Combined Electrophysiology and Microdialysis on Hippocampal Slice Cultures Using the Physiocard® System

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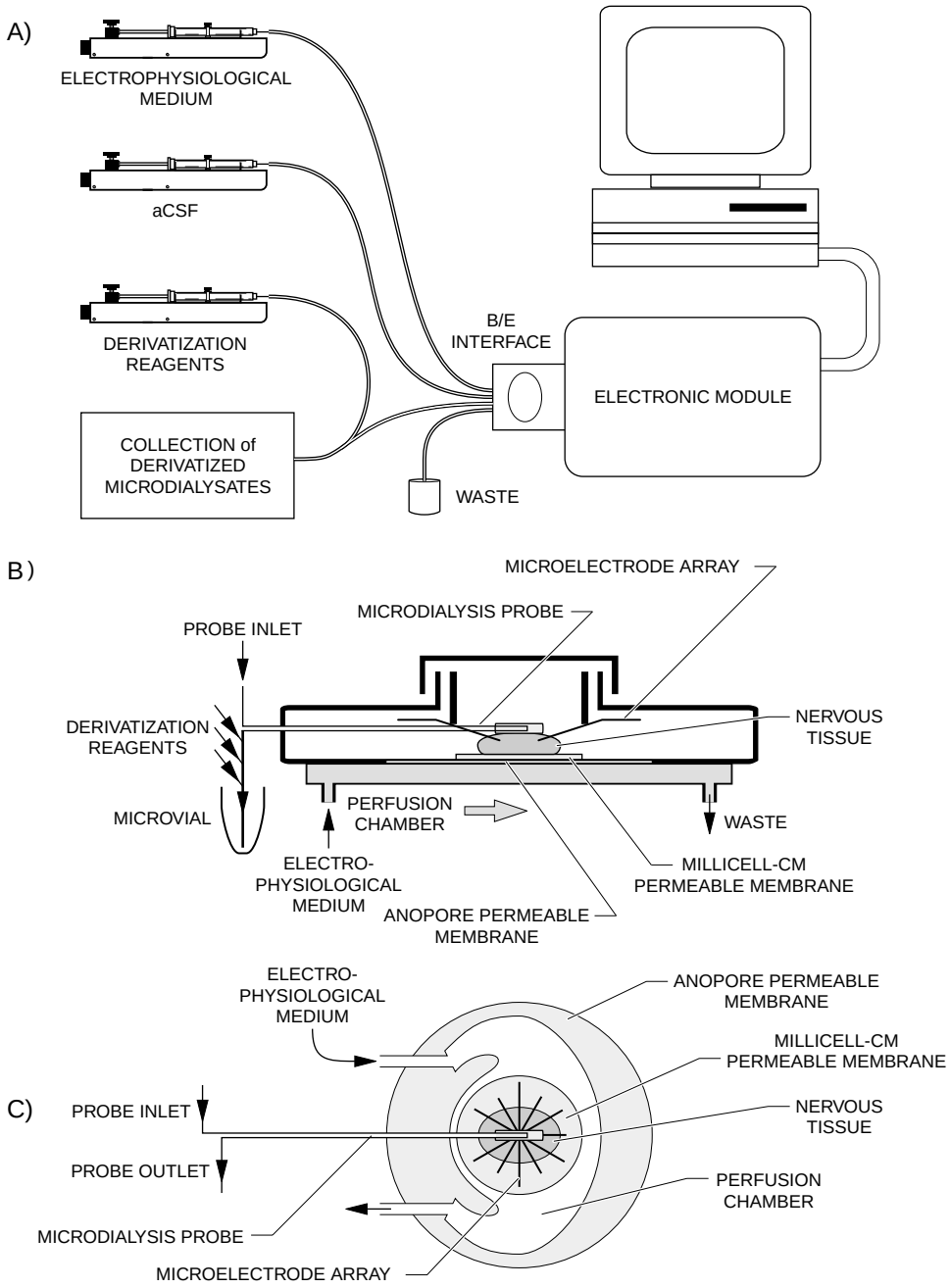
This paper describes a new device (Physiocard®) for in vitro exploration of brain slices. The system is composed of four parts: 1) a biological/electronic interface (B/E interface) carrying an array of thirty microelectrodes, 2) a microdialysis probe, 3) an electronic module designed to receive the B/E interface, and 4) a computer with a data acquisition card. Physiocard allows the combination of extracellular electrophysiological recordings from hippocampal organotypic slice cultures and the sampling of extracellular fluid from a restricted region of the slice using the microdialysis probe. Slice cultures survive in optimal condition when adequate medium is perfused through the B/E interface. Glutamate (Glu) concentrations are monitored in microdialysis samples by capillary electrophoresis with laser-induced fluorescence detection coupled to a continuous flow derivatization system, while evoked field potential responses (EvFPR) are simultaneously recorded using the multielectrode array of the B/E interface. Because of the high temporal resolution of the system, rapid changes in Glu concentration and EvFPR can be recorded following different pharmacological treatments. The Physiocard system allows new insights for many studies on the central nervous system by carrying combined electrophysiological and microdialysis approaches. It can be used for various neurophysiological studies or screening tests as a complement to in vivo approaches.

