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Changes in diffusion parameters, energy-related metabolites and glutamate in the rat cortex after transient hypoxia/ischemia

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Abstract

It has been shown that global anoxia leads to dramatic changes in the diffusion properties of the extracellular space (ECS). In this study, we investigated how changes in ECS volume and geometry in the rat somatosensory cortex during and after transient hypoxia/ischemia correlate with extracellular concentrations of energy-related metabolites and glutamate. Adult male Wistar rats ($n = 12$) were anesthetized and subjected to hypoxia/ischemia for 30 min (ventilation with 10% oxygen and unilateral carotid artery occlusion). The ECS diffusion parameters, volume fraction and tortuosity, were determined from concentration–time profiles of tetramethylammonium applied by iontophoresis. Concentrations of lactate, glucose, pyruvate and glutamate in the extracellular fluid (ECF) were monitored by microdialysis ($n = 9$). During hypoxia/ischemia, the ECS volume fraction decreased from initial values of 0.19 ± 0.03 (mean ± S.E.M.) to 0.07 ± 0.01 and tortuosity increased from 1.57 ± 0.01 to 1.88 ± 0.03. During reperfusion the volume fraction returned to control values within 20 min and then increased to 0.23 ± 0.01, while tortuosity only returned to original values (1.53 ± 0.06). The concentrations of lactate and glutamate, and the lactate/pyruvate ratio, substantially increased during hypoxia/ischemia, followed by continuous recovery during reperfusion. The glucose concentration decreased rapidly during hypoxia/ischemia with a subsequent return to control values within 20 min of reperfusion. We conclude that transient hypoxia/ischemia causes similar changes in ECS diffusion parameters as does global anoxia and that the time course of the reduction in ECS volume fraction correlates with the increase of extracellular concentration of glutamate. The decrease in the ECS volume fraction can therefore contribute to an increased accumulation of toxic metabolites, which may aggravate functional deficits and lead to damage of the central nervous system (CNS).

Keywords: Hypoxia; Extracellular space; Microdialysis; Ischemia; Diffusion

The diffusion of neuroactive substances through the extracellular space of the CNS is the underlying mechanism of extrasynaptic (volume) transmission, which is an important mode of communication between nerve cells [1]. Diffusion in the ECS obeys Fick's law but is constrained by two factors: extracellular volume fraction α, which is the ratio of the ECS volume to total tissue volume and tortuosity λ, a parameter describing the impact of tissue geometry on diffusion compared to a free diffusion medium. Tortuosity is defined as $\lambda = (D/\mathrm{ADC})^{1/2}$, where ADC is the apparent diffusion coefficient in the brain and D is the free diffusion coefficient. The absolute values of the ECS difusion parameters can be determined by the real-time iontophoretic method using tetramethylammonium (TMA^+)-selective microelectrodes [15,16].

It has been shown that many pathological states result in changes in extracellular space volume and geometry, significantly affecting signal transmission [22,23]. Among those of major clinical relevance and experimental interest are conditions leading to brain hypoxia or ischemia. Acute hypoxia or ischemia, and also some other acute neurological disorders that involve cell membrane depolarization (cortical spreading depression, status epilepticus and hypoglycaemia), cause excessive transmembrane ionic shifts that are accompanied by the movement of water from the extracellular to the intracellular compartment (cytotoxic edema). Rapid cellular swelling inevitably results in a shrinkage of the ECS, the impaired diffusion of substances

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through the ECS and the greater accumulation of toxic metabolites. In turn, these consequences can contribute to functional deficits and CNS damage. Experimentally, ischemia-evoked changes in the ECS diffusion parameters in the brain cortex in vivo have, so far, been studied only in a model of global anoxia induced by cardiac arrest. These studies have revealed a dramatic decrease in ECS volume fraction and an increase in tortuosity, occurring only a few minutes after the interruption of the blood supply to the brain [25,27]. In the present study, we have examined the ECS diffusion parameters in the somatosensory cortex of adult rats during transient hypoxia combined with unilateral common carotid artery occlusion and also during subsequent reperfusion. The obtained data were correlated with changes in the energy-related metabolites lactate and glucose, the lactate/pyruvate-ratio and glutamate, monitored by intracerebral microdialysis.

Adult male Wistar rats (300–350 g) were anesthetized by an intraperitoneal injection of urethane (1.5 g/kg, Sigma-Aldrich Chemie GmbH, Seelze, Germany). The animals were intubated and connected to a ventilator (CIV 101, Columbus Instruments, Columbus, OH, USA), relaxed with pancuroniumbromide (0.6 mg/kg, Pavulon, Organon, Netherlands), and ventilated with air. The body temperature was maintained at 36–37 °C by a heating pad. The somatosensory cortex of the rat was partially exposed by a burr hole 2–3 mm caudal from the bregma and 2–3 mm lateral from the midline. A transient hypoxia/ischemia of 30 min duration was induced by reducing the inspiratory oxygen content to 10% (in nitrogen) and unilateral clamping of the common carotid artery. Following the hypoxic period, the animals were again ventilated with air ($pO_2 = 21\%$). The control animals were sham-operated and ventilated with air throughout the experiment. In order to measure in the ipsilateral somatosensory cortex, diffusion and microdialysis measurements were not performed simultaneously.

All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were carried out in accordance with the European Communities Council Directive of 24.November 1986 (86/609/EEC) and approved by the local Institutional Animal Ethics Committee.

The ECS diffusion parameters were studied by the real-time iontophoretic method described in detail previously [13]. Briefly, an extracellular marker that is restricted to the extracellular compartment, such as tetramethylammonium ions (TMA^+, MW = 74.1 Da) to which cell membranes are relatively impermeable, is released into the extracellular space by iontophoresis and its local concentration measured with a TMA^+-selective microelectrode (TMA^+-ISM) located about 100–200 μm from the release site. The concentration of TMA^+ in the ECS is inversely proportional to the ECS volume. Double-barrelled TMA^+-ISMs were prepared by a procedure described in detail previously [21]. The tip of the ion-sensitive barrel was filled with a liquid ion exchanger (Corning 477317); the rest of the barrel was backfilled with 150 mM TMA^+ chloride. The reference barrel contained 150 mM NaCl. The shank of the iontophoretic pipette was bent so that it could be aligned parallel to that of the ion-selective microelectrode and was backfilled with 150 mM TMA^+ chloride. An electrode array was made by gluing a TMA^+-ISM to an iontophoretic micropipette with a tip separation of 100–200 μm. The iontophoresis parameters were +20 nA bias current (continuously applied current to maintain a constant electrode transport number), and a +180 nA current step of 24 s duration, to generate the diffusion curve. The TMA^+ diffusion curves were generated at regular intervals of 5 min. Before tissue measurements, diffusion curves were first recorded in 0.3% agar (Sigma-Aldrich, Steinheim, Germany) dissolved in a solution containing 150 mM NaCl, 3 mM KCl, and 1 mM TMACl. The diffusion curves were analysed to obtain the electrode transport number (n) and the free TMA^+ diffusion coefficient (D) by curve-fitting according to a modified diffusion equation using the VOLTORO program [15]. Diffusion curves were then recorded in the somatosensory cortex at a depth of 1200–1500 μm. Knowing n and D, the values of extracellular volume fraction α and tortuosity λ could be obtained from the diffusion curves.

The technique of microdialysis is based on sampling fluid via a double-lumen probe with an integrated semipermeable membrane in which the equilibration of substances in the extracellular space and perfusion fluid takes place by diffusion according to the concentration gradient. We used a double-lumen microdialysis probe with a membrane length of 2 mm, an outer diameter of 0.5 mm and a cut-off at 20,000 Da (CMA 12, 2 mm membrane length, CMA Microdialysis, Sweden). The inserted microdialysis catheter was connected by low-volume fluorinated ethylene propylene (FEP)-tubing (1.2 μl/10 cm) to a precision infusion pump (CMA 102, CMA Microdialysis, Sweden) in order to maintain a constant dialysate flow. The microdialysis catheter was continuously perfused with a dialysate containing 147 mmol/l NaCl, 2.7 mmol/l KCl, 1.2 mmol/l $CaCl_2$ and 0.85 mmol/l $MgCl_2$ (Perfusion fluid CNS, CMA Microdialysis, Sweden) at a flow rate of 2 μl/min. After a stabilisation period of 60 min following insertion into the brain, microdialysate samples were collected in 10 min intervals and immediately frozen at −40 °C until analysed. Thawed and centrifuged dialysate samples were analysed enzymatically with a CMA 600 Microdialysis Analyser (CMA/Microdialysis, Sweden) for lactate, pyruvate, glucose and glutamate concentrations.

The exchange of substances across the microdialysis membrane is limited by the total area of the membrane, the perfusion flow rate, the characteristics of the diffusing substance and the diffusion constant in the tissue surrounding the probe [24]. The recovery rate expresses the relation between the concentration of the substance in the microdialysis probe effluent and the concentration in the medium [14]. Before and at the end of the experiments, the recovery rates for each probe were determined by continuing the perfusion at the same settings in a calibration solution containing known concentrations of the different analytes. The calibration solution contained 2.50 mmol/l lactate, 250 μmol/l pyruvate, 5.55 mmol/l glucose, 250 mmol/l glycerol and 25 μmol/l glutamate (Calibrator, CMA Microdialysis, Sweden). The concentrations in the calibration solution were compared with the concentrations of the in vitro microdialysis samples to determine the relative recovery for each substance. The measured experimental values were weighted by the relative recovery to estimate the in vivo extracellular concentration of the substances in the immediate vicinity of the probes. In

vitro recovery rates were 24.3 ± 1.6% for lactate, 23.1 ± 0.6% for pyruvate, 13.7 ± 0.8% for glutamate and 14.1 ± 0.8% for glucose ($n = 14$). All results are presented as weighted concentrations.

The concentration of a metabolite in the extracellular fluid is clearly affected by changes in the extracellular space volume fraction. A decrease in α would, in the absence of any changes in metabolite supply or utilization, result in an increase in the measured metabolite concentration. Similarly, an increase in α would cause the measured metabolite concentration to decrease. To take into account the effects of changes in α, we have expressed our results as both the actual measured metabolite concentrations and also as the concentrations corrected for changes in α relative to its pre-hypoxic/ischemic baseline values. However, the physiological concentrations are those without a correction factor.

The results of the experiments are expressed as the mean ± standard error of the mean (S.E.M.). Differences within and between groups were evaluated using Student's paired *t*-test. Values of $p < 0.05$ were considered significant.

The mean values of extracellular volume fraction α and tortuosity λ during normoxia were $\alpha = 0.19 \pm 0.03$ and $\lambda = 1.57 \pm 0.01$ ($n = 12$, mean ± S.E.M.), which are similar to the values observed in rat cortex previously [13,27]. During 30 min of hypoxia-ischemia, α gradually decreased, reaching a minimum of 0.07 ± 0.01 at the end of the hypoxic/ischemic insult (Fig. 1A). The tortuosity simultaneously increased to 1.88 ± 0.03 (Fig. 1B). After the release of carotid artery occlusion and the beginning of normoxic ventilation, both α and λ started to return to normal values, reaching them within 20 min of the recovery period. During the next 20 min, α continued to increase to 0.23 ± 0.01 while λ decreased to 1.53 ± 0.06, then both parameters remained unchanged at these levels until the end of the 90-min recovery phase (Fig. 1A and B).

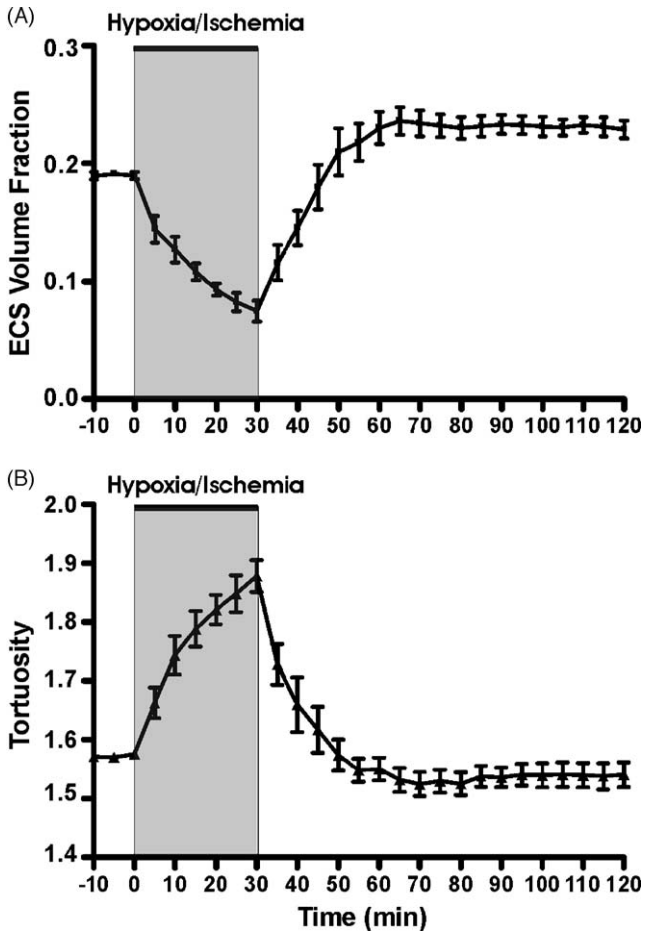

Fig. 1. The time course of changes in extracellular space volume fraction α (A) and tortuosity λ (B) during transient hypoxia/ischemia and subsequent reperfusion.

After a stabilisation period of 60 min following probe insertion, the basal cortical level of lactate and the lactate/pyruvate ratio remained stable at 0.99 ± 0.06 mmol/l and 23.44 ± 1.85, respectively ($n = 9$). There were no statistical differences compared to the control group ($n = 5$). Combined hypoxia/ischemia led to an immediate rise in lactate dialysate levels, reaching a plateau of 3.01 ± 0.62 mmol/l within 20 min (Fig. 2A). The lactate/pyruvate ratio showed a similar time course during hypoxia/ischemia, reaching a plateau of 64.79 ± 11.24 (Fig. 2B). After the release of carotid occlusion and reoxygenation, lactate levels and the lactate/pyruvate ratio decreased, reaching control values within 30–40 min. Taking into account the effect of the changes in ECS volume fraction, the calculated extracellular concentrations of lactate during hypoxia/ischemia would be 30–50% lower than those actually measured (Fig. 2A).

Before the induction of hypoxia/ischemia, we found stable basal glucose and glutamate levels of 2.94 ± 0.18 mmol/l and 6.85 ± 0.97 μmol/l, respectively ($n = 9$), without any significant differences compared with control animals ($n = 5$). Unilateral carotid occlusion and a reduction in inspiratory oxygen content led to a steep decrease in glucose dialysate concentrations, reaching a minimum of 1.45 ± 0.23 mmol/l after 20 min of hypoxia/ischemia. During the reoxygenation period extracellular glucose concentrations returned to control levels within 20 min and then slowly decreased, reaching a value of 2.05 ± 0.17 mmol/l at the end of the experiment. The glucose concentrations during hypoxia/ischemia would be even lower if we take into account the accompanying changes in ECS volume fraction. During reperfusion, the glucose concentration corrected for the increase in α reached initial values within 20 min and remained at this level until the end of the experiment (Fig. 2C). Extracellular glutamate levels increased during hypoxia/ischemia, reaching maximum values of 59.30 ± 15.90 μmol/l at the end of the hypoxic/ischemic insult. During reperfusion the extracellular glutamate levels decreased, reaching control values 90 min after reperfusion. The concentration of glutamate corrected for the increase in α would be lower with the greatest increase seen within the first 10 min (Fig. 2D).

The aim of this study was to investigate changes in the diffusion parameters of the ECS and the extracellular concentrations of energy-related metabolites and glutamate in the rat somatosensory cortex during transient hypoxia/ischemia and reperfusion. This allowed us to analyse the relationship between the dynamic changes in the diffusion properties of the brain cortex and energy metabolism.

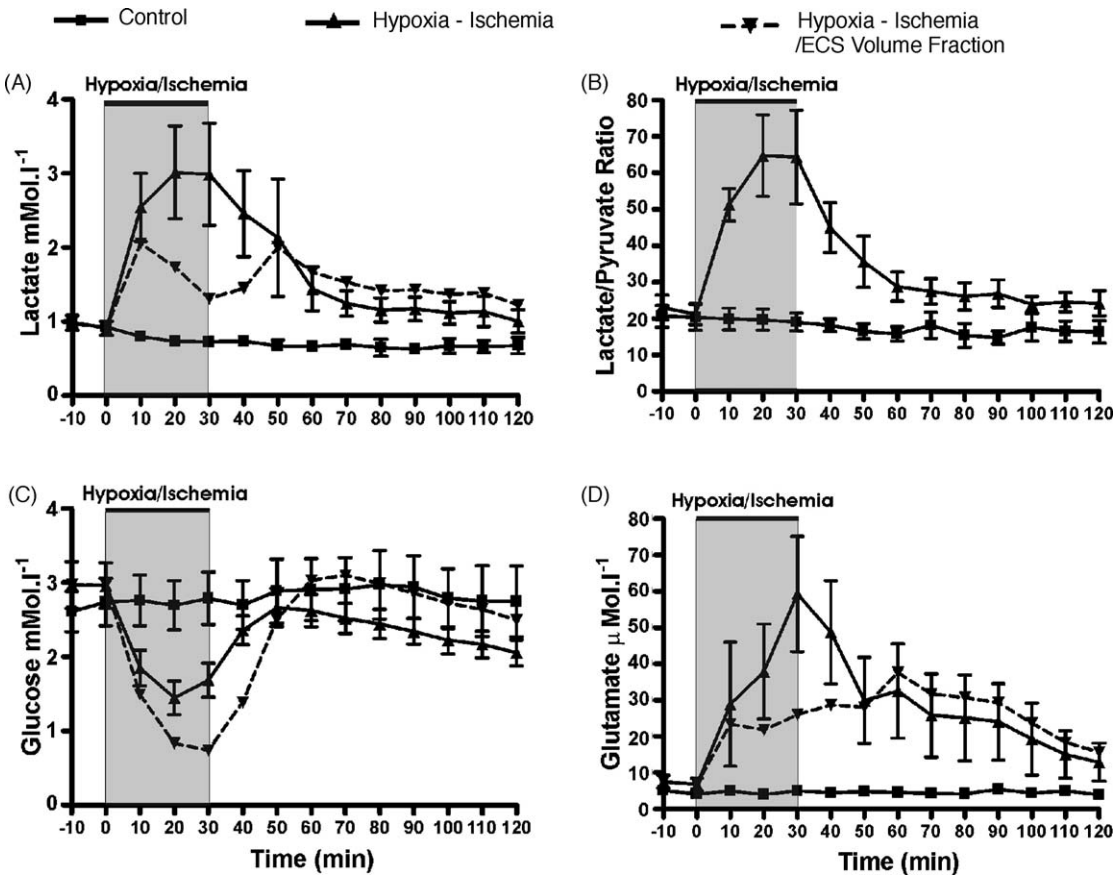

Fig. 2. The time course of changes in the concentration of extracellular lactate (A), the lactate/pyruvate ratio (B), and the concentrations of extracellular glucose (C) and glutamate (D) during transient hypoxia/ischemia and subsequent reperfusion, compared to controls. The stated concentrations, representing the actual physiological concentrations, may be underestimated. The time courses of the concentrations of the evaluated metabolites corrected for changes in ECS volume are presented as dashed lines, and they show how much of the concentration change is due to the ECS volume change.

Previous studies using a model of terminal anoxia in the rat cortex have shown a fast decrease in ECS volume and an increase in tortuosity within a few minutes following cardiac arrest [25,27]. The ultimate changes in ECS diffusion parameters were associated with an abrupt elevation of $[K^+]_e$ and an acid shift in pH_e [27] and correlated well with a reduction in the apparent diffusion coefficient of water (ADC_w) as measured by diffusion-weighted MRI [25]. Also, another study demonstrated a temporary reduction in ADC_w during transient hypoxia/ischemia with subsequent renormalization during reperfusion [10]. The present study found a continuous decrease in α and increase in λ during a hypoxic/ischemic insult, with final values similar to those previously found in terminal anoxia [25,27]. Similarly as during terminal anoxia, we observed that the changes in ECS diffusion parameters were accelerated by ischemic depolarization, which usually occurred between 5 and 10 min after the onset of hypoxia/ischemia, suggesting that ionic shifts were also responsible for the initial cellular swelling in this model. A similar time course in the reduction of the ECS size, measured by the electrical impedance technique, was reported during transient hypoxia/ischemia in the parietal cortex of 4-week-old rats [17].

During reperfusion the tortuosity renormalized within 20 min, while the ECS volume fraction increased and remained elevated about 20% above original normoxic values. This increase in the size of the ECS corresponds well with the findings of an increased signal in T1 weighted images and an elevated water content in the brain cortex of 4-week-old rats after a hypoxic-ischemic insult [17,18]. The authors concluded that changes in T1, but not T2, weighted MRI best serve as an indicator of edema associated with an elevation in water content. Also, a temporary increase in ADC_w without significant changes in the signal of T2 weighted MRI has been found in the parietal cortex of adult rats during reperfusion after hypoxia-ischemia [10].

To monitor changes in cerebral energy metabolism we used microdialysis, which is considered a highly sensitive technique for determining regional metabolic tissue concentrations [24], but it has some methodical limitations. Changes in the extracellular space volume during hypoxic conditions may have effects on microdialysate concentrations and probe efficiency. Relative recovery can change in the same probe during different physiological and pathological conditions [9]. Compared to in vitro calibration the in vivo recovery of substances strongly depends on the surrounding tissue properties especially extracellular volume fraction and tortuosity as well as various release, uptake and clearance processes [4]. Based on these findings the calculation of the interstitial concentrations based entirely on the in vitro recovery can be underestimated and possibly

could not predict tissue interstitial concentration accurately [3,4,9]. However, we suggest that the changes in extracellular microdialysate levels reflect the time course of this dynamic process.

In our experiments we found a steep increase in extracellular lactate concentration immediately after the onset of hypoxia/ischemia, which has also been seen previously [19]. In the past decades, lactate has been considered a dead-end waste product of anaerobic glycolysis, contributing to acidosis and tissue damage. Recent studies, however, have shown that lactate can be utilized by neurons as an energy source during aerobic conditions [5] and can even support neuronal survival and function during glucose deprivation in organotypic hippocampal slice cultures [7]. Another study demonstrated a beneficial effect of lactate during the initial phase of reperfusion [8]. This finding supports studies suggesting that lactate is used as a preferred substrate for the immediate restoration of neuronal ATP after hypoxia [20]. In our experiments, during reperfusion, we have seen a decrease in extracellular lactate concentration, reaching control values within 90 min of reoxygenation. This indicates a return to a sufficient oxygen supply and the uptake of lactate, possibly by neurons. A second marker for anaerobic metabolism is the tissue-specific L/P ratio, because it is closely correlated with the redox potential of cells [6]. Our results show a steep increase in the L/P ratio during hypoxia/ischemia, indicating a reversal of the cytosolic redox potential and a switch to anaerobic glycolysis. During reperfusion the L/P ratio normalized, which again corresponds with a return to the aerobic pathway of energy production.

The concentration of glucose in the ECF is a balance between supply and utilization, and possibly both mechanisms are involved in the decrease seen during hypoxia/ischemia in our experiments. During recovery the glucose concentration returned to initial values within 20 min and then slightly decreased again. This small drop is probably caused by a dilution effect of vasogenic edema.

It has been shown that the extracellular concentration of glutamate increases during brain ischemia, and the excessive activation of its receptors is believed to be a major cause of ischemia-related neuronal injury [2]. In our experiments, the concentration of glutamate in the ECF started to increase soon after the onset of hypoxia/ischemia and continued to increase to a level 10-fold above control values at the end of the insult. We have also shown how the ECS volume decrease contributes to the increase in the extracellular glutamate concentration. The activation of glutamate receptors may result in rapid cellular swelling [11]. However, only very high concentrations (10^{-2} M) were shown to cause a substantial decrease in the ECS volume in the isolated spinal cord of rat pups under normoxic conditions [26]. Because such concentrations are not achieved even under pathological conditions, it was suggested that glutamate-induced astrocytic swelling in vivo could be indirect and mediated by glutamate's effects on neuronal cells, such as increases in the extracellular potassium concentration promoted by neuronal depolarization [12].

In conclusion, we have demonstrated that transient hypoxia/ischemia causes similar changes in ECS diffusion parameters as does global anoxia. The observed reduction in ECS volume, reflecting cytotoxic edema, correlates well with the time course of the elevation in extracellular glutamate concentration. We have also shown the impact of the ECS volume on the concentrations of substances diffusing through the ECS, evidencing to what degree ECS shrinkage contributes to the increased concentrations of toxic metabolites.

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