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Analysis of glutamate in striatal microdialysates using capillary electrophoresis and laser-induced fluorescence detection

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Abstract

High-performance liquid chromatography (HPLC) with electrochemical detection has been used routinely to analyse the neurochemical constituents of brain microdialysates. However, conventional HPLC analysis requires large injection volumes and hence lengthy dialysis sampling times. Capillary electrophoresis (CE) is a rapid high-resolution separation technique with the ability to routinely handle very small sample volumes. If CE is coupled to a high-sensitivity detection system, such as laser-induced fluorescence (LIF), it becomes a powerful and rapid separation technique for the analysis of small-volume microdialysis samples.

These preliminary studies report reduced separation times for the excitatory amino acid glutamate, prederivatised with naphthalene 2,3-dialdehyde, and demonstrate its detection within small-volume brain microdialysis samples. The limit of detection for this system was 10^{-8} *M*.

Characterisation of striatal microdialysis samples comprised infusions of Ca^{2+}-free artificial cerebrospinal fluid (aCSF) and Tetrodotoxin (TTx) (10 m*M*) to demonstrate that the detected transmitter is of neuronal origin and released in a calcium-dependent manner.

Removal of calcium from aCSF resulted in a decrease in glutamate in dialysis samples. Glutamate release significantly decreased ($p < 0.05$) to ca. 40% of preinfusion control levels after 60 min and this level was maintained throughout the sampling period. These data suggest that glutamate release is, to some degree, a calcium-dependent process. TTx infusion (10 μM) produced a significant ($p < 0.05$) reduction in glutamate release to ca. 10% of preinfusion levels. It would therefore appear that glutamate release is dependent on neuronal activity. In summary, we have demonstrated the establishment of CE–LIF and microdialysis for the measurement of glutamate.

1. Introduction

The excitatory amino acids are quantitatively one of the most important group of neurotransmitters in the brain with ubiquitous occurrence and universal neuronal excitatory properties [1]. Glutamate is one of these amino acids with such characteristics. Glutamate is involved in various metabolic functions, is released from neurones in response to neuronal stimulation and is subsequently taken up by both neurones and glia [2]. Recently glutamate has been implicated in the pathogenesis of neurological and psychiatric disorders such as Alzheimers disease [3], Parkinson's disease [4] and schizophrenia [5]. The rapid and sensitive in vivo monitoring of excitatory amino acids, in particular glutamate, in response to potential neuroactive molecules is therefore of particular interest in the search for possible treatments for these conditions. Brain mi-

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crodialysis [6] is a technique which can monitor in vivo changes in release of many neurotransmitters such as dopamine [7], serotonin [8] and amino acids [9].

Analysis of the neurochemical constituents of brain microdialysates has conventionally used high-performance liquid chromatography (HPLC) with electrochemical detection. However, conventional HPLC analysis requires large injection volumes and hence lengthy dialysis sampling times. Capillary electrophoresis (CE) is a rapid high-resolution separation technique with the ability to routinely handle very small sample volumes (typically 1–5 nl). If CE is coupled to a high-sensitivity detection system, such as laser-induced fluorescence (LIF), it becomes a powerful technique for the analysis of small-volume microdialysis samples.

We now report a rapid and sensitive method for the analysis and detection of glutamate in rat striatal dialysates by CE–LIF.

2. Experimental

2.1. Chemicals

All chemicals were analytical grade and purchased from BDH (Poole, UK). Tetrodotoxin was purchased from Sigma (Poole, UK). Naphthalene 2,3-dialdehyde (NDA) was purchased from Aldrich (Poole, UK).

2.2. Microdialysis

Animals

Male Sprague–Dawley rats (280–350 g Charles River) were used in all experiments. All animals were group housed in cages with food and water available ad libitum. Following surgery the animals were singly housed in Plexiglass cages (30 cm^2) with food and water available ad libitum.

Surgical procedure

Following induction of anaesthesia with gaseous administration of halothane (5%) (Fluothane, Zeneca, Macclesfield, UK) the animals were secured in a stereotaxic frame with ear and incisor bars. Anaesthesia was maintained by continuous administration of halothane (1–2%). A microdialysis guide cannula (CMA/Microdialysis, Sweden) was implanted and cemented to the skull using dental acrylic. Coordinates for the striatum were taken according to Paxinos and Watson [10]: RC, 0.4; L, 3.7 (reference point taken from bregma); V, 2.8 mm from the dura. The wound was sutured and the animals left to recover for 24 h.

Microdialysis procedure

The dialysis probe (4 mm × 0.5 mm O.D.) (CMA/Microdialysis) was implanted via the guide cannula into the striatum of the unrestrained rat 24 h post surgery and was perfused with artificial cerebrospinal fluid (aCSF) (NaCl 125 m*M*, KCl 3.0 m*M*, $MgSO_4$ 0.75 m*M* and $CaCl_2$ 1.2 m*M*, pH 7.4) at a flow-rate of 0.5 μl/min. A 3-h stabilisation period was allowed following probe implantation after which time dialysis sampling was carried out by a modification of the method of Routledge et al. [11]; 20-min samples were collected throughout the experimental period. Five preinfusion control samples were collected and all subsequent values were expressed as a percentage of the preinfusion control level. Characterisation studies comprised continuous infusions of either Ca^{2+}-free aCSF (NaCl 125 m*M*, KCl 3.0 m*M* and $MgSO_4$ 0.75 m*M*, pH 7.4) or Tetrodotoxin (TTx) (10 μM in aCSF).

2.3. Capillary electrophoresis

Apparatus

All analyses were performed on a Beckman P/ACE 2050 with He–Cd LIF (442 nm) (Omnichrome, CA, USA). Separations were performed in fused-silica capillaries (47 cm × 375 μm O.D. × 50 μm I.D.) (Composite Metal Services, Hallow, Worcester, UK) with an applied voltage of 0.64 kV/cm. Sample was applied to the capillary via a high-pressure injection system. Data were collected and integrated using Xchrom (VG Data Systems, Altrincham, UK).

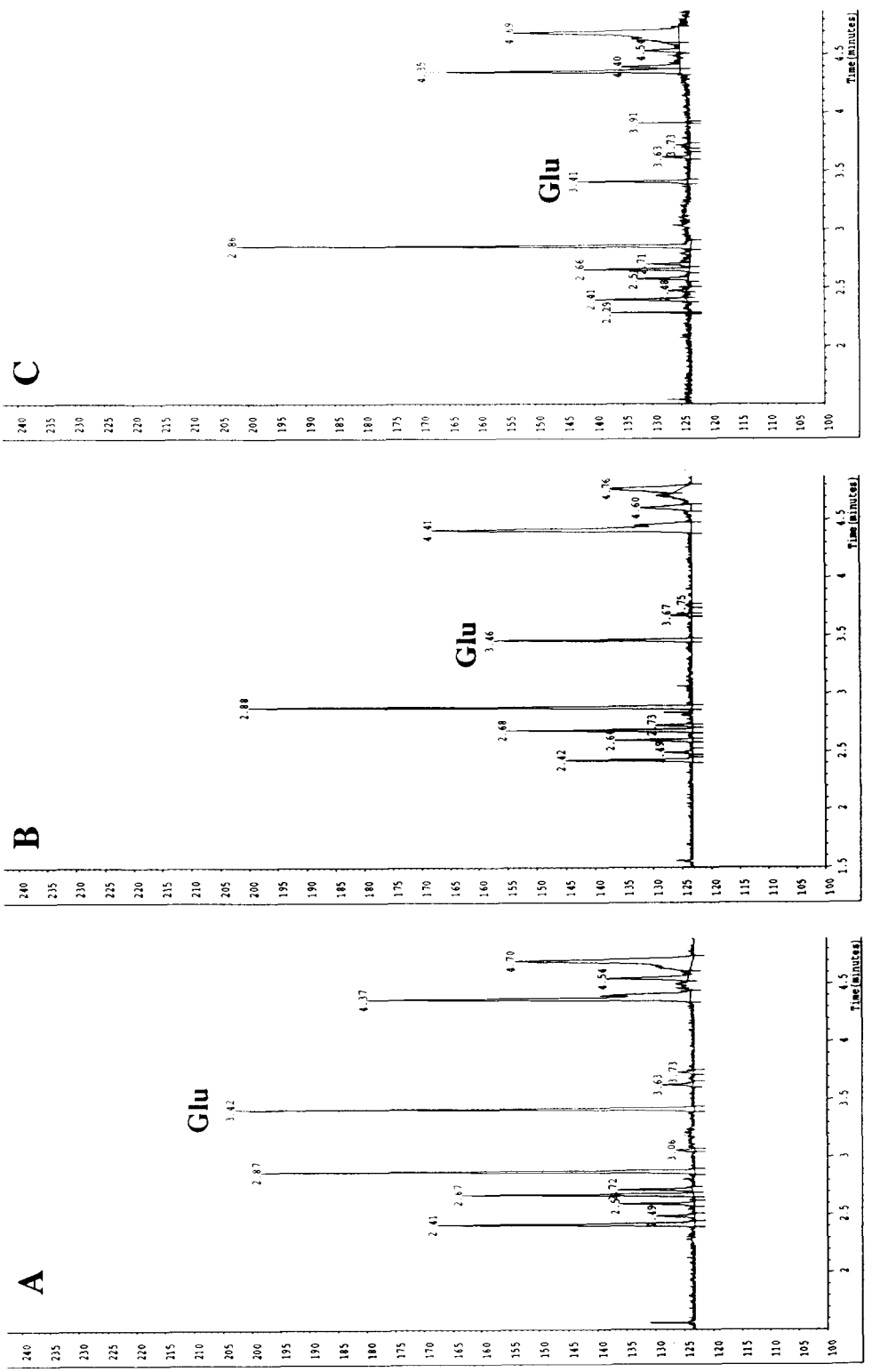

Fig. 1. Electropherograms demonstrating decreasing glutamate in response to infusions of Ca^{2+}-free aCSF. (A) Preinfusion control sample, (B) 100 min post infusion, (C) 280 min post infusion.

Separation procedure

Separations used 30 m*M* sodium carbonate with 20 m*M* sodium dodecyl sulphate (SDS) pH 9.5 (pH using 1 *M* NaOH). Total run time was 5 min. The capillary was rinsed with 0.1 *M* NaOH (2 min) and running buffer (2 min) between analyses.

Derivatisation procedure

All samples were prederivatised with NDA by a modification of the method of Hernandez et al. [12]. Dialysate or standard samples (10 μl) were mixed with 50 m*M* boric acid buffer pH 9.5, 10 m*M* sodium cyanide and 30 m*M* NDA in methanol (1:5:2:1) and left to stand for 5 min at room temperature before analysis.

Analysis of data

Results were analysed by ANOVA followed by post hoc analysis where appropriate using the Super ANOVA software application (Abacus Concepts, Berkeley, CA, USA) on an Apple Macintosh PC.

3. Results

Samples were analysed by micellar electrokinetic capillary chromatography (MECC) and separation conditions were optimised to produce high resolution of the excitatory amino acid glutamate and as many of the other dialysate constituents as possible. Reproducibility was good with separations of glutamate maintained with as little as a 1% variation in retention time (3.26 ± 0.01 min). Standard calibrations revealed a linear relationship between concentration and peak area over the concentration range 10^{-6}–10^{-8} *M* for derivatised glutamate (slope = $5.6 \cdot 10^{7}$ and correlation coefficient = 1). The limit of detection for glutamate was 50 amol on capillary which corresponds to a concentration of 10^{-8} *M*.

The average recovery of the probe, for glutamate, was calculated to be 18.5 ± 1.4%. Basal levels of glutamate within dialysates, ranged between 1 and 10 μ*M*.

Infusions of Ca^{2+}-free aCSF resulted in a decrease in glutamate concentrations in dialysis samples (Fig. 1). Glutamate release showed a significant decrease ($p < 0.05$) to 40% of preinfusion control levels 60 min post infusion and this reduced level was maintained throughout the sampling period (Fig. 2).

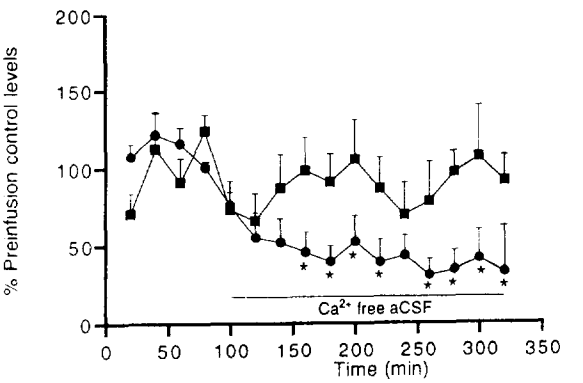

Fig. 2. Effects of Ca^{2+}-free aCSF infusion on striatal glutamate release. ■ = Vehicle controls; ● = Ca^{2+}-free aCSF treatment. Data are expressed as the mean ± standard error of the mean (SEM) for $n = 6$ animals. * demonstrates statistical significance ($p < 0.05$) from vehicle controls.