Monitoring neuronal release via the analysis of extracellular concentration of neurotransmitters complements electrophysiological studies. However, this combination is rather difficult to achieve with in vivo models, mainly because of the relative inaccessibility of the central nervous system (CNS) to prolonged and localized manipulation. Any biological response results from the interaction of multiple neurons, synapses, neurotransmitters, and the implied contribution of a large number of cerebral areas. Many groups have explored in vitro alternatives to separating and studying the different events. The use of organotypic cultures represents an interesting model to perform such studies. Brain organotypic cultures are used in a wide variety of physiological, pharmacological and biochemical studies to mimic in vivo brain functions in a simpler way (1). Some years ago, one of the authors described a technique for preparing hippocampal organotypic cultures in which brain slices are maintained on a porous and transparent membrane at the interface between a culture medium and a controlled atmosphere (2). These cultures do not spread as monolayers but retain a three-dimensional organization while preserving the different cell types. Dendritic processes of pyramidal neurons and the time course of synaptic development are similar to those observed in situ at comparable developmental stages (2-4).

Glutamate (Glu) has been shown to play important roles in hippocampal functions such as learning, memory, or plasticity, as well as in the excitotoxicity phenomenon seen in many different kinds of brain injury situations. One approach to Glu monitoring was performed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIFD), an alternative to conventional analytical techniques (like LC). CE-LIFD has already been described for the determination of excitatory amino acids (EAA) [Glu and/or aspartate (Asp); (5-11)], noradrenaline [NA; (12)], both GABA and Glu (13),

F1

General scheme of the Physiocard system. (A): Schematic representation of this system composed of 1) a B/E interface carrying a multielectrode array, 2) a microdialysis probe connected to a continuous flow derivatization system, 3) an electronic module designed to receive the B/E interface, to stimulate and to amplify the responses originating from the network of electrodes in contact with the tissue, while each electrode can be assigned to stimulation or recording, and, 4) a computer with an acquisition card and software (WCP) to record and analyze the electrophysiological responses. (B): Detailed scheme of the B/E interface (lower part containing the perfusion chamber and the upper part which allows the downward movement of the microelectrode array and the microdialysis probe), bearing the hippocampal slice culture and the continuous flow derivatization system of the dialysates. (C): Overhead view showing the microelectrode array and the probe on a hippocampal slice.



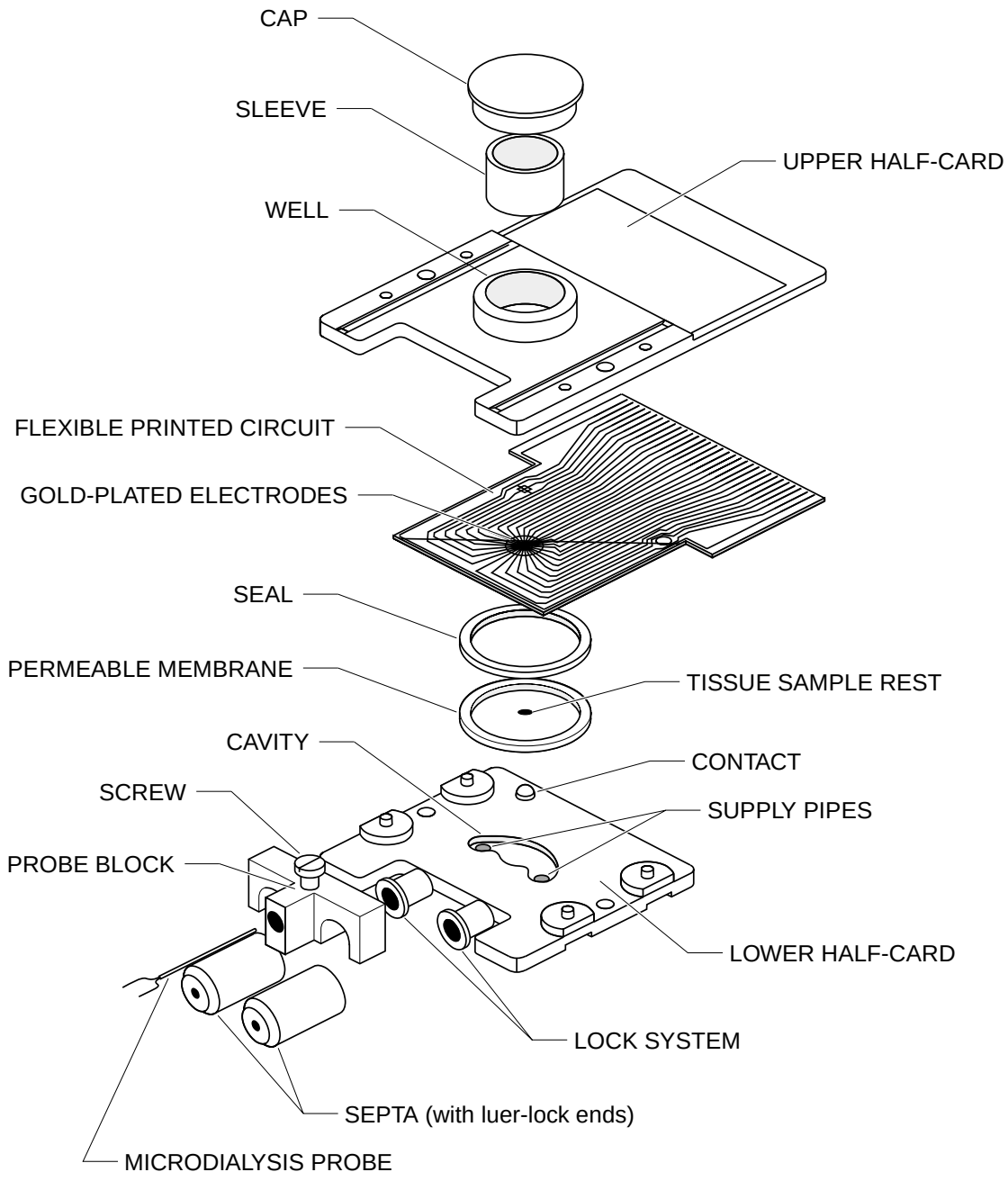
and both NA and Glu (14) in brain microdialysis samples. Due to the nanoliter volumes needed for injection, CE-LIFD especially has been shown to improve temporal resolution (45-s to 2-min fractions) of the microdialysis approach (9,10,14).

