

Changes in Extracellular Space Volume and Geometry Induced by Cortical Spreading Depression in Immature and Adult Rats

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Summary

Changes in extracellular space (ECS) diffusion parameters, DC potentials and extracellular potassium concentration were studied during single and repeated cortical spreading depressions (SD) in 13-15 (P13-15), 21 (P21) and 90-day-old (adult) Wistar rats. The real-time iontophoretic method using tetramethylammonium (TMA⁺)-selective microelectrodes was employed to measure three ECS parameters in the somatosensory cortex: the ECS volume fraction α (α = ECS volume/total tissue volume), ECS tortuosity λ (increase in diffusion path length) and the nonspecific TMA⁺ uptake k' . SD was elicited by needle prick. SD was significantly longer at P13-15 than at P21 and in adults. During SD, α in all age groups decreased from 0.21-0.23 to 0.05-0.09; λ increased from 1.55-1.65 to 1.95-2.07. Ten minutes after SD, α (in adults) and λ (all age groups) increased compared to controls. This increase persisted even 1 hour after SD. When SD was repeated at 1 hour intervals, both α and λ showed a gradual cumulative increase with SD repetition. Our study also shows that cortical SD is, as early as P13, accompanied by severe ECS shrinkage and increased diffusion path length (tortuosity) with values similar to adults, followed by a long-lasting increase in ECS volume and tortuosity when compared to pre-SD values.

Key words

Cell swelling • Cortex • Diffusion • Spreading depression • Extracellular space

Introduction

Cortical spreading depression (SD) (Leão 1944, Bureš *et al.* 1974), implicated in the pathