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Dual asymmetric-flow microdialysis for *in vivo* monitoring of brain neurochemicals

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ABSTRACT

Microdialysis is an extensively used technique for both *in vivo* and *in vitro* experiments, applicable to animal and human studies. In neurosciences, the *in vivo* microdialysis is usually performed to follow changes in the extracellular levels of substances and to monitor neurotransmitters release in the brain of freely moving animals. Catecholamines, such as dopamine and their related compounds, are involved in the neurochemistry and in the physiology of mental diseases and neurological disorders. It is generally supposed that the brain's energy requirement is supplied by glucose oxidation. More recently, lactate was proposed to be the metabolic substrate used by neurons during synaptic activity. In our study, an innovative microdialysis approach for simultaneous monitoring of catecholamines, indolamines, glutamate and energy substrates in the striatum of freely moving rats, using an asymmetric perfusion flow rate on microdialysis probe, is described. As a result of this asymmetric perfusion, two samples are available from the same brain region, having the same analytes composition but different concentrations. The asymmetric flow perfusion could be a useful tool in neurosciences studies related to brain's energy requirement, such as toxin-induced models of Parkinson's disease.

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1. Introduction

Microdialysis is a technique for sampling the chemistry from most brain regions, tissues and organs with limited tissue traumas [1,2]. The idea of this technique, developed in 1972 [3] and popularized by Ungerstedt and Pycok [4], was to implant a thin dialysis tube into the tissue to mimic the function of a blood vessel artificial capillary, whereby substances may be both topically recovered from and supplied to a tissue [5]. Although based on a very simple principle, microdialysis is one of the most widely used techniques for *in vivo* and *in vitro* sampling of the chemical substances in extracellular fluids of animal tissues or cultured cells. The microdialysis probe employs the dialysis principle according to whom a semi permeable membrane, introduced into a tissue or placed into contact with a moist surface, separates two fluid compartments. The probe is perfused with a liquid and the low molecular weight compounds diffuse down their concentration gradients in both directions. The collected perfusion fluid can be analyzed with different analytical systems such as spectrophotometer [6], microsensors [7] or HPLC with electrochemical [8], UV and fluorescence [9] detectors. The sensitivity of the used analytical technique is the limit of microdialysis [10].

A prerequisite of many neurological studies is an accurate and continuous monitoring of *in vivo* concentrations of substances in the brain extracellular space. The combination of microdialysis with highly sensitive analytical techniques allows the measurement of a lot of neuroactive compounds [11].

Catecholamines and their related compounds are involved in the neurochemistry and in the physiology of mental diseases and neurological disorders.

A significant reduction of striatal content of dopamine (DA) [8], a catechol-like neurotransmitter implicated in cognitive functions [12] and in reward pathways [13], and of its catabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) [14] and 3-methoxytyramine (3-MT) [15], is a hallmark in Parkinson's disease [16,17]. Catecholamines biochemical dynamics have been studied extensively both *in vivo* and *in vitro* [18–23].

The mammalian brain is an expensive energy-consuming organ [24]. Most of brain's energy consumption is due to neuronal activity (about 80%) and other processes such as neurotransmitter recycling and axonal and dendritic transport [25]. At the moment, there is a controversy concerning the energy substrate used by the activated neurons and metabolic changes during neuronal activation [26,27]. Under normal physiological conditions, glucose is the major source

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of energy for the mammalian brain [26,28]. Magistretti and Pellerin [27] proposed that glucose is metabolized mainly in astroglial cells with production of lactic acid. Lactate is secreted in the extracellular space, promptly uptaked by neurons and reduced to pyruvate which is actively involved in ATP synthesis. Increased brain levels of pyruvate and lactate/pyruvate ratio, reflect, respectively, an increase in brain metabolic activity and a relative increase in anaerobic metabolism [29]. Several studies demonstrated that oxidative stress and mitochondrial dysfunction play an important role in the development of neurodegenerative diseases such as Parkinson's disease [30], Huntington's disease, Alzheimer's disease and cancer [31].

In this paper, a novel microdialysis approach for simultaneous monitoring of catecholamines, indolamines, glutamate and energy substrates in the striatum of freely moving rats, is described. The asymmetric perfusion represents an innovative perspective in microdialysis experiments, where the asymmetry of flow rate could be used as a tool to prepare (concentrate/dilute) the sample directly *in vivo* from the same cerebral site. This allows the quantification of different classes of neurochemicals, present in the collected samples, without any manipulation, using two different analytical systems. The two inlets of the dual microdialysis probe [17], once inserted into right striatum of a rat, were perfused with an artificial extracellular fluid using an asymmetric flow rate. The collected samples, resulting from the same brain region, were analyzed. Catecholamines, glutamate and indolamines were determined by LC while the energy substrates were quantified by ISCUS analyzer. This technique could be a useful tool in neurosciences studies related to neurochemical impairment and energy requirement [30,31].

2. Materials and methods

2.1. Chemicals and solutions

Glucose (Gluc), lactate (Lac), pyruvate (Pyr), ascorbic acid (AA), glutamate, uric acid (UA), L-DOPA, DOPAC, dopamine (DA), HVA, 5-HIAA, 3-MT, serotonin (5-HT), D-amphetamine (D-amph), K_2HPO_4, KH_2PO_4, EDTA, methanol (MeOH, HPLC grade) and sodium octylsulphate (OSA) were purchased from Sigma–Aldrich (Milano, Italy). Stock solution of AA, glutamate, catecholamines and indolamines were prepared in phosphoric acid and stored at −80 °C. UA was dissolved in MilliQ water (1 mg/10 ml) with 100 μl of NaOH 0.1 N. Immediately before use, the stock solutions were diluted in Ringer in order to obtain appropriate concentrations.

Glucose, lactate and pyruvate stock solutions (1 M) were freshly prepared dissolving drugs in water. D-Amph solution was freshly prepared dissolving drug in saline (0.9% NaCl) few minutes prior treatments.

The composition of the ringer solution used was as follows, in mM: NaCl 147.0, KCl 4.0, $CaCl_2$ 1.2, $MgCl_2$ 1.0 (pH 6.0).

2.2. Animals

Male Wistar rats, weighing 250–350 g, were used in all experiments. The animals were maintained under standard animal care conditions (12:12 h light/dark cycle, lights coming on at 7 a.m.; room temperature 21 °C), with food and water *ad libitum*. Before starting any experiment, the health of the rat was assessed according to published guidelines [32]. All procedures are specifically licensed under the European Community directive 86/609 included in Decreto No. 116/1992 of the Italian Ministry of Public Health.

2.3. Microdialysis probe

The striatal microdialysis probe has been developed by our research group [17] and combines two independent probes (P1 and P2) of concentric design (Fig. 1). The dual probe has two separate inlets with two corresponding separate outlets, which permits a separate sample collection from the same cerebral site. The probes were made using two sections of plastic-coated silica tubing (diameter 0.15 mm; Scientific Glass Engineering, Milton Keynes, UK) each placed in the centre of semi-permeable polyacrylonitrile dialysis fiber (molecular cut-off weight of 12 kDa, Filtral 16 Hospal Industrie, France). Each probe has a final diameter of 0.3 mm. The tips of the dialysis fibers were sealed and joined using quick-drying epoxy glue. The two sections of silica tubing serve as inlets; the outlets were also made with a section of plastic-coated silica tubing, positioned in the centre of the polythene tubing. The semi-permeable membrane will be coated with epoxy leaving an active length of 4 mm. The diameter of the final probe is approximately 0.6 mm.

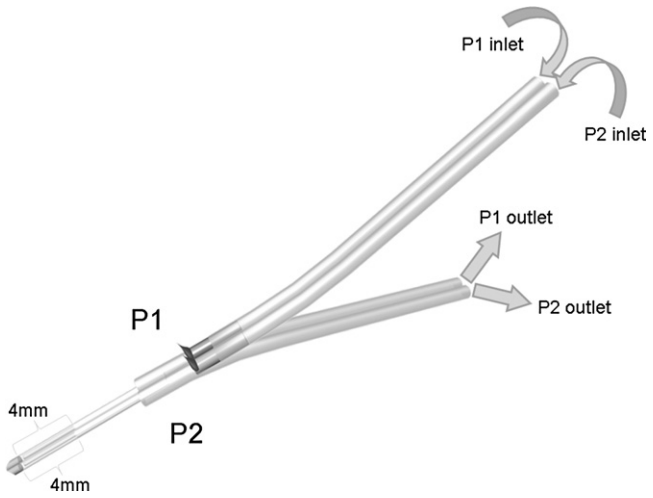

Fig. 1. Three dimensional drawing of a dual striatal probe with two separate inlets and two corresponding outlets. Each probe (P1 and P2) has a final diameter of 0.3 mm and an active length of 4.0 mm.

2.4. Stereotaxic surgery

Stereotaxic surgery was performed under chloral hydrate (400 mg/kg given intraperitoneally) anaesthesia. Microdialysis probes were implanted in the right striatum using the specific stereotaxic coordinates from the atlas of Paxinos and Watson [33]: A/P +0.5, M/L −2.5 from bregma and D/V −7.0 from dura. Body temperature during anaesthesia was maintained at 37 °C (by means of an isothermal-heating pad). Following surgery, the animals were placed in large Plexiglas cylinders (45 cm in diameter), and maintained in a temperature- and light-controlled environment, with free access to food and water. Experiments were carried out 24 h after probe implantation with the animal in its home bowl. This arrangement allows the rats free movement.

2.5. Microdialysis in vitro procedures

Before the probe was placed into the rat striatum, the experimental set-up was assembled as illustrated in Fig. 2 and an *in vitro* calibration was performed as follows: the microdialysis probe was placed in a mixed standard solution containing DA(1 μM), DOPAC (3 μM), HVA (3 μM) glutamate (10 μM), glucose (1 mM), lactate (1 mM) and pyruvate (1 mM). Each of the two microdialysis chambers (P1 and P2 in Fig. 2) was perfused with ringer solution by two microinfusion pumps (CMA/100, Solna, Sweden) at a maximum flow rate of 3.0 μl/min. The pump channels were connected to the probe inlets by a length of polyethylene tubing.

A first series of experiments were performed to obtain the best microdialysis set-up for the analysis of energy metabolism,

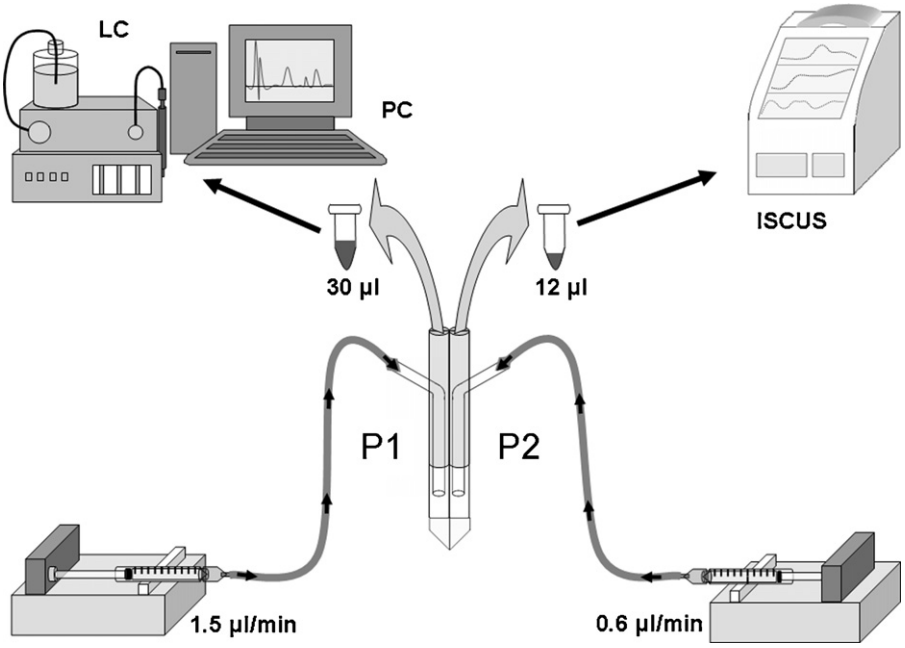

Fig. 2. Schematic representation of typical microdialysis experiment using the dual asymmetric flow. The asymmetric perfusion of dual probe, by two high precision pumps, resulted in two samples different in their concentrations and volumes. Samples were analyzed by mean LC-EC (catecholamines and indolamines), LC-FD (glutamate) and ISCUS Analyzer (glucose, lactate and pyruvate).

in relation with probe's recovery, sample volume and analytical system requirement. The dual probe was perfused with a ringer solution using the same flow rate on P1 and P2 sides. Five different rates were tested, 1.5–1.25–1–0.75–0.5 μl/min, and the relative recovery of each analyte was calculated. The first sample was collected after 30 min of stabilization, then 3 dialysates were collected, for each flow, every 20 min. The collected samples were analyzed by ISCUS Analyzer (CMA Microdialysis). A new mixed standard solution was used every time the flow was changed, in order to prevent oxidation events.

In order to evaluate the effect of a dual asymmetric flow on energy substrates recovery, continuous dialysis was performed fixing the flow rate on P1 side of the probe at 0.6 μl/min and varying the flow rate on P2 (0.5–0.75–1–1.25–1.5 μl/min). Relative recovery was calculated for each analyte. Two separate dialysate samples were manually collected in 250 μl micro-centrifuge tubes connected to the outlets. The first sample was collected after 30 min of stabilization (time 0), then dialysates were collected at 20 min intervals for 1 h. The collected samples were analyzed by ISCUS Analyzer (CMA Microdialysis). Again, a new mixed standard solution was used every time the flow was changed, in order to prevent oxidation events.

Similarly, to evaluate the effect of a dual asymmetric flow on catecholamines and glutamate, continuous dialysis was performed fixing the flow rate on P1 side of the probe at 1.5 μl/min and varying the flow rate on P2 (0.5–0.75–1–1.25–1.5 μl/min). Relative recovery was calculated for each analyte. The samples collected from P1 were analyzed by liquid chromatography with electrochemical detection (LC-EC) and with fluorimetric detection (LC-FD).

2.6. Microdialysis in vivo procedures

A parallel series of microdialysis experiments were carried out *in vivo* 24 h after striatal probe implantation as described above. Continuous dialysis was performed fixing the flow rate on P1 at 0.6 μl/min and varying the flow rate on P2 (1.5–1.2–0.9–0.6 μl/min). The collected samples were both quantified by ISCUS Analyzer and relative recovery was calculated. The first microdialysate was collected after 60 min of stabilization (time 0), then 3 dialysates were collected at 20 min intervals for each flow variation.

In a separate series of experiments, once defined and fixed the best-performing fluxes, a single dose of D-amph (2 mg/kg/2.5 ml) was administered intraperitoneally 60 min after the start of the microdialysis experiments. After 60 min of stabilization (time 0), dialysates were collected, at 20 min intervals, for 60 min prior to the start of experiments and for 3 h after systemic pharmacological treatments. Subsequently, microdialysates were stored in freezer (−70 °C) until analysis.

2.7. LC analysis of microdialysis samples

DA, 3-MT, DOPAC, HVA, AA and UA were quantified by high performance liquid chromatography with electrochemical detection (LC-EC) as previously described [17,34] using an Alltech 426 HPLC pump equipped with a Rheodyne injector (mod. 7725), a column 15 cm × 4.6 mm i.d. (Tosoh Haas ODS80TM C18), an electrochemical detector BAS mod. LC4B and a PC-based analog-to-digital converter system (Varian Star Chromatographic Workstation).

The LC-EC mobile phase was citric acid 0.1 M, Na acetate 0.1 M, EDTA 1.0 mM, MeOH 9% and sodium octylsulphate 50 mg/l (pH 2.9); the flow rate fixed at 1.3 ml min^{-1}.

Glutamate was quantified by LC with fluorimetric detection (at 360 and 450 nm – excitation and emission wavelengths) using a Varian 9010 gradient system following precolumn derivatization by *o*-phthaldialdeyde. Derivatized glutamate was separated on Ultrasphere C 18 3 μm high speed 75 mm × 4.6 mm Beckman column. A gradient was run from 25 mM Na_2HPO_4–acetonitrile (95:5) pH 6.6 with H_3PO_4 to 25 mM Na_2HPO_4–acetonitrile (70:30) in 20 min at a flow rate of 1.2 ml min^{-1}.

2.8. Spectrophotometric analysis

The micro-spectrophotometric determination of glucose, lactate and pyruvate was performed in 10 min by the ISCUS Clinical Microdialysis Analyzer (CMA Microdialysis). ISCUS is a selective

analyser designed for small samples volumes obtained when sampling with microdialysis. Analytes are enzymatically oxidized by an oxidase (GOx, LOx, POx) into byproducts and hydrogen peroxide: peroxidase (POD) catalyzes the colorimetric reaction with hydrogen peroxide formed, reagent (phenol, 4-chloro-phenol, TOOS) and 4-amino-antipyrine (4-AA) quinoneimine producing a colored compound red-violet. The absorbance of the colored compound is read at a wavelength of 530 nm and compared with a standard for determining the concentration of analytes in the dialysate.

2.9. Histology

Following the experiments, rats were sacrificed with an overdose of chloral hydrate (800 mg/kg intraperitoneally). The location of each microdialysis probe was confirmed by post-mortem histology. Brains were fixed in 10% formal saline solution and 50 μm coronal sections were made with a cryostat. The slices were stained with cresyl violet and examined under a microscope.

2.10. Statistic analysis

The concentrations in the dialysate were expressed in nM (DA, 3-MT), μM (AA, UA, DOPAC, HVA, glutamate and pyruvate) or mM (glucose and lactate) and given as mean ± SEM. The percent recovery of the probe (for every neurochemical in the mixed standard solution) was calculated in each sample using the following formula:

$$\text{Recovery}\ (\%) = \frac{[\text{microdialysate}]}{[\text{calibration solution}]} \times 100 \quad (1)$$

then averaged ($n = 3$) and given as mean ± SEM. Calibration results were plotted as microdialysate concentrations or percent recovery *vs*. perfusion flow rate; Pearson's correlation and linear regression were calculated.

Drug effects on neurochemicals were statistically evaluated in terms of changes in absolute dialysate concentrations. Statistical significance was assessed using analysis of variance (ANOVA) for differences over time. Statistical differences were determined by paired *t*-tests with Newman–Keuls multiple comparison adjustment. *P* values equal to or less than 0.05 were considered significant.

3. Results

3.1. Experimental in vitro characterization and optimization of the dual asymmetric flow

Preliminary studies were carried out in order to investigate the optimal perfusion flow rate and the recovery capability of the probe for catecholamines, glutamate and energy substrates. The i*n vitro* characterization of the dual asymmetric flow was attained placing the probe in a mixed standard solution as described in Section 2.5. A three-point calibration was performed on five different flow rates ranging from 1.5 μl/min to 0.5 μl/min (0.25 μl/min decrement) in order to obtain the recovery dependence on flow rate for glucose (13.71–42.83%), lactate (21.68–53.39%) and pyruvate (11.28–46.59%). As illustrated in Fig. 3, the relative recovery was inversely correlated with flow rate (Pearson *r*: −0.986 for Gluc, −0.995 for Lac and −0.999 for Pyr) and showed an excellent linear response ($r^2 = 0.989$ for Gluc, $r^2 = 0.991$ for Lac and $r^2 = 0.994$ for Pyr) in the studied range. In the present study the perfusion flow rate for energy metabolism compounds was fixed at 0.6 μl/min, which combines the required volume sample for ISCUS spectrophotometric analysis (12 μl/20 min) and the best recovery values (41.1 ± 1.23% for glucose, 52.3 ± 2.12% for lactate and