The present paper describes experiments using a new multirecording device (Physiocard), performing continuous electrophysiological/biochemical studies on organotypic cultures kept alive outside an incubator. The electrophysiological part is formed by a biological/electronic interface (B/E interface) which carries the culture and is inserted into an electronic module. A thirty-microelectrode array is integrated into the B/E interface to enable electrical stimulation and recording of the neuronal activity. Simultaneously, electrical activity is monitored by the multielectrode array, while variations in extracellular concentrations of neurotransmitters are monitored by a microdialysis probe. Both are adjusted into close contact with the upper surface of the slice culture. Extracellular electrophysiological recordings, Glu concentration monitoring, and some drug effects obtained with this system in hippocampal organotypic slice cultures are reported here.

Experimental

Preparation of Organotypic Cultures

The organotypic cultures were prepared as previously described



F2

Design of the biological/electronic interface of the Physiocard system. The device comprises two half-cards, the upper one and the lower one, which fit together to form the operational unit. The lower half-card has a cavity which contains the culture medium, comprising two supply pipes, each with a lock system at the end. The cavity is surmounted by a transparent and permeable membrane on which the Anopore disc and the tissue sample rest. The Anopore disc is 8 mm in diameter and a porous, translucent membrane. The device is air-tight when a seal is added and the two half-cards are secured together by clips. The upper half-card comprises an outlying well within which a sleeve slides. The sleeve can be lowered when the cap is screwed down. A microdialysis probe is threaded into the small hole of the probe block, which fits onto the lock system. The probe is positioned adjacent to the surface of the lower card and locked into the required position by the screw. Two septa secure the probe block by luer-lock fittings. The lowering of the sleeve, by progressively pressing on the flexible printed circuit, moves the gold-plated electrodes and the microdialysis probe downward so it can touch the surface of the tissue explant. When the two half-cards are adjusted together, the reference electrode (a golden ring) is automatically connected to the electronic ground through a contact.

(2). Hippocampi were dissected from seven-day-old Wistar rats and slices (400 µm-thick) were prepared using a McIlwain tissue chopper. The slices were transferred into a cold dissection medium [100% MEM (minimal essential medium) + 10 mM Tris, pH 7.35]. Slices were then laid down onto porous and transparent Millicell-CM membranes which were kept in 6 well-plate dishes filled with 4 mL of a culture medium consisting of 50% MEM (+ 4 mM $NaHCO_3$ + 5 mM Tris), 25% horse serum and 25% Hank's solution, pH 7.2.

Cultures were kept in a 5% CO_2 incubator set at a temperature of 37°C during the first 4 days and at 33°C for the following days before being used. Under those conditions, survival of cultures was optimum. One- to two-week-old cultures were used for all subsequent studies.

The Physiocard System

This system is fully described in a forthcoming paper (15). It is composed of four parts (***F1A***): 1) the B/E interface carrying a multielectrode array, 2) a microdialysis probe, 3) the electronic module designed to receive the interface, and 4) a computer with an acquisition card. Three Baby-Bee syringe drives managed by a single Queen Bee electronic controller (BAS, West Lafayette, U.S.A.) are used to perfuse the B/E interface with electrophysiological medium and the probe with artificial CSF (aCSF), and to add derivatization reagents to collected microdialysates (see

below). A general view of the B/E interface is represented in ***F2***. It is formed by a lower card carrying the culture. A low-volume perfusion chamber contained in this part of the card allows continuous perfusion (20-100 µL/min) with a modified Ringer's solution and ensures good survival conditions for the culture. This cavity is delimited by a solid, permeable, and transparent membrane (Anopore 0.2 µm, Whatman, Maidstone, U.K.). Electrical activity is recorded by a multielectrode array (30 gold electrodes) integrated into the upper part of the B/E interface enabling stimulation and recording of neurons over periods of up to 72 hours. The stimulation paradigm for evoked responses is given by the computer. Spontaneous activity can also be recorded. The recordings can be performed by up to six different electrodes. This part of the interface is inserted into the electronic module.

