincide with any peak in the normal dialysate electropherogram (see Fig. 7).

2.6. In vivo assays

2.6.1. Subjects

Male albino rats of the Wistar strain and weighing between 250 and 300 g were individually housed with water and food ad libitum.

2.6.2. Experimental groups

Ten animals were subject to surgery to implant a *guide shaft*, which allowed the microdialysis probe to be inserted into the target area (primary somato-sensory cortex) 7 days after surgery. However, one rat had the *guide shaft* blocked and another one was excluded from the results (and not considered in the numbers reported in brackets) because of its wrong placement (see below). Animals ($n = 8$) were divided in two groups, one group (experimental, $n = 4$) received whisker stimulation, whereas the other group (control, $n = 4$) had no whisker manipulation but was otherwise treated as the experimental group.

2.6.3. Surgery

Subjects were anesthetized with ketamine (100 mg/kg) and sodium penthotal (10 mg/kg) and a guide shaft made of 10-mm-long, 21-gauge stainless steel tubing was aimed to the parietal cortex at 30° angle in the coronal plane according to the following coordinates: 2.3 mm posterior to bregma, 3.0 mm lateral to the midsagittal suture and 2.0 mm ventral to the surface of the skull. Subjects recovered for 6 days before performing the experiments.

2.6.4. Experiment procedure

After recovery from surgery, a probe attached to the mixing reactor and to the swivel joint was inserted in the guide shaft. Then the animal was released in a plastic chamber were it could move freely and was left undisturbed for 7 h after which the experiment was performed. The rat was gently held by hand to prevent accidental whisker stimulation. After 10 s baseline the experimental rats were stimulated by moving its contralateral whiskers for 10 s. The control group received no whisker stimula-

tion. After a further 26 s the collection tube was cut off and processed as described before.

2.7. Statistics

Each rat data was normalized as a percent of the average of its first 10 basal glutamate concentrations. Normalized data were analyzed using two-way ANOVA with time treated as a within subject variable and treatment (stimulation or control) treated as a between subject variable. Levels at specific time points were compared by the test of within-subject contrast (SPSS 8.0 for Windows statistical package). Significance level was set at $P < 0.05$.

2.8. Histology

The perfusion site was histologically verified according to the atlas of Paxinos and Watson [18]. After the microdialysis session each animal was overdosed with chloroform and the brains perfused intracardially with 0.9% saline followed by 4% formaldehyde solution. For visualization, frozen brains were sectioned on a cryomat at 40 μm. Data from subjects with incorrect probe placement were excluded from analysis.

3. Results

3.1. In vitro assays

Fig. 4 shows the result of the in vitro assay. A threefold increase in glutamate level is observed at the 10th s and during the next 2 s glutamate levels saturate the detector.

3.2. In vivo experiments

Fig. 5 shows the average time course of glutamate levels in control and experimental groups. In both groups basal glutamate concentrations are similar and show little variation ($3.70 \pm 0.5 \times 10^{-6}$ M). Upon the 12th s a glutamate level increase is observed in the experimental group, after which glutamate level
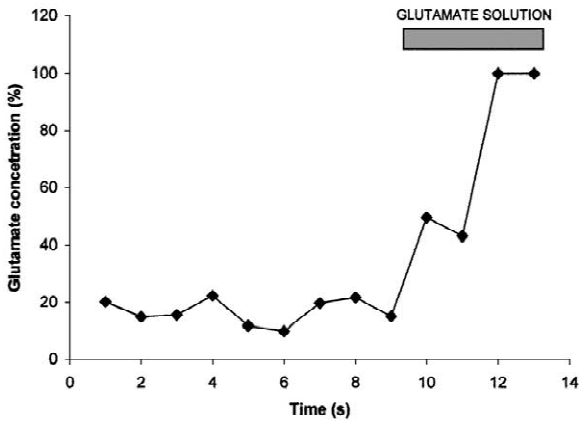
Fig. 4. Typical glutamate recovery temporal course in the in vitro experiment. The results show a 300% increase in glutamate concentration in 1 s, glutamate increases further in the next 2 s. The maximum level corresponds to the saturation of the analyser.

Fig. 6. The black rectangle represents the target area, while the dashed line rectangle represents the probe position of the one rat (from the experimental group) which was excluded from the statistical analysis.

cantly higher than that from controls ($F(1, 7) = 11.5$ $P < 0.01$).

decreases back to basal levels. The glutamate concentration in the control group showed no change during the time course of the experiment. Two-way ANOVA repeated measure test showed that the sensorial stimulation (treatment factor) significantly affected glutamate concentration over time (time $\times$ treatment factor, $F(27, 189) = 4.4$, $P < 0.0001$). The test of within-subject contrast further demonstrates that, at the 12th s, the glutamate concentration in the group subject to sensorial stimulation were signifi-

3.3. Histology

Fig. 6 shows the average probe position of the animals included in the statistical analysis. One animal from the experimental group had a probe position outside the target area and was excluded from the analysis. This animal showed no glutamate concentration change in the experimental time
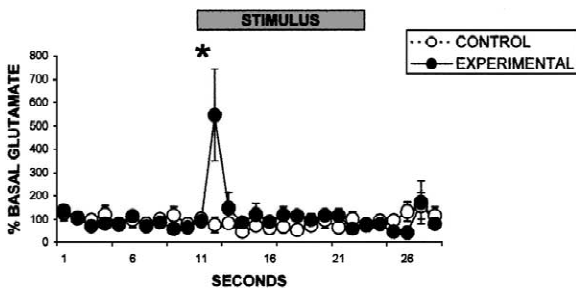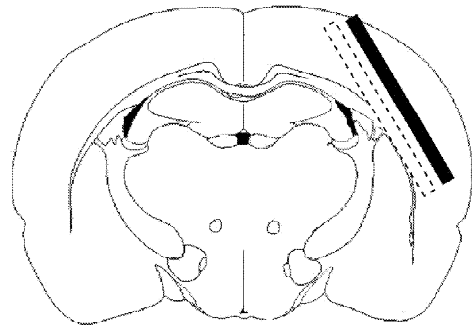

Fig. 5. Average glutamate level time course in control (opened circles) and experimental (filled circles) groups. Upon the 12th s a significant glutamate level increase is observed in the experimental group, after which glutamate concentration decreases back to basal levels. *Two-way ANOVA followed by within-subject contrast test: $P < 0.01$.
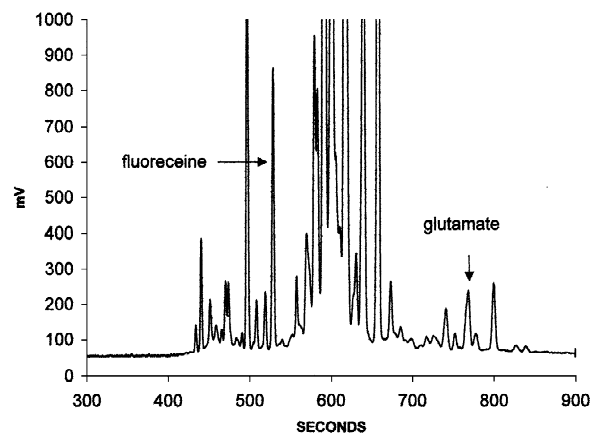

Fig. 7. Typical electropherogram showing glutamate and fluorescein signals. In these experiments fluorescein was used as internal standard.

course, supporting an anatomical specificity for the event reported.

4. Discussion

The use of CZE introduces the possibility of analyzing attomole amounts in nanoliter injection volumes. In theory, this low volume would allow microdialysis with sub-second sampling times [5]. Two previous studies have reported brain micro-dialysis with temporal resolution under 30 s. Lada et al. reported a 12-s resolution utilizing an online derivatizing procedure, the outflow of which was automatically transferred to a separation capillary by a flow-gated interface. In this procedure temporal resolution was limited by band broadening during sampling, transfer to the assay system, derivatization time, and separation time [5]. By contrast, in the present protocol band broadening was reduced by cutting the sample collection tube shortly after ending the sampling.

As another antecedent, Tucci et al. reported a 6-s resolution utilizing online mixture of the dialysate with an FITC-containing derivatizing solution. The outflow of the mixing reactor was collected every 6 s with a borosilicate tube by capillarity. Samples were stored in a water saturated chamber and allowed to react in the dark for 18 h, diluted and then analyzed by CZE [6]. In this protocol, temporal resolution was no longer limited by derivatization and separation times but only by band broadening during sampling and transfer to the assay system. When online derivatization is combined with offline CZE analysis, the major difficulty in improving BM temporal resolution is the collection, separation and sorting of nanoliter samples. Our design facilitates these operations. The 30-nl mixing reactor permits samples to be derivatized online without compromising temporal resolution and avoiding painstaking manipulation of such small volumes. Accumulation in the 99 μm I.D. collection tube permits sample storing in an ordered manner and enables subsequent sample separation. The collection tube also facilitates the use of this protocol in behavioral experiments with fully awake animals. In the present experiment the rat was gently held by hand during sensorial stimulation to prevent whisker stimulation from a different non-

attempted source. However, many ''in vivo'' experiments may benefit from the present protocol without holding the animals. The major drawback of this protocol is the laborious and time-consuming construction of the assembly which is destroyed in each experiment; this limitation may be overcome by a pluggable collection tube.

The in vitro assay shows a 300% increase in glutamate level with respect to basal in only 1 s and further increase in the next second. This assay demonstrates that it is possible to detect glutamate concentration changes within 1 s, although the total concentration change might take at least 3 s to be accurately measured.

In vivo assays showed a significant increase in glutamate levels within a single second in experimental rats. There is no difference between experimental and control groups in the time previous to stimulation or after it. Interestingly the glutamate level increase was observed only in 1 s at the beginning of the stimulation time, and rapidly decreased even when stimulation continued. During this time glutamate levels were slightly, although not significantly, over controls. This result shows for the first time the detection of a glutamate level increase within only 1 s.

It is worth remarking on the differences between the in vitro and the in vivo assays. In the former we observed that at least 3 s are needed for the probe perfusion liquid to equilibrate with the external solution, while in the in vivo assay we detected a glutamate increase in only 1 s. Besides the difficulties in comparing results from such different matrixes (biological and in vitro), these results suggest that both glutamate release and reuptake took less than 1 s, and that the probe might not have been able to account for the total concentration change that occurred in such a short time.

This observation seems to be quite relevant, since in our laboratory several brain areas have been probed to monitor glutamate concentrations over the time associated with the activation of neuronal circuits and the increase in glutamate concentrations have been seen during the first intervals measured. For instance, electrical stimulation of the prefrontal cortex increased glutamate release in the nucleus accumbens, a glutamate projection area, in the first 30-s interval [19]; glutamate levels were significantly

raised 30 s after nociceptive stimulation [20]. Moreover, the levels of glutamate significantly decreased 6 s after artery clamping [6]. The present results suggest that these events could occur in a much shorter time scale as, in those cases, 30 or 6 s was the allowed temporal resolution. Classical studies on spinal cord reflexes have shown that glutamate neurons' synaptic delay is as short as 0.5 ms [21]. Therefore, the approach of monitoring extracellular amino acid concentration's temporal course requires improvement in temporal resolution. Basically, the online mixing reactor and the a posteriori sampling separation and sorting described here offer new technical possibilities to enhance the understanding of neurotransmitter release mechanisms.

Acknowledgements

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