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High temporal resolution for in vivo monitoring of neurotransmitters in awake epileptic rats using brain microdialysis and capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A method for high temporal resolution monitoring of five neurotransmitters, dopamine (DA), noradrenaline (NA), gamma-aminobutyric acid (GABA), glutamate (Glu), L-aspartate (L-Asp), in freely-moving rats using microdialysis and capillary electrophoresis with laser-induced fluorescence detection (CE-LIFD) was developed. An on-line device, including microdialysis and derivatization with naphthalene-2,3-dicarboxaldehyde, mixes the dialysate with derivatization reagents directly in the collection tube, i.e. with no reactor. Thereafter, collected derivatized samples are analyzed off-line with an automated CE system coupled to a LIFD using a 442 nm excitation. The sampling time was limited by the minimal volume required for the analysis by the automated CE system used: neurotransmitters could be determined in 667 nl dialysates (940 nl after derivatization), i.e. in samples collected every 20 s with a flow rate of 2 μl/min. The detection limits at the dialysis probe were 3×10^{-9}, 1×10^{-9}, 1.9×10^{-8}, 4.2×10^{-7}, 2.1×10^{-7} mol/l for DA, NA, GABA, Glu and L-Asp, respectively. The protocol was validated using in vitro/in vivo tests and the performances—repeatability, linearity, characteristics of the probes—were determined.

Finally, the high temporal resolution allowed the simultaneous monitoring of these neurotransmitters in rats with genetic absence epilepsy and revealed, for the first time, increases in GABA concentrations concomitantly with the seizures, detected when our new microdialysis method was combined to electroencephalographic recordings.
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1. Introduction

Studying rapid neurochemical events requires techniques enabling measurements with a sub-minute time scale. So far, biosensors or in vivo electrochemistry have been used to monitor such rapid changes in extracellular levels of neurotransmitters (for review, Kehr, 1999). However, with such a method, only one compound can be monitored at once, thus, limiting the use of biosensors when one wants to study interactions between several neurotransmitters. In contrast, microdialysis allows the simultaneous monitoring of several molecules since it is coupled to a separation-based analytical technique. However, microdialysis has been previously limited in many cases by its poor temporal resolution when combined with the conventional high performance liquid chromatography which requires relatively large volumes of samples and therefore a 10–30 min sampling duration. In order to analyze sub-microliter dialysates obtained with a sub-minute sampling, our group and others have successfully

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used capillary electrophoresis coupled to laser-induced fluorescence detection (CE-LIFD) which allows to detect attomoles of neurotransmitters in low volume samples (see reviews, Parrot et al., 2003a; Kennedy et al., 2002). For example, such an approach led to determine glutamate (Glu) and aspartate (Asp) in microdialysates collected every 1 s–1 min or to monitor catecholamines every 10 s–1 min. However, some limits in these previous works can be pointed out. First, most works dealing with high time resolution microdialysis coupled to CE were carried out on anesthetized rats (e.g. Lada et al., 1998; Bert et al., 2002). Since many anesthetics affect extracellular levels of neurotransmitters by impairing the neuronal excitability (Rozza et al., 2000; Pan and Lai, 1995), the use of awake animals seems to be better from a neurobiological point of view, especially when studying physiologically-related (e.g. states of vigilance, epileptic seizures) or behavior-induced release of neurotransmitters (e.g. Silva et al., 2001). Another criticism is that previous studies were mainly focused on Glu (e.g. Pérez et al., 2000; Rossell et al., 2003) or excitatory amino acids (e.g. Lada et al., 1998; Parrot et al., 2003b) and only one work reported data dealing with interactions between two classes of neurotransmitters (Bert et al., 2002). Third, the groups which have introduced high temporal resolution for microdialysis monitoring using CE-LIFD analysis (see review Parrot et al., 2003a) used no commercially available CE systems, but rather prototypes, “home-made” devices (e.g. Lada et al., 1997; Robert et al., 1998). In that case, the protocols cannot be easily set-up in neuroscience laboratories without the help of a specialized workshop for making the on-line CE system.

In order to overcome the three disadvantages mentioned above, the present work was aimed to perform high temporal resolution microdialysis on awake rats for the simultaneous monitoring of five neurotransmitters, dopamine (DA), noradrenaline (NA), γ-aminobutyric acid (GABA), Glu and aspartate (Asp) using two successive analyses performed on a commercially available CE instrument. As neurotransmitters are not fluorescent at available laser wavelengths, the system has to include an on-line derivatization of samples with naphthalene-2,3-dicarboxaldehyde, which reacts quickly with the primary amine moiety of neurotransmitters (Zhou et al., 1995). Besides, the new system must have a minimal dead volume in order to limit diffusion of the solutes in microdialysis tubings. The first step of the work was to design a new device for simultaneous microdialysis/derivatization compatible with frequent collection in freely-moving rats. The second step was to test the ability to analyze off-line derivatized microdialysates collected with high frequency and exhibiting low volumes, using a commercially available CE system. The performances, i.e. linearity, repeatability, detection limits, of the entire system, e.g. from the dialysis to the analysis steps, were determined through the measurements of two classes of neurotransmitters, catecholamines and amino acids, within the same sample.