Implantation of the electrodes and the microdialysis probe. A Millicell-CM membrane carrying the hippocampal slice is placed above the Anopore membrane of the lower part of the B/E interface. A side-by-side microdialysis probe (225 µm o.d., 2 mm active dialysis length) is delicately adjusted on the surface of the brain slice. The flexible array of thirty microelectrodes is placed into close contact with the upper surface of the slice culture by screwing down the cap, which pushes down the sleeve and thus moves down on the flexible array to contact the nervous tissue (***F1B-C***; ***F2***). Most of the time, two electrodes located in the CA_3 region of the hippocampus are used as stimulation electrodes and those located in the CA_1 area are used as recording electrodes. Typically, 2-4 recording electrodes are used for each experiment. The microdialysis probe is perfused (1 µL/min) with aCSF. In vitro probe recovery is about 8% for Glu due to the limited surface contact between the dialysis membrane and the tissue. The inlet of the dialysis probe is connected to the perfusion pump, whereas the outlet is attached to the continuous flow derivatization system described below. The outlet of this system is inserted into a 100-µL glass microvial (Alltech, Deerfield, U.S.A.) and 2-min fractions are collected. After a 2-min reaction time at room temperature, they are stored at -20°C for several hours in hermetically sealed Eppendorf tubes.

CE-LIFD Analysis of Dialysates

CE-LIFD system. Capillary electrophoresis was performed on a Spectraphoresis 100 module purchased from Thermo Separation Products (Les Ulis, France) equipped with a LIF detector from Zeta Technology (Toulouse, France). The LIF detector is directly derived from the instrument described by Hernandez and coworkers (16), with some differences in lens position and the presence of a ball lens (17). The excitation was performed by an Omnichrome (Chino, U.S.A.) helium-cadmium laser (model 4056-30M) at a wavelength of 442 nm having a maximal output of 64 mW, while only 10 mW were used to perform the excitation. The emission intensity was measured at a wavelength of 490 nm. Fluorescence was detected by a photomultiplier tube and electrical current was generated.

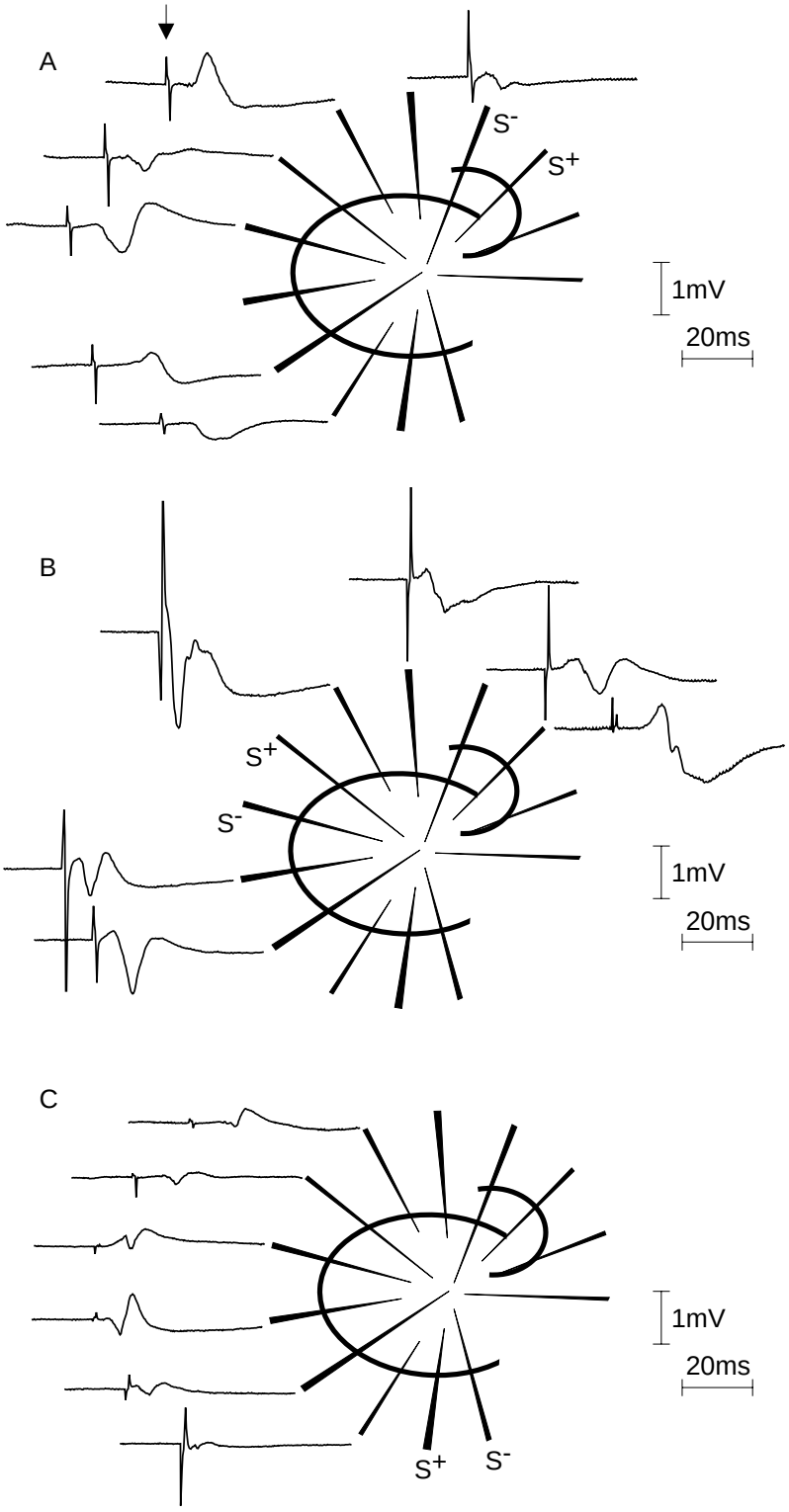
Separations were carried out with a fused-silica capillary of 50 µm i.d. and 375 µm o.d. having a total length of 70 cm and an effective length of 35 cm (defined as the length from the point of injection to the point of detection). On-column LIFD was carried out through a 5-mm-wide window opened by removing the polyimide cover of the capillary. The capillary was sequentially flushed (3 x 10 min) with 1 M, 0.1 M sodium hydroxide and water prior to use. Before each electrophoretic run, the capillary was flushed with the separation buffer (90 s). Hydrodynamic injections were made by applying vacuum (250 mbars) at the detection end of the capillary for a fixed period of time (0.5 s). The injection volumes were calculated according to the Hagen-Poiseulle formula (400 pL). The separation buffer used was 100 mM borate buffer (pH 9.2) and the running voltage was 30 kV. Peak identification was based on migration time and quantification by comparing peak area with standard solution.