Infusions of TTx (10 μ*M*) also resulted in a significantly ($p < 0.05$) decreased glutamate in dialysis samples (Fig. 3), reaching a maximum of 10% of preinfusion control levels (Fig. 4).

4. Discussion

These data demonstrate that separation and detection of glutamate in brain microdialysate samples is possible using CE–LIF. The linearity of the derivatisation and detection appears to be effective to a minimum of 10^{-8} *M* glutamate (amol levels on capillary) and the reproducibility of this system also appears to be excellent; since basal levels are well within the limit of detection the routine analysis of glutamate using this system is now possible. Also the 5 min run time is an improvement on conventional HPLC analyses using *o*-phthalaldehyde (OPA) derivatisation [13].

Ca^{2+} dependence and tetrodotoxin sensitivity are regarded as fundamental criteria for determining the neuronal origin and mechanisms of

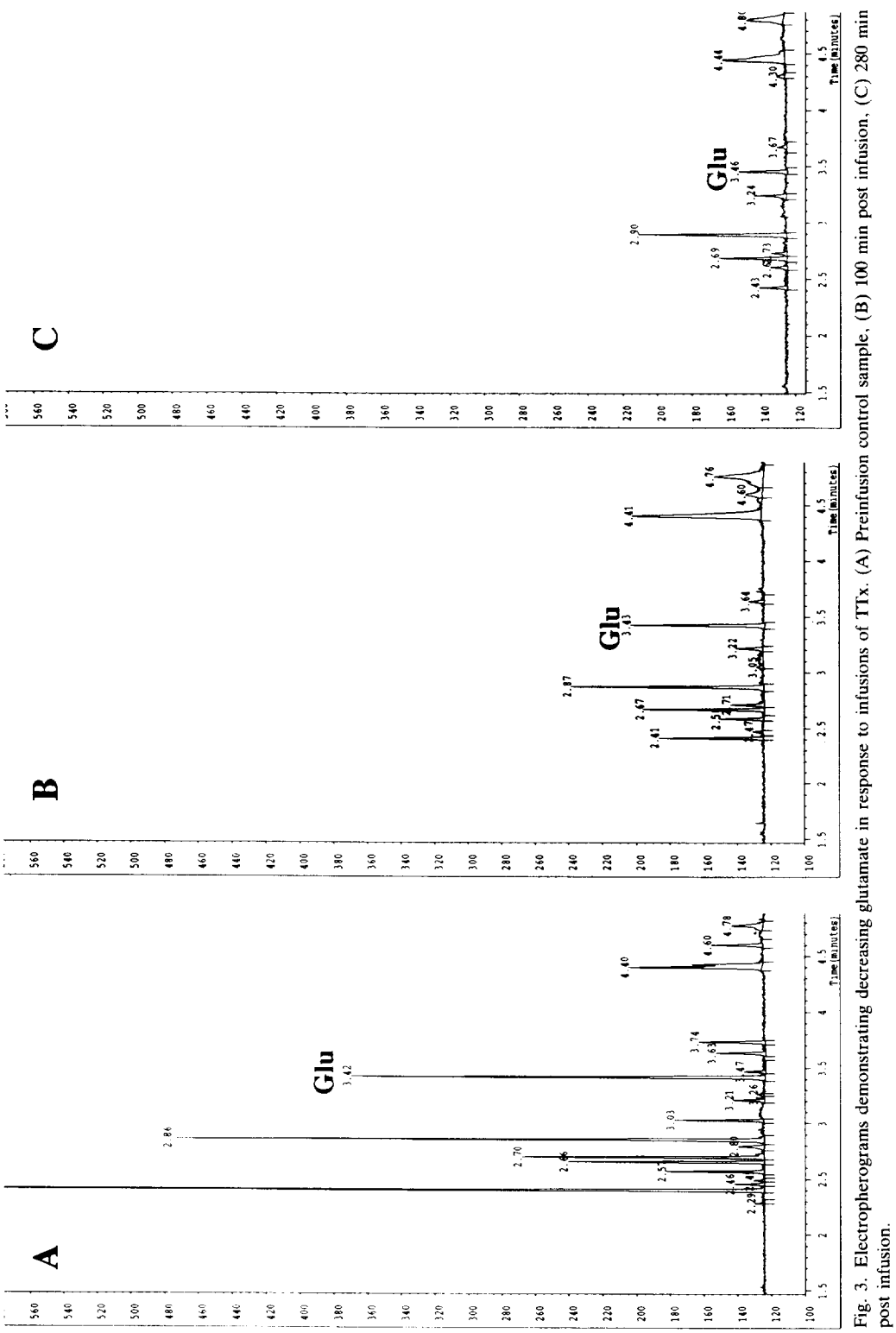

Fig. 3. Electropherograms demonstrating decreasing glutamate in response to infusions of TTx. (A) Preinfusion control sample, (B) 100 min post infusion, (C) 280 min post infusion.

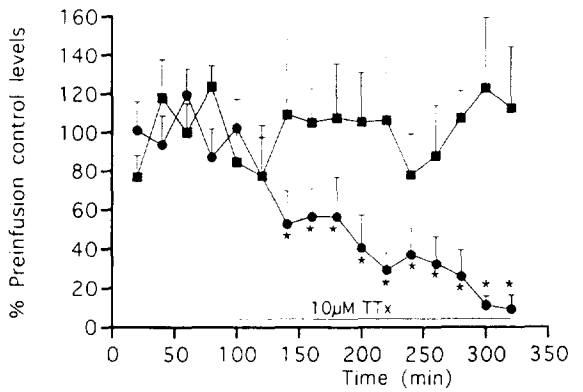

Fig. 4. Effects of TTx infusion on striatal glutamate release. ■ = Vehicle controls; ● = TTx treatment. Data are expressed as the mean ± SEM for $n = 6$ animals. * demonstrates statistical significance ($p < 0.05$) from vehicle controls.

release of neurotransmitters and therefore these are the standard manipulations frequently used to characterise microdialysis studies [14].

Removal of calcium from the infused aCSF resulted in a decrease in glutamate in dialysis samples suggesting that glutamate release is, to some degree, a calcium-dependent process. It is known that non-synaptically released pools of glutamate exist throughout the brain and the size of these pools may vary; thus it would be predicted that all sampled glutamate may not necessarily be released in a Ca^{2+}-dependent manner [15]. Therefore, in the present study 40% of the glutamate sampled from the striatum may have been from non-synaptically released sources or released by a Ca^{2+}-independent mechanism. Although separation was optimised for glutamate other unknown components were detected (retention times 2.41, 2.49, 2.59, 2.68, 2.72, 3.64, 4.54 min) (Figs. 1 and 3). These components show similar decreases in response to the Ca^{2+}-free aCSF suggesting that many of these molecules are released by a Ca^{2+}-dependent mechanism.

TTx is a Na^+ channel blocker which prevents neuronal impulse flow and consequently neurotransmitter release. Infusions of TTx resulted in a marked and significant decrease in glutamate levels; these data suggest that glutamate release in the striatum is dependent on neuronal activity. TTx, like Ca^{2+}-free aCSF infusions, induced decreases in many of the other components observed from electropherograms and it appears likely that these molecules may be other amino acids (e.g. aspartate, glycine, taurine and serine) and monoamine neurotransmitters (e.g. dopamine and noradrenaline).

In summary, we have demonstrated the establishment of CE–LIF and microdialysis for the measurement of glutamate. The present study utilised these techniques for the analysis and characterisation of glutamate release from the striatum of the freely moving rat. Decreases in dialysate glutamate concentrations following infusions of Ca^{2+}-free aCSF and TTx demonstrated that release is mainly from neuronal sources and that both Ca^{2+}-dependent and -independent mechanisms of release may be involved. The main advantage of CE–LIF is its ability to assay small sample volumes within a short analysis time making it very suitable for use with microdialysis. This technique has the potential to measure not only amino acids but many other constituents of brain dialysates, and to monitor these molecules simultaneously in response to drugs and other stimuli.

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