Finally, this new technique was used in vivo to monitor extracellular concentrations of neurotransmitters in awake rats from a strain with genetic absence epilepsy (Genetic Absence Epilepsy Rats from Strasbourg or GAERS). These rats display spontaneous, recurrent generalized non-convulsive seizures which are characterized by spike-and-wave discharges (SWD) measured on the electroencephalographic recording (EEG) and lasting from 20 s to 1 min. These seizures occur about every minute when the animals are in a state of quiet wakefulness (Danober et al., 1998). In this model, several hypotheses concerning the possible variations of neurotransmitters in the thalamic relay nuclei, a critical structure in the generation of absence seizures, are still to be determined because of a lack of sensitive methods to detect changes of extracellular levels of neurotransmitters within less than 60 s (Danober et al., 1998). In addition, this model offers the possibility to observe spontaneous seizures for which onsets and ends can be easily determined on the EEG. In addition, no overt behavioral changes occur during the seizures, thus allowing reliable collection of dialysates. Using this model, a coupling of 20 s sampling microdialysis with EEG recordings was performed to determine the possible associations between variations in neurotransmitter levels and the occurrence of seizures.

This new method will allow to monitor extracellular concentrations of several neurotransmitters in awake rats using high sampling rate microdialysis. It represents a new tool for studying, under physiological conditions, short-lasting changes in neurotransmitter concentrations and/or interactions between neurotransmitters.

2. Materials and methods

2.1. Microdialysis/derivatization probes

In all experiments, concentric microdialysis probes were constructed in our laboratory from regenerated cellulose dialysis tubing (Spectra/Por hollow fiber; molecular weight cut-off 6000 Da, 225 μm o.d., length: 2 or 3 mm, Spectrum Medical Industries, Los Angeles, CA, USA) and fused-silica capillary tubings (38 cm long, 40 μm i.d., 105 μm o.d., Polymicro Technology, Phoenix, AZ, USA). In most experiments, the body of the probe consisted in a 26-G stainless steel tubing; in experiments using awake rats, it was glued on a flat probe holder (Harvard, USA) adaptable to a CMA 12 canula-guide (CMA, Stockholm, Sweden). An on-line derivatization system at the outlet of the probe, which was different from those previously described (Robert et al., 1996; Bert et al., 2002), has been optimized in order to mimimize the outlet dead volume. In the new system, the derivatization reaction takes place in the collecting vial: the capillary tubing at the outlet of the probe was glued lengthwise to another capillary (75 μm i.d., 150 μm o.d.) bringing a mixture of derivatization reagents directly into the collection tube (see Fig. 1). The latter capillary was introduced and fixed with glue in a polyethylene tubing (300 μm i.d., length 2.5 cm). On the other side of the polyethylene tubing, three

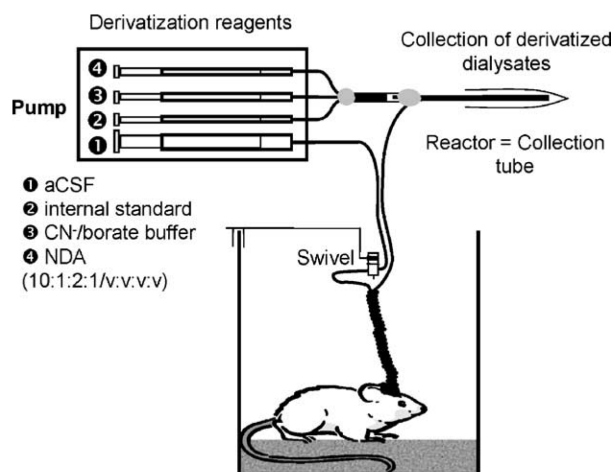

Fig. 1. Schematic representation of the set-up for microdialysis/on-line derivatization for an awake rat. The outlet capillary of the microdialysis probe was glued lengthwise to a capillary bringing derivatization reagents directly in the collection tube, i.e. with no dead volume reactor. In that case, the dead volume was only due to the tubing between the probe and collection tube. In experiments using anesthetized rats, no swivel was used.

capillaries (75 μm i.d., 150 μm o.d.) corresponding to the three reagents were introduced and fixed with glue. The derivatization reagents were delivered with the same syringe pump as the artificial cerebrospinal fluid (aCSF) (Harvard Model PHD 2000 Infuse), since this pump can give access to different flow rates depending of the diameter of the syringe when equipped with a multiple-syringe holder. The aCSF was perfused at 2 μl/min using a 500 μl syringe. Internal standard (IS) solution [0.1 mM cysteic acid (Cys) in 0.117 M perchloric acid], and naphthalene-2,3-dicarboxaldehyde (NDA) solution (2.925 mM in acetonitrile/water, 50:50, v/v) were perfused at 0.2 μl/min using 50 μl syringes. Borate/sodium cyanide (NaCN) solution [mixing solution (100:20, v/v) of 500 mmol/l borate buffer pH 8.7, and 87 mmol/l NaCN in water] was perfused at 0.4 μl/min using a 100 μl syringe.

After being flushed with water, the probes were continuously perfused at 2 μl/min (500 μl syringe) with aCSF (145.0 mM NaCl, 2.70 mM KCl, 1.0 mM $MgCl_2$, 1.20 mM $CaCl_2$, 0.45 mM NaH_2PO_4, 2.33 mM Na_2HPO_4, pH 7.4). In vitro recovery of 3 mm membrane probes at room temperature was estimated to be 4.3 ± 0.4%, 4.0 ± 0.7%, 6.7 ± 0.3%, 6.9 ± 0.3% and 8.4 ± 0.4% (mean ± S.E.M., $n = 10$) for DA, NA, GABA, Glu and Asp, respectively.

2.2. *Surgical procedures and microdialysis experiments*

Rats were housed up to four per cage in a constant temperature room (21 ± 1 °C) and maintained on a 12:12 h light/dark cycle (lights on at 06.00 a.m.), with food and water ad libitum. The care and use of laboratory animals were in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC). Rats were anesthetized and placed in a stereotaxic frame (David Kopf, USA). Body temperature was maintained close to 37.5 °C using a heated underblanket (Harvard Instruments, USA).

2.2.1. *Experiments using anesthetized rats*

Male Sprague-Dawley rats (350–400 g, Harlan, France) were anesthetized with urethane (1.4 mg/kg i.p.). The skull was exposed and, after drilling one appropriate hole, a 3 mm dialysis probe was implanted in the right striatum at the following coordinates relative to bregma: anterior 0 mm, lateral 3.5 mm, ventral 6.5 mm below the brain surface according to the atlas of Paxinos and Watson (1998). The collection of six 20 s basal samples was initiated 3 h after the probe implantation. After the sixth sample was collected, drug application was begun. Fixed volumes of drugs diluted in aCSF, 167 and 83 nl, were administered by applying air-pressure with a 10 ml syringe over 20 and 10 s, respectively, to an injection micropipette (glass tubing with an internal diameter of 0.3 mm pulled and broken back to an external diameter of 50 μm). The injection micropipette was attached to the external surface of the microdialysis probe and placed laterally at 150 μm and vertically at 2 mm up from the tip of the microdialysis membrane. Such a procedure prevented hydrodynamic disturbances which are created when drugs are applied by reverse dialysis (Bert et al., 2002). Stock solutions of 40 mmol/l PDC (from Tocris, UK) and 10 mmol/l nipecotic acid (from Sigma) were prepared in aCSF and stocked at −40 °C. Final solutions of 10 mM PDC and 1 mM nipecotic acid were diluted in aCSF.

2.2.2. *Experiments on awake rats*

Male Wistar rats (350–400 g) from the Genetic Absence Epilepsy Rats from Strasbourg strain were used. These rats display recurrent generalized non-convulsive absence seizures characterized by bilateral and synchronous SWD on the EEG, concomitant with a behavioral arrest (Danober et al., 1998). They were anesthetized with chloral hydrate (400 mg/kg i.p.). The skull was exposed and, after drilling appropriate holes, a guide-canula was implanted in the left lateral thalamus at the following coordinates: anterior −3.5 mm, lateral 2.0 mm, ventral 6 mm below the skull surface, with bregma as the reference (Paxinos and Watson, 1998). The rats were equipped with three or four monopolar stainless-steel cortical electrodes connected to a female micro-connector. The guide-canula and electrodes were secured with acrylic cement. Post-operative care consisted in applying Soframycine® (Roussel Diamant, France) under the skin and sulfanilamide (Sigma, France) on the wound margin. After surgery, rats were housed in individual cages with food and water ad libitum. The microdialysis experiment was performed 10 days after guide-canula/electrode implantation. After verification of the EEG signal, the 2 mm dialysis probes were implanted into the guide-canulae and the animals were placed in the experiment chamber, the inlet of the probe being connected to a liquid swivel (Harvard, mouse model, USA) in order to allow free movements to the animals (see Fig. 1). The collection of 20 s samples was initiated 3 h after

microdialysis probe insertion. The EEG connector was plugged in before the beginning of microdialysis collection. Microdialysates were stored at −20 °C until analysis. Following collection, the dye methylene blue 1% was perfused through the dialysis probe and rats were immediately sacrificed in order to verify the placement of the probe. Rats were discarded when any fraction of the active part of the probe was located outside the brain area of interest.

2.3. Analytical procedure

After collection, derivatized samples were analyzed for catecholamine and/or amino acid contents using an automatic capillary zone electrophoresis P/ACE^{TM} MDQ system (Beckman, USA) equipped with a ZETALIF laser-induced fluorescence detector (Picometrics, France). The excitation was performed by a He–Cd laser (Liconix, USA) at a wavelength of 442 nm. Emission wavelength was 490 nm. Separations were carried out in fused-silica capillaries (50 μm i.d., 375 μm o.d., Composite Metal Services, Worcester, England). Capillary flushing and hydrodynamic injections were performed by applying pressure at the capillary inlet. Each day, before analyses were begun, the analysis capillary was sequentially flushed for 15 min with 1% NaOH, then for 15 min with purified water, and finally for 5 min with the appropriate running buffer. Two separation procedures were successively used: (i) first, the catecholamine content was analyzed using a procedure adapted from Bert et al. (1996), i.e. a 51 cm long capillary, having an effective length of 38.5 cm, with 110 mmol/l phosphate buffer pH 7.05 ± 0.02, applied voltage of 25 kV, hydrodynamic injection (2 psi, 20 s) of the sample followed by the injection of 0.2 mol/l orthophosphoric acid (1.5 psi, 10 s), temperature 30 °C, acquisition 60 Hz; the capillary was flushed 3 × 1 min with 1% NaOH, water and running buffer between each analysis; (ii) subsequently, amino acids including GABA, Glu and L-Asp were analyzed (for details, see Sauvinet et al., 2003).