Continuous flow derivatization system. The principle of this on-line derivatization system with very low dead volume has been previously described (14,18). Its construction was further simplified and consisted of four 10 cm x (105 µm o.d., 40 µm i.d.) fused-silica capillary tubes inserted into polyethylene tubing (300 µm i.d., 700 µm o.d., 1 cm long) (PE 10). The ends of the silica tubes were glued to the PE 10 with a 1-mm interval, 3 mm of the PE 10 tubing being empty and used as a reaction chamber. The dialysates were first mixed with 0.1 M perchloric acid containing the internal standard [10^{-5} M α-amino-adipic acid (AAD)], then with the sodium cyanide solution in borate buffer [prepared by mixing (40:100 vol.) a 43 mM cyanide solution in water with borate buffer (500 mM, pH 8.7)] and finally with the naphthalene-2, 3-dicarboxaldehyde (NDA; Fluka, Buchs, Switzerland) solution [2.5 mM in water:acetonitrile (50:50 vol.)]. The probe and the cyanide/borate solution were infused with 100 µL Hamilton syringes whereas perchloric acid and NDA solutions were infused with 50 µL Hamilton syringes. The respective perfusion flow rates were 1 µL/min, 0.2 µL/min, and 0.1 µL/min (perchloric acid and NDA). Prior to collection, equilibration of the derivatization system was performed for 10 min.

System performance. The method has been previously described (14,18) and was improved for this study. The detection limit was 40 nM for Glu (signal-to-noise ratio 2:1). Intra-assay reproducibility was assessed by performing a

F3

Electrophysiological responses obtained using the multi-electrode array of the Physiocard system as a function of the location of stimulation and recording electrodes in different areas of one hippocampal slice. (A) Stimulation electrodes in CA_4 area; (B) Stimulation electrodes in CA_3 area; (C) Stimulation electrodes in CA_1 area. Representative EvFPR obtained with the different recording electrodes are presented. Each one shows the stimulation artifact (arrow) and the biological response.



continuous flow derivatization of a standard solution containing 0.8 mM Glu (including AAD as the internal standard). Two-minute fractions were collected (n=7), stored at -20°C, and CE-LIFD analysis was performed to determine Glu concentration; the relative standard deviation was 2.8% for peak area (after correction with AAD).

Results and Discussion

Electrophysiological Responses of CNS Slices

Most of the known systems for in vitro electrophysiological recordings involved a recording chamber placed in a Faraday cage and needed a classic electrophysiological setup (antivibration table, head stage, amplifier, stimulation unit, isolation unit, oscilloscope, micro-manipulator...). Our technique simplifies those arrangements (see ***F1***), which are concentrated either in the B/E interface or in the electronic module.

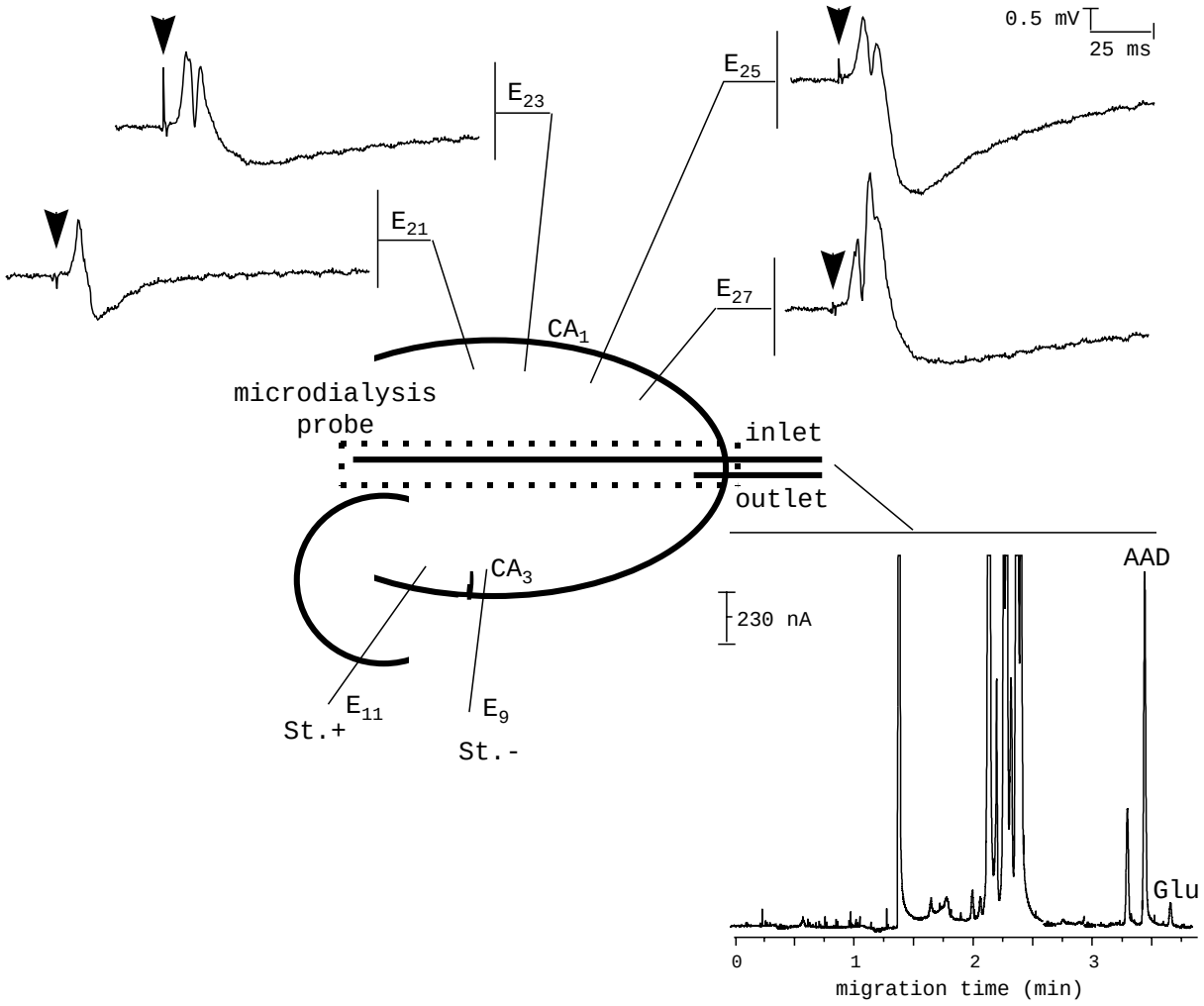
We used a multielectrode array and a recording chamber (part of the B/E interface) that can simultaneously fulfill biological and electrical requirements (chemically inert, sterilizable and transparent to allow visual control). The array showed no noticeable deterioration even after repeated use and allowed recording with a good signal-to-noise ratio.

With this system, one can perform simultaneous stimulation, continuous recording of neuronal activity for several hours, and visual assessment of the position of the electrodes. Both spontaneous field potentials responses and evoked field potentials responses (EvFPR) can be recorded with this system (***F3***). This device provides about the same results as with the classical extracellular electrophysiological setup, but more recordings — and thus a better monitoring of the events occurring in the slice — can be made. Both negative field potentials recorded in the dendritic areas and positive responses in the somatic layers were obtained in the CA_1 region by stimulation of a group of CA_3 cells, as previously reported (2,4). Basal EvFPR amplitude was typically in the 0.5-2.5 mV range.

Drug delivery can be performed by perfusion of the lower chamber and effects of the tested molecules on synaptic activity are automatically recorded and analyzed by a computerized data acquisition system. The system has been previously characterized using molecules with well-known effects on synaptic activity such as tetrodotoxine (blocker of Na^+ voltage de-

F4

General implantation scheme of electrodes and microdialysis probe on an organotypic slice culture using the Physiocard system with typical electropherogram and EvFPR recordings obtained under these conditions. The arrowheads indicate the stimulation artifacts.



pendent channels), dexamethasone (antiepileptic glucocorticoid), NBQX (kainate/AMPA receptor antagonist), bicuculline (GABA-A antagonist) and kainate (15). The effects are similar to those obtained by other research groups using conventional electrophysiological approaches.

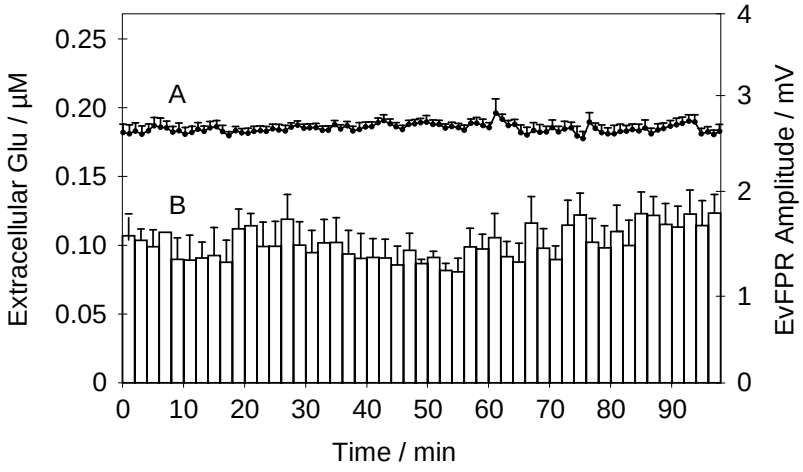
As an example of such applications, ***F3*** shows EvFPR recorded from the same one-week-old hippocampal organotypic slice culture and obtained with three different stimulation paradigms (***F3A***: stimulation in CA_4, ***F3B***: stimulation in CA_3, ***F3C***: stimulation in CA_1) and recordings in different regions of the hippocampus. This experiment was performed to demonstrate the variety of evoked signal recordings available when stimulation electrodes are changed during the experiment. Furthermore, it confirms that the synaptic delay (time from the end of the stimulation artifact to the origin of the signal) increases with the distance between the recording electrodes and the stimulation ones. This increase is probably due to differences in the cable properties of the tissue and in the propagation of signals along it.

Combined Microdialysis and Electrophysiology Recordings on CNS Slices

The laminar arrangement of the cell fields and fiber pathways in the hippocampal formation makes this brain region particularly suitable for the stimulation of specific pathways. Since CA_1 pyramidal cells receive the vast majority of their afferent inputs from CA_3 pyramidal cells via Schaffer collaterals, it is of particular interest, from an electrophysiological point of view, to stimulate in the CA_3 area while recording in the CA_1 region. Furthermore, the microdialysis probe can easily be implanted between these two regions (***F4***), in the apical dendritic field of CA_1, (i.e., in a synaptic region). With this arrangement, the release of neurotransmitters like Glu can be monitored after different stimulation patterns and/or simultaneously to the recording of synaptic responses of CA_1 neurons evoked by stimulation of the CA_3 area. These advantages are emphasized by the use of the Physiocard electrophysiological system. Several recording and stimulation electrodes, as well as microdialysis probes, can be placed under visual control in the desired areas of the hippocampal slice be-

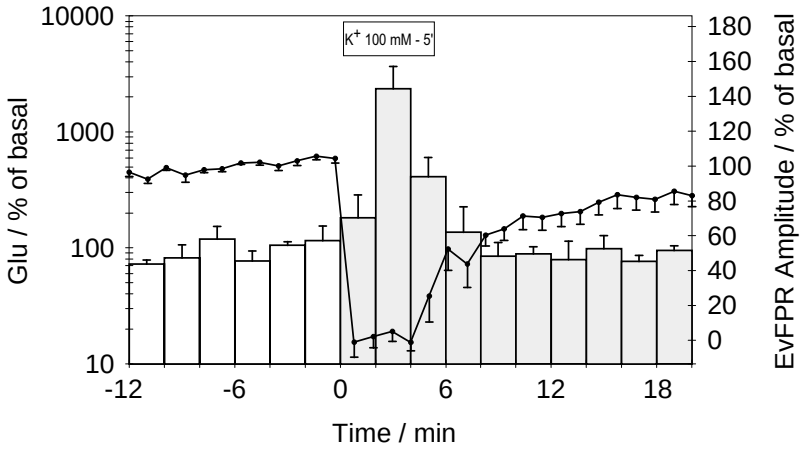
F5

Time course of Glu concentrations in 2-min fractions of hippocampus dialysates (A) and of EvFPR amplitudes (B) in basal conditions. Data are presented as means ± SEM (n=5 for Glu, n=11 for EvFPR). Each column represents Glu concentration in a 2-min fraction and each point the EvFPR amplitude recorded every minute.



F6

Effects of K^+-induced depolarisation on Glu concentrations in 2-min fractions of hippocampus dialysates and on EvFPR amplitude. A 5-min K^+-perfusion (100 mM) was performed. Glu concentrations are presented as logarithmic values. Means ± SEM of the baseline values: Glu, 8.1 ± 0.2x10^{-8} M (n=3), EvFPR amplitudes, 1.13 ± 0.24 mV (n=12). Each column represents Glu concentration in a 2-min fraction and each point the EvFPR amplitude recorded every minute.



cause of the transparency of this device. Finally, in contrast with a conventional electrophysiological setup, several extracellular recordings (usually 2-4 and up to 6) can be easily performed without disturbing the probe.

CE-LIFD analysis of 2-min hippocampal microdialysis samples showed peaks with migration times corresponding to those of Glu and AAD in standard solution (***F4***). Even if the CE-LIFD system allows the separation and detection of Asp in standard solutions, the extracellular basal levels of this excitatory amino acid were below detection during control conditions.

The amplitude of EvFPR was used as an indication of the overall electrical activity of neurons in the organotypic culture. Because of the use of the B/E interface and the electronic module, several simultaneous electrophysiological recordings can be easily obtained from different electrodes located in CA_1 (***F4***).

The concentrations of Glu collected from the hippocampus reached a stable baseline (about 10^{-7} M) within 45-60 min after implanting the microdialysis probe and remained stable throughout the experiment (***F5***). The amplitudes of EvFPR were also stable (about 2.5 mV) throughout the experiment (***F5***).

The perfusion of high-K^+ solution (100 mM for 5 min) induced an increase in extracellular Glu that peaked at +2 min (x25, n=3) and a strong decrease in EvFPR amplitude that was maximal (-99%, n=12) between +1 and +4 min becoming -25% after +20 min (***F6***). Temporal patterns of Glu output and EvFPR variations were thus almost similar. These results are in agreement with previous reports since high-K^+ perfusion induced both a blockade of action potential generation, leading to a decrease in EvFPR amplitude, and a reversal of Glu uptake, causing an increase in extracellular Glu concentrations (19).

In vivo simultaneous monitoring of neuronal activity and extracellular neurotransmitter concentrations has been previously described using a system with a microelectrode incorporated within a microdialysis probe which permits Glu determination and global neuronal activity to be monitored through the recording of both electroencephalogram and extracellular current potential (20-22). The present in vitro approach implies a more direct monitoring of synaptic responses in the different brain subregions, mainly because experiments were carried out on brain slices, allowing more precise positioning of the probe and electrodes under visual control.

An interesting in vitro approach combined electrophysiology and a push-pull cannula (24,25) to study acute hippocampal slices functioning with precise temporal monitoring (1- or 5-min), but this sampling technique requires high flow rates (25 to 50 µL/min). Since the dialysis membrane acts as a mechanical barrier to turbulence in the fluid flow, cell damage in the nervous tissue is greatly reduced using the microdialysis technique. However, at the present time, such a combination has been previously reported in a limited number of studies (25).

The reduction of microdialysis sample volumes needed for neurotransmitters analysis using CE-LIFD with a continuous flow derivatization system permits work with slow perfusion rates and/or an increase in temporal resolution. Up to 0.5-min fractions can be collected (18), a characteristic that allows for a better monitoring of the variations in extracellular neuro-

transmitters concentrations. Such a high sampling rate has been previously reported with on-line systems for DA monitoring using microbore HPLC-ED [1-min fractions; (26)], and for Glu and Asp monitoring using CE-LIFD [45-s to 2-min; (9,10)]. In addition to slow perfusion rate and high temporal resolution, the present CE-LIFD method enables the monitoring of different neurotransmitters in a single microdialysis sample without hampered temporal resolution (14). This is in contrast with conventional analytical methods like HPLC, in which such a simultaneous determination of neurotransmitters involves poor temporal resolution (typical sampling times of 10-30 min) or the use of fast perfusion rates (2-3 µL/min) in order to collect sufficient sample volumes for the two subsequent analyses (27-30).

Conclusion and Perspectives

The novelty of the present system derives from the combination of electrophysiology and microdialysis monitoring with a high sampling rate in an in vitro model of organotypic slice cultures. Until now, such a combination was performed either on acute slices or using superfusion or push-pull techniques for neurotransmitter collection. Physiocard's use of well-organized slices recovered from the dissection trauma, creates a system which mimics the in vivo situation and provides optimal conditions for slice survival. Moreover, because the multi-electrode array and the microdialysis probe can be simultaneously positioned, this system can continuously measure and analyze biochemical/electrophysiological activities with excellent time and spatial resolution and little tissue damage.

This system offers a unique opportunity to obtain information on nervous tissue in reaction to drug treatments or to physiological or pharmacological alterations. Various neurophysiological studies can thus be performed to investigate neural network mapping, post-lesion nervous recovery, delayed neuronal death, neurodegenerative disease, epilepsy, neurotoxicity, or neuropharmacology analysis.

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