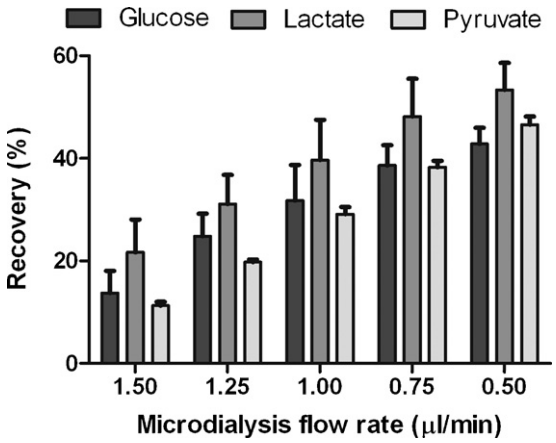

Fig. 3. Energy substrates recovery dependence on perfusion flow rate. A three-point calibration was performed using five different flow rates (steps) from 1.5 μl/min to 0.5 μl/min and relative recovery for each compound was calculated at every step resulting inversely correlated with the flow rate.

45.1 ± 1.11% for pyruvate), also in agreement with *in vivo* results (see next paragraph).

A second series of *in vitro* experiments were carried out in order to evaluate the effect of different combined flows on recovery capability of the probe. During the experiment the perfusion flow rate on P1 side of the microdialysis probe was held steady, while on P2 side was modified (ranging between 0.5 and 1.5 μl/min) and the relative recovery on P1 side was calculated for every rate.

Fig. 4 shows the effect of an asymmetric perfusion on relative recovery of catecholamines, glutamate (Fig. 4A) and energy metabolism (Fig. 4B). Catecholamines and glutamate recovery was achieved fixing the flow rate on P1 side at 1.5 μl/min and relative recovery was calculated ranging from 30.03% to 28.6% for dopamine, from 25.8% to 24.9% for DOPAC, from 25.6% to 24.6% for HVA and from 30.8% to 28.7% for glutamate, in agreement with the literature when using a 4 mm dialysis membrane [35]. Relative recovery of energy metabolism compounds was obtained fixing at 0.6 μl/min rate on P1 side and changing the perfusion rate on P2. It ranged, respectively, from 41.1% to 40.8% for glucose, from 52.0% to 51.8% for lactate and from 45.1% to 44.8% for pyruvate. The same procedures, performed on P2 side, confirmed the obtained results on P1 side (data not shown). Alteration of perfusion flows, in all tested combinations, did not affect the recovery capability of the opposite sides of the probe, that, acting as two separate entities, are not mutually influenced.

In vitro recovery values of all analytes at 1.5 μl/min and 0.6 μl/min were calculated on each dialysis device prior to *in vivo* experiments.

3.2. In vivo validation of dual asymmetric flow

In vivo tests were performed in order to confirm the independence between the two side of the probe and to obtain the best flow rate perfusion concerning energy metabolism analytes in microdialysis experiments. Fig. 5 shows flow rate dependence of concentrations of glucose, lactate and pyruvate recorded in the striatum of freely moving rats ($n = 3$). As described in materials and methods paragraph, the microdialysis experiments were carried out on freely moving rats 24 h after striatal probe implantation, fixing the perfusion flow rate on P1 side at 0.6 μl/min and changing the rate on P2 side from 1.5 μl/min to 0.6 μl/min with a 0.3 μl/min decrement. Absolute values of glucose, lactate and pyruvate, calculated as mean ± SEM, were inversely correlated with flow rate and ranging, respectively, between 0.256 ± 0.004 mM and 0.626 ± 0.004 mM for Gluc ($r^2 = 0.945$); 0.348 ± 0.006 mM

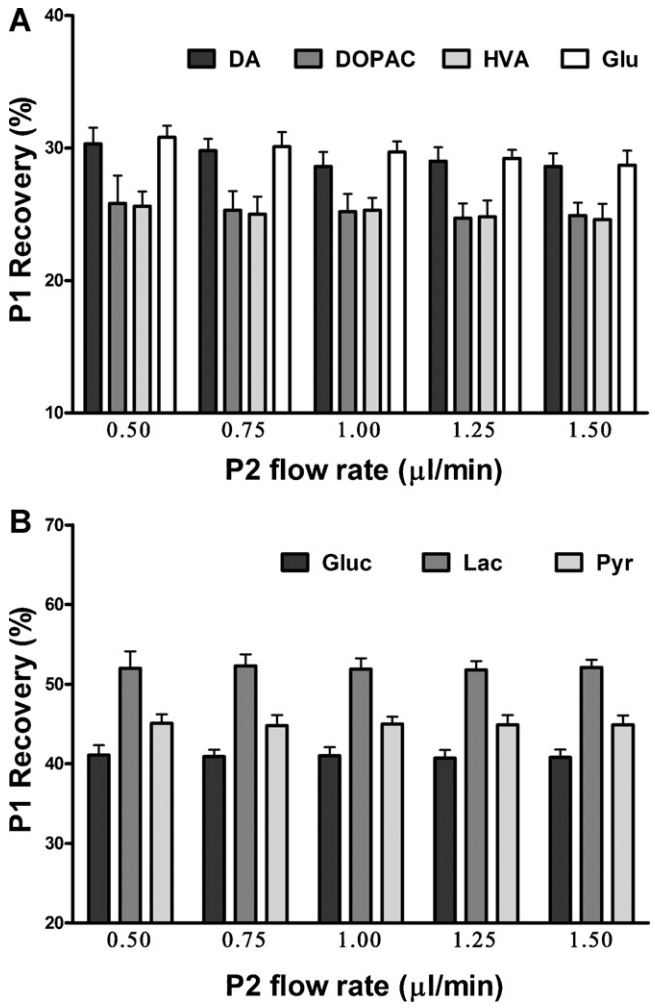

Fig. 4. Effect of dual asymmetric flow on relative recovery of catecholamines, glutamate (panel A) and energy substrates (panel B). Continuous dialysis was performed holding steady the perfusion flow rate on P1 side of the probe and varying the flow rate on P2. Catecholamines, glutamate and energy substrates determination were performed fixing the flow rate at 1.5 μl/min and 0.6 μl/min, respectively. Changes of flows, in all tested combinations, did not affect the recovery capability of the two sides of the probe.

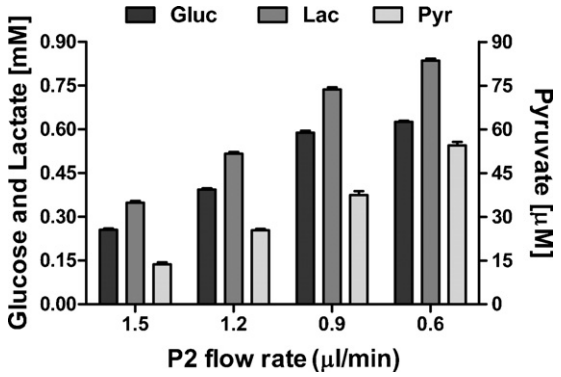

Fig. 5. *In vivo* flow rate dependence of striatal concentrations of glucose, lactate and pyruvate in freely moving rats. The flow was held steady at 0.6 μl/min on P1 and varied on P2. The recovery on P2 was inversely correlated with flow rate (ranging, respectively, between 0.256 ± 0.004 mM and 0.626 ± 0.004 mM for Gluc, 0.348 ± 0.006 mM and 0.835 ± 0.007 mM for Lac; 13.67 ± 0.72 μM and 54.45 ± 1.24 μM for Pyr).

and 0.835 ± 0.007 mM for Lac (r^2 = 0.979); 13.67 ± 0.72 μM and 54.45 ± 1.24 μM for Pyr (r^2 = 0.981) (Fig. 5).

Although the recovery of P2 samples decreased with increasing flow rate perfusion, no statistically significant alteration in analytes concentrations was observed on P1 samples when P2 flow was modified. At 0.6 μl/min fixed flow rate recorded values (n = 12) were: glucose 0.623 ± 0.004 mM, lactate 0.841 ± 0.006 mM and pyruvate 54.23 ± 1.72 μM.

3.3. Baseline levels and D-amphetamine induced changes on striatal DA, 5HT and their metabolites, AA, UA and glutamate.

Three microdialysis samples were collected after a period of stabilization (1 h) and before D-amphetamine administration. Baseline levels of striatal neurochemicals were calculated as mean ± SEM of data obtained from all animals used in this study. For each compound baseline values were respectively: AA 5.18 ± 0.38 μM, UA 1.70 ± 0.10 μM, DOPAC 0.97 ± 0.03 μM, DA 4.27 ± 0.17 nM, 5-HIAA 0.79 ± 0.03 μM, HVA 1.14 ± 0.06 μM, 3-MT 3.62 ± 0.19 nM and glutamate 5.47 ± 0.14 μM. No baseline levels of NE, L-DOPA and 5-HT were detected in striatal microdialysates.

A single dose of D-amph were systemically (2 mg/kg i.p., n = 3) administered to rats. D-Amphetamine increased (P < 0.05) dialysate levels of DA (maximum increase to 2506% of baseline after 100 min), 3-MT (maximum increase to 2454% of baseline after 120 min), AA (maximum increase to 302% of baseline after 140 min) and UA (maximum increase to 202% of baseline after 180 min). Conversely, D-amphetamine decreased (P < 0.05) dialysate levels of DOPAC + HVA (maximum decrease to 70% of baseline after 200 min) and glutamate (maximum decrease to 63% of baseline after 200 min, Fig. 6D) while 5-HIAA levels were unaffected (data not shown).

3.4. Baseline levels of striatal glucose, lactate, pyruvate and effects of systemic administration of D-amphetamine on energy metabolism

As shown in Fig. 6 spectrophotometric analysis of striatal dialysate revealed an immediate change in the levels of glucose (Fig. 6A), lactate (Fig. 6B) and pyruvate (Fig. 6C) after systemic administration of D-amph.

Baseline levels of striatal energy metabolism analytes, calculated as mean ± SEM of data obtained from four microdialysis samples collected after a period of stabilization (1 h) and before D-amph administration, were respectively: 0.372 ± 0.01 mM for glucose; 0.653 ± 0.02 mM for lactate and 82.06 ± 3.05 μM for pyruvate. A single dose of D-amphetamine (2 mg/kg i.p.) produced a statistically significant increase (P < 0.05) on dialysates levels of glucose, showing a maximum increase to 160% of baseline values after 140 min, and on dialysates of lactate and pyruvate with a maximum increase, respectively, to 177% and 179% of baseline values after 120 min.

4. Discussion

4.1. The dual asymmetric flow

Microdialysis is a technique whereby substances may be both recovered from and supplied to a tissue. According to microdialysis theory, different resistances that substances encounter by diffusing back and forth across the membrane *in vitro* [36–39] and *in vivo* [40], leads to a underestimation of the real *in vivo* concentration of the studied analytes.

The recovery of substances can be further distinguished in two types: absolute recovery (positively correlated with the flow rate)

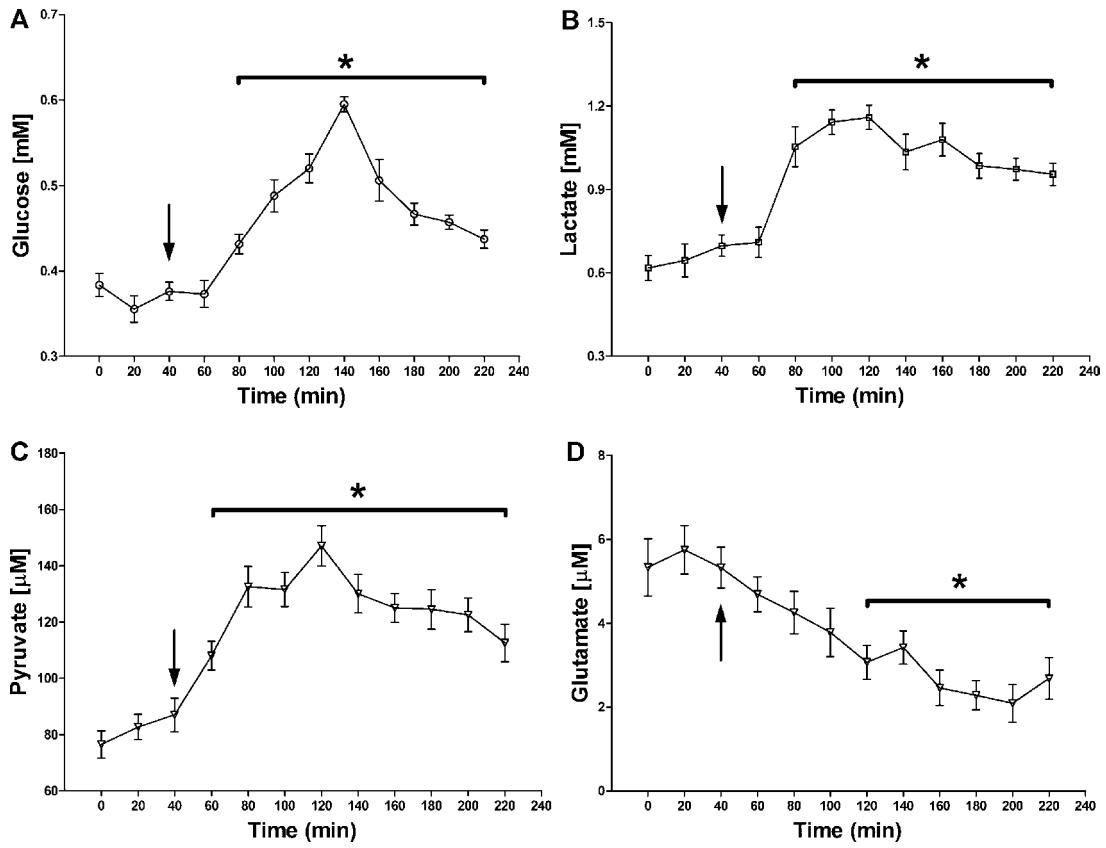

Fig. 6. Effect of systemic administration of a single dose of D-amphetamine (2 mg/kg) on striatal levels of glucose (A), lactate (B), pyruvate (C) and glutamate (D). After intraperitoneal drug administration (arrows), a statistically significant increase ($^*P < 0.05$) of levels of energy metabolism compounds on dialysates were observed, showing a maximum increase of +160%, +177% and +179%, respectively, for glucose, lactate and pyruvate compared to baseline values. A statistically significant decrease (−63% of baseline values, $^*P < 0.05$) of glutamate was observed.

and relative recovery (inversely correlated with the flow rate). The increase of the recovery is a way to compensate the microdialysis limit, usually set by the sensitivity of the analytical technique [10]. High perfusion flow rate results in an increase of the absolute recovery [41] and is useful if an analytical technique with a good sensitivity is used (LC-EC, LC-FD). On the other hand, when a less sensitive analytical technique is used (spectrophotometric method, ISCUS) a reduction of the flow rate results in an increase of the relative recovery, i.e. the concentration of the sample [5].

In our study, a striatal probe (Fig. 1) which combines two independent microdialysis probes and may permit separate dialysate sample collection from the same intrastriatal site, was used. The two separate inlets and two separate outlets allow asymmetrical flow rate perfusion between the P1 and P2 sides of the probes during the microdialysis experiment, thus resulting in two samples with the same analytes composition but different in their concentrations and volumes as well as required by the analytical system used for their determination. *In vitro* and *in vivo* tests were performed in order to evaluate the effect of different combined flows on recovery and to achieve the recovery capability of the probe for catecholamines, glutamate and energy substrates. The different combinations of flow rate studied did not affect the recovery capability of the two sides of the probe, that result not mutually influenced and act as two separate entities. The best performing asymmetrical flow rate combination for *in vitro* and *in vivo* microdialysis experiment resulted 1.5 μl/min for catecholamines and glutamate and 0.6 μl/min energy substrates, when a 4 mm dialysis membrane (the longest possible membrane for striatal implant) and 20 min sample rate (the longest permissible sample time in order to minimize oxidative events) were used.

When experimental conditions are completely under control, in particular the concentration of neurochemicals, the *in vitro* results showed a proportional increase of relative recovery lowering the perfusion flow rate. On the contrary, striatal glucose and lactate did not increase proportionally when lowering the flow rate from 0.9 to 0.6 μl/min suggesting that *in vivo* conditions are only in part related to the microdialysis setup. Because of the concentration of neurochemicals in the extracellular compartment is actively controlled by neural cells (mainly neurons and astrocites in physiological conditions), the explanation of these fluctuations is not so simple and further studies are necessary in this direction.

4.2. Striatal DA, 5HT and their metabolites, AA, UA and glutamate changes after systemic administration of D-amphetamine

In the present study, systemic administration of D-amphetamine (2 mg/kg i.p.) increased DA and decreased its acidic metabolite levels in striatal dialysate from freely moving rats. As known, D-amphetamine increases DA release in terminal fields of dopaminergic neurons. Both newly synthesized and vesicular DA are released by D-amphetamine into the striatal extracellular compartment [42,43]. The increase in released DA and the decrease in DOPAC and HVA extracellular levels are in agreement with previous results in freely moving rats [9,44]. The increase in 3-MT extracellular levels [20] and behavioural changes [9,45] further confirmed DA release. D-Amphetamine also decreases DA oxidative metabolism by inhibiting MAO [43] and depletion of newly synthesized DA, the main substrate for MAO [46]. XO activity is strictly related to DA oxidative metabolism [47].

$O_2^{\bullet -}$ generated by XO participates in the enzymatic DA oxidation [9,48].

After D-amphetamine i.p. administration, an increase in extracellular UA and a concomitant decrease in DOPAC has been observed (data not shown); it is likely that D-amphetamine does not increase XO metabolism, but simply mobilizes UA from intraneuronal pools [49] acting at nigral sites [9]. In agreement with the literature [50], systemic D-amphetamine administration did not affect serotonin oxidative metabolism (5-HIAA). D-Amphetamine increased AA and decreased glutamate concentrations (data not shown). These findings are consistent with the functioning of an AA/GLU heteroexchange system [51,52], in which AA release is linked to impulse traffic, transmitter release and GLU uptake [9].

4.3. Striatal energy metabolism changes after systemic administration of D-amphetamine

A statistically significant increase of glucose, lactate and pyruvate levels on striatal dialysates of freely moving rats was observed after intraperitoneally administration of D-amphetamine (2 mg/kg i.p.) compared to baseline values. The oxidation of glucose supplies the brain's energy requirement [53], then the determination of its concentration in the extracellular fluid (ECF) of the brain is important for evaluation of brain's energetic and metabolism. Brain ECF glucose concentration remains controversial and varies if obtained from awake or anesthetized animals. The concentration of glucose in the ECF of awake, freely moving rats varies by brain area studied and depends on several factors such as the area being measured and the level of brain activity in that area at time of measurement [54]. Different studies, using zero-net-flux and other methods, report glucose concentration in brain's ECF ranging between 0.35 and 3.3 mM [55–61]. Stimuli such as tail pinch or restraint, induce motor activity and leads to a physiological striatal activation in rodents. Glutamate uptake into astrocytes, during synaptic activity, leads to increased glucose use and then in glycolysis in these cells, resulting in lactate production and its release to extracellular fluid. Hence, lactate was taken up by neurons and used as energy substrate in Kreb's cycle after its conversion to pyruvate. This hypothesis, though controversial and not yet fully accepted, is known as astrocyte-neuron lactate shuttle (ANLSH) and was proposed by Magistretti and Pellerin [27]. It was also shown that intracellular ascorbic acid stimulates lactate uptake by neurons and inhibits glucose use in these cells [24].

5. Conclusion

We proposed an innovative microdialysis approach for the simultaneous monitoring of catecholamines, indolamines, glutamate and energy substrates in freely moving animals using a different perfusion flow rate on microdialysis probe, resulting in two samples from the same brain region that will be analyzed by two different methods. The asymmetric flow could be useful to correlate striatal neurotransmitter levels, such as DA depletion, with brain energy requirement in toxin-induced models in rodents of Parkinson's disease, such as MPTP, 6-hydroxydopamine or alpha synuclein.

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