2.4. Statistical analysis

Regression equations were calculated by the least-squares linear regression method. Linear regression analysis was performed to test the significance of linearity of the system. For in vivo validation experiments, data are given as mean ± S.E.M. expressed as a percent of the six values preceding the first drug administration. Comparisons between treated and control groups were achieved on % transformed data using MANOVA. For GAERS experiments, data are given as mean ± S.E.M. expressed as a percent of the values corresponding to intercritic periods. After having taken into account the dead time of the collection system, only dialysates collected entirely during a seizure or a interictal period were used in the analysis. Differences of concentrations between 20 s ictal and interictal periods were tested using the Wilcoxon test. The level of significance was set at $p < 0.05$ for all comparisons.

3. Results

The first part of this study was designed to develop and validate a new system of high sampling rate microdialysis, derivatization of sub-microliter microdialysates and subsequent off-line analysis of derivatized samples using a commercially available CE-LIFD instrument. In the second part, the defined protocol was applied to study the variations of the extracellular concentrations of five major neurotransmitters during epileptic seizures in awake rats.

3.1. Performances of the microdialysis/derivatization/analysis system

3.1.1. In vitro tests

3.1.1.1. Minimal sample volume, repeatability and linearity. As the collected microdialysates have to be analyzed with a commercial, automatic CE system, the minimal sample volume required for the CE analysis had to be determined. Indeed, sample injections are currently made at one side of the capillary by applying a pressure. The volume of the sample must be sufficient to (i) allow the capillary end to plunge into it, preventing the injection of air microbubbles with sample, and (ii) to avoid any significant loss by evaporation when a series of dialysates (i.e. at least 30 samples) is placed in the CE sample rack before being injected.

In a first series of experiments, when the flow rate for aCSF was fixed at 1 μl/min—rate classically used in microdialysis experiments—20 s derivatized samples (470 nl volumes) can be collected and analyzed. However, it appeared that a batch of more than 10 samples could not be successively analyzed with sufficient repeatability, probably due to evaporation loss in the sample rack of the CE system (data not shown). Second, in order to use the automatic CE system for routine experiments, the perfusion rate of aCSF was enhanced to 2 μl/min. In this case, a derivatized 20 s sample corresponds to a 940 nl volume, which appeared to be the minimal volume required for reliable, reproducible injections. Indeed, the % R.S.D. ($n = 10$) are to be around 10% for the five neurotransmitters tested (Table 1). Besides, thirty 940 nl samples, stored at +4 °C in the CE system, could be successively analyzed for amino acid contents with a sufficient repeatability and without any intervention of the operator (Table 1).

Thereafter, the entire system (probe-derivatization device and CE-LIFD) was validated in vitro, by dipping the probe in different standard solutions and analyzing the sampled microdialysates. The regression coefficient of the calibration obtained with standard solutions showed a reliable linearity of the system including sampling, derivatization and analysis (Table 1). The detection limits at the dialysis probes, i.e. the minimal concentrations of standard solutions which can be detected with our whole microdialysis/derivatization/analysis system, were 3×10^{-9}, 1×10^{-9}, 1.9×10^{-8}, 4.2×10^{-7}, 2.1×10^{-7} mol/l for DA, NA, GABA, Glu and L-Asp (signal-to-noise ratio = 2), respectively. As the analysis of low volume samples could be easily

Table 1
Quantitative parameters for the monitoring of dopamine, noradrenaline, GABA, glutamate and L-aspartate in standard solutions using the microdialysis/derivation system coupled to an off-line analysis with a commercial CE system

	DA	NA	GABA	Glu	Asp
Repeatability (% R.S.D.)[a]					
$n = 10$	11.9	12.0	9.7	9.8	9.9
$n = 30$	15.1	17.0	10.9	7.6	9.3
Range (mol/l)[b]	10^{-9} to 10^{-6}		10^{-8} to 10^{-5}	10^{-7} to 10^{-4}	
Linearity (r)[c]	0.9953	0.9927	0.9953	0.9996	0.9994
Detection limit (mol/l)[d]					
At the probes[e]	3×10^{-9}	1×10^{-9}	1.9×10^{-8}	4.2×10^{-7}	2.1×10^{-7}
CE system[f]	2.5×10^{-10}	0.6×10^{-10}	0.9×10^{-9}	2.0×10^{-8}	1.1×10^{-8}

Samples were collected every 20 s, i.e. corresponding to 940 nl after derivatization.
[a] Probe plunged in a standard solution with 5×10^{-8} mol/l DA and NA, 5×10^{-7} mol/l GABA, 5×10^{-6} mol/l Glu and L-Asp, collection at the outlet of the microdialysis/derivatization system (10 or 30 samples) was begun 2 min after the plunge.
[b] Number of concentrations: 7.
[c] Probe plunged successively in standard solutions of various concentrations, collection was begun 2 min after each plunge.
[d] Extrapolations (signal-to-noise ratio: 2) based on calibration curves.
[e] Minimal concentrations of standard solutions detected with the microdialysis/derivatization/CE system.
[f] Minimal concentrations detected in 940 nl standards by the CE system only.

performed off-line, stability of microdialysates during storage, i.e. between collection and analysis, was tested. Samples could be kept at −20 °C for at least 2–3 days (data not shown) if the seal of the collection tubes was guaranteed. After the opening of the tube for analysis, each sample had to be analyzed rapidly, i.e. during the same working day because of the risk of evaporation.

3.1.1.2. Characteristics of the probe system. Microdialysis experiments on awake animals required long dialysis tubings in order to let the animals move freely. However, the dead volume of these tubings, i.e. between the active dialysis membrane and the outlet of the derivatization system, had to be minimized when a frequent collection was performed. Indeed, if the interval of time between dialysis and collection was superior to the sampling time, solutes could diffuse more between sample plugs. In our case, the length of the outlet tubing needed for our experimental animal chamber was 38 cm; the calculated dead volume of the 38 cm long × 40 μm i.d. outlet tubing was 0.47 μl and corresponded to a theoretical delay of 14 s with a 2 μl/min flow rate. However, the dead time had to be determined experimentally in order to correlate accurately the neurochemical results to the physiological events for in vivo studies (behavior, electroencephalographic recordings, etc. . . .). Fig. 2 shows an example of determination of the dead time corresponding to the dead volume of one

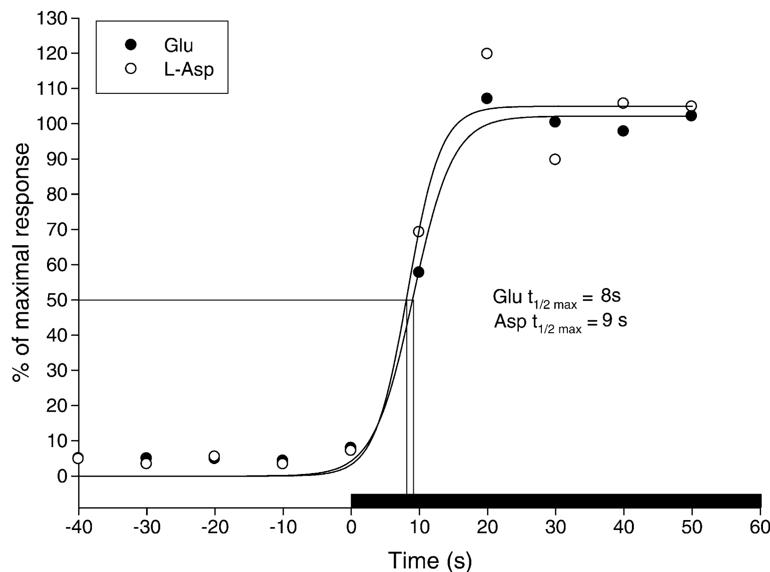

Fig. 2. Determination of the time of response. Representation of the fitted curves corresponding to the increases in Glu and Asp concentration after the probe was plunged in a standard solution containing 5×10^{-6} mol/l Glu and Asp (black bar). Data are expressed as percent of the maximal response. $t_{1/2\max}$ corresponds to the half time between the beginning of the dialysis in Glu/Asp-concentrated solution and the reach of the maximal response. Samples were collected every 10 s.

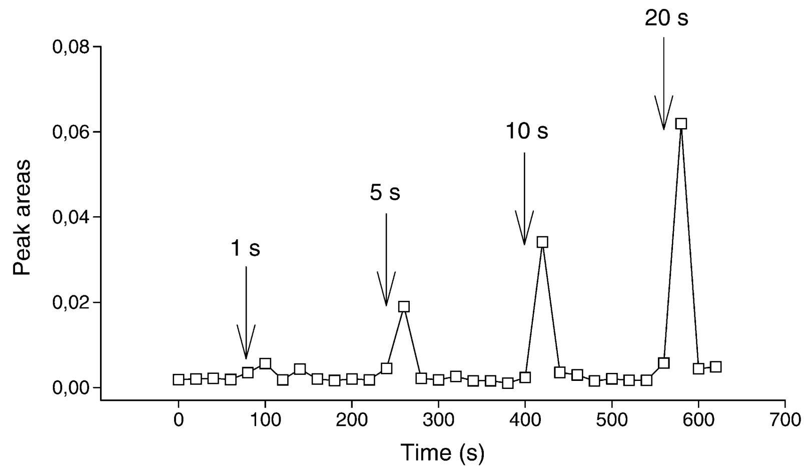

Fig. 3. Amplitude of response. The probe was plunged four times in a standard solution containing 5×10^{-6} mol/l Glu, for 1, 5, 10 or 20 s ($n = 2$). Between each dip, the probe membrane was placed in aCSF. Note that the response is detected on the sample following the plunge, the dead volume being not taken into account.

probe. Each probe was first dipped into an aCSF solution and thereafter into a standard solution containing 5×10^{-6} mol/l Glu and Asp. Samples were collected every 10 s, instead of 20 s (such small volume could be analyzed since the number of samples was limited, see Section 3.1.1.1). The plot of the concentration of neurotransmitters in microdialysates versus time was drawn (Fig. 2) and the regression equation allowed the calculation of the half-time of maximal signal, which represents the half of our dead volume. The dead time varied from 18 to 23 s (20.4 s ± 1.0, mean ± S.E.M., $n = 5$) between experiments and was always higher to the theoretical one (for recall, 14 s). One can estimate that a 20 s variation in concentrations can be detected on the following microdialysate. That assumption was also verified in another experience whose aim was to determined if short variations in neurotransmitters around the probe membrane could be detected using our new system. For this purpose, the probe was dipped for 1, 5, 10 or 20 s in a standard solution containing 5×10^{-6} mol/l Glu (Fig. 3). The results showed that all the variations could be measured. It appeared that the peaks of increases were proportional to the duration of the dips ($r = 0.9834$). These data suggest that our dialysis system allows to detect very short-lasting variations (1–5 s). Moreover, no diffusion phenomenon was observed as a 20 s variation was seen in just one sample. In conclusion, our dialysis system permits to monitor very short-lasting changes and the time spent by the solutes for crossing the membrane was not a limiting factor for monitoring accurately short-lasting events.

3.1.2. In vivo tests

Experiments on anesthetized rats were carried out in order to test the ability of the system to detect in vivo rapid variations in neurotransmitters with a high sampling rate microdialysis. For this purpose, two drugs known to increase the concentrations of neurotransmitters were administered for short periods of time and their effects on neurotransmitter levels were monitored. Fig. 4 shows the results obtained with amino acids. In this case, PDC, a Glu/Asp uptake inhibitor, administered for 20 s, induced an enhancement of both Glu and Asp levels (Fig. 4, A1, B1) whose maximum was reached 20 s after PDC application. Thereafter, the concentrations of Glu and Asp returned to baseline. An increase in GABA levels also occurred as previously described (Segovia et al., 1997); however, the profile of enhancement was different from those of excitatory amino acids, since the GABA levels reached a plateau within the 40 first seconds (Fig. 4, C1). Four minutes after this 20 s application, PDC was again applied but for 10 s. This induced a new, but smaller increase in both Glu and Asp levels, but unaltered the level of GABA (Fig. 4, A1, B1, C1).

Nipecotic acid, a GABA uptake blocker, administered for 20 s, induced an increase in GABA levels which was maximum at +20 s and then returned to baseline (Fig. 4, C2). This drug failed to alter the concentrations of Glu and Asp (Fig. 4, A2, B2). Nipecotic acid was also administered a second time for a 10 s period and still led to an increase in GABA levels without change in the concentrations of Glu and Asp (Fig. 4, C2, A2, B2).

3.2. In vivo monitoring in awake spontaneously epileptic rats

In the study presented above, we showed that short pharmacologically-induced changes could be monitored. Afterwards, we sought to demonstrate that our approach could detect short-lasting neurochemical changes linked to pathophysiological states. To this aim, we chose to evaluate the variations of neurotransmitter levels associated with absence epilepsy seizures in GAERS strain. In this animal model, the epileptic episodes are characterized by SWD on the EEG, last from 20 s to 1 min and occur every 1–3 min. The concentrations of DA, NA, GABA, Glu and Asp, were monitored every 20 s while the occurrence (ictal periods) or non-occurrence (interictal periods) of seizures were determined by EEG recordings in freely-moving GAERS. The

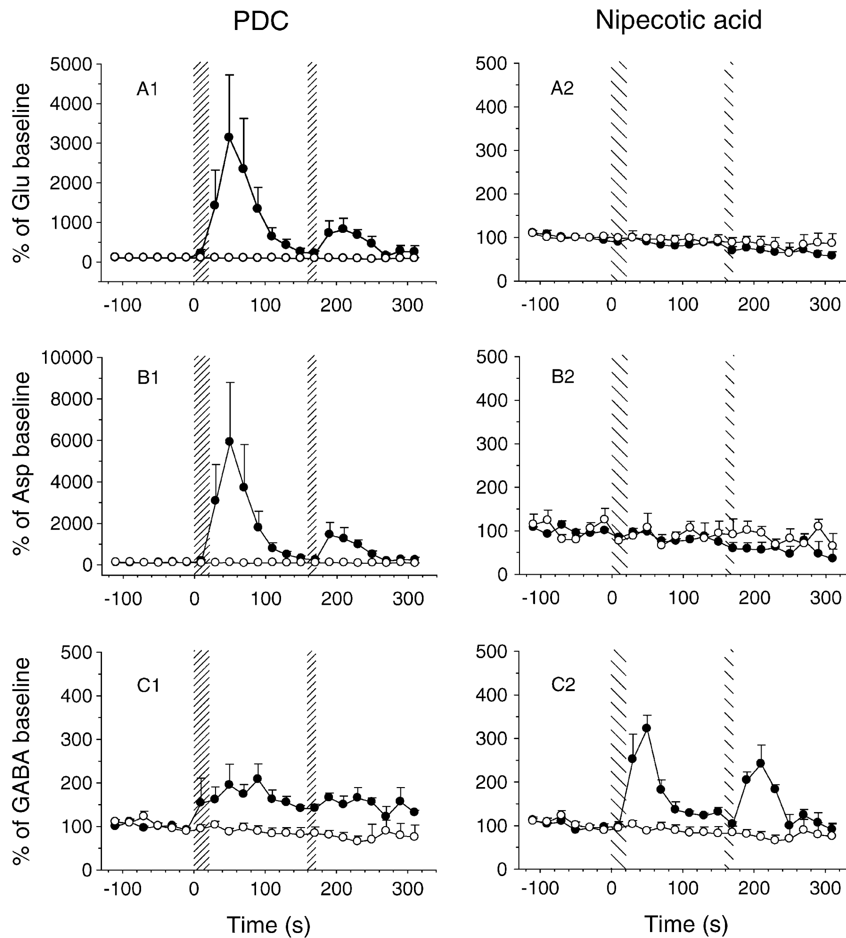

Fig. 4. In vivo validation. Effect of injected nanovolumes of 10 mM PDC (left) or 1 mM nipecotic acid (right) through a micropipette on Glu (top), Asp (middle) and GABA (bottom) levels monitored in the striatum of anesthetized rats with a 20 s microdialysis sampling rate. Black symbols represent experiments with drug application and white symbols, those with aCSF ejection (control experiments). Drug volumes (167 and 83 nl) were delivered over a 20 and 10 s period, respectively (hatched bars). Data are expressed as percent (mean ± S.E.M.) of the six baseline values preceding drug or vehicle injection. Each plot represents five experiments. Statistical analysis revealed significant differences between treated and control rats as soon as the drugs were applied (MANOVA, not shown).

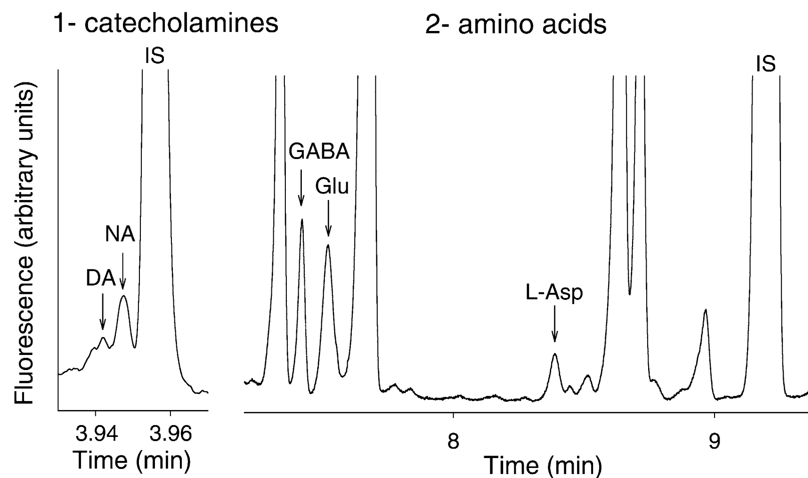

Fig. 5. Analyses by CE-LIFD. Electropherograms of a microdialysate from thalamus of a freely-moving rats, first analyzed in catecholamines separation conditions (left) and then in amino acid separation conditions (right). Concentrations of DA, NA, GABA, Glu and L-Asp in the microdialysate were 1×10^{-10}, 4×10^{-10}, 18.9×10^{-9}, 50.6×10^{-9} and 11.7×10^{-9} mol/l, respectively. Internal standard (IS) was 3,4-dihydroxybenzylamine for catecholamines and cysteic acid for amino acids.

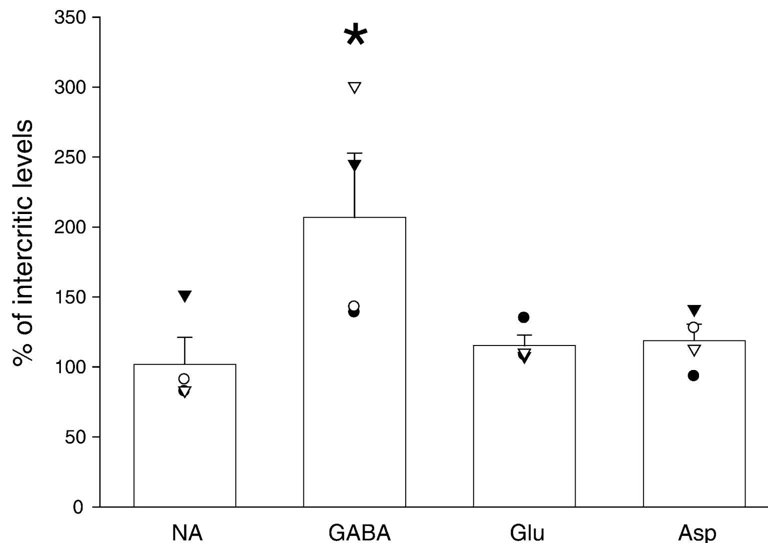

Fig. 6. Variations in thalamic concentrations of NA, GABA, Glu and Asp when epileptic seizures occur in GAERS strain. White bars represent the concentration of neurotransmitters in “seizure” samples expressed as percent (mean ± S.E.M., $n = 4$) of values for which no seizure is detected. Statistical analysis revealed significant differences between “interictal” and “seizure” fractions (* $p < 0.05$, Wilcoxon test). Scatter plots represent data from individual animals. The dead volume of the dialysis system was determined for each experiment in order to analyze the fractions of interest.

thalamus, one of the brain area in which SWD are generated (Danober et al., 1998), was chosen in this study. An example of typical electropherograms obtained from a 20 s brain microdialysate from a freely-moving GAERS is shown in Fig. 5. They correspond to the analysis of five neurotransmitters, DA, NA, GABA, Glu and L-Asp present in the lateral nuclei of the thalamus. DA concentration was under the limit of quantification in most microdialysates, so that seizure-associated changes in DA could not be investigated.

Fig. 6 presents the neurochemical data obtained in four rats. The levels of GABA during the seizures (ictal period) increased by +107% as compared to interictal levels (* $p < 0.05$, Wilcoxon test). Concentrations of NA, Glu and Asp were not significantly changed. Histological analysis revealed that all four probes were located in the posterior and ventral posteromedial nuclei of the thalamus.

4. Discussion

This paper describes, for the first time, a method which allows to monitor short-lasting variations in the extracellular concentrations of five neurotransmitters in awake, freely-moving animals using microdialysis and CE-LIFD analysis. Until now, the very few groups which have been successful in performing high temporal resolution monitoring used mainly home-made CE systems. Our first consideration was to use a commercially available, automatic CE-LIFD system for analysing very low volumes obtained through high temporal microdialysis sampling. To our knowledge, volume dialysates analyzed so far using a commercial, automatic CE system were not less than 5 μl (see Qu et al., 2001; Zhang et al., 2001). The limiting factor in our protocol was the volume required by the CE apparatus: we found that 940 nl corresponded to the minimal volume required for the analysis of large series of samples with a good repeatability.

The second step was to test the performances of the coupling of our CE-LIFD instrument to a new microdialysis/derivatization device which allows collection and derivatization of sub-microliter dialysates. With such a device, the 940 nl minimal volume for CE-analysis corresponds to derivatized samples collected every 20 s, the probe being perfused at a 2 μl/min flow rate. Under these conditions, criteria of linearity and repeatability are fully satisfied. The limits of detection for Glu and Asp at the probes are similar to those obtained by Lada et al. (1997). In order to monitor accurately short-lasting variations in neurotransmitters in freely moving rats, the dead volume of the device, i.e. the volume of tubings between the site of dialysis and the outlet where the collection occurs, had to be minimized. Classical dialysis set-ups for freely-moving animals have a liquid swivel which leads to high dead volume. We suppressed the swivel at the outlet of the probe (while keeping it at the inlet side) and we further minimized the dead volume by using a single capillary for the outlet of the probe system. However, as this dead volume cannot be negligible, it must be determined with precision in order to know the delay between any change in neurotransmitter around the membrane probe and the response obtained at the outlet. The time lag (18–23 s) corresponding to the dead volume was in the same magnitude as the sampling rate (20 s). As a consequence, a variation occurring during the collection of one sample can be detected in the fraction collected in the following sample (see Fig. 3).

This approach which has been developed and fully validated should represent an efficient tool for the monitoring of short-lasting changes in the extracellular concentrations

of neurotransmitters in physiological studies. This is shown in the experiment on epileptic rats, exhibiting short-lasting seizures (20 s–1 min). The present work shows that, in GAERS strain, a significant increase in GABA levels in the relay nuclei of the thalamus was associated with absence seizures. This is in agreement with previous electrophysiological and pharmacological studies suggesting that an increase in GABA neurotransmission in the thalamic relay nuclei may participate in the triggering of absence seizures by lowering the membrane potential of thalamocortical neurons and thus allowing the occurrence of rhythmic bursts of action potentials (see reviews, Danober et al., 1998; McCormick and Contreras, 2001; Crunelli and Leresche, 2002). This hypothesis could be addressed only with a high temporal resolution dialysis technique, because of the short duration of these seizures. Our results will have to be confirmed with an increased number of animals, and the specificity of the structure will need to be verified by comparing these data with those obtained in adjacent nuclei (e.g. intralaminar nuclei of the thalamus).

In conclusion, we have developed and validated a technique for the monitoring of the extracellular concentrations of five neurotransmitters in freely moving animals. We have shown that the analysis of low volume microdialysates can be performed off-line using a commercially available CE-LIFD system. Consequently, this technique can be set-up in neurosciences laboratories which have no access to a specialized workshop making custom-made analysis instruments. Furthermore, one advantage of our technique is to discouple microdialysis sampling from the CE analysis. Indeed, the off-line analysis can be useful if any breakdown of the CE-LIFD system occurs; in this case, the sub-microliter microdialysates can be stored up to 3 days before to be analyzed. In contrast, if on-line analysis is used, samples cannot always be saved and data can be lost. Thus, off-line analysis offers more flexibility for planning the experiments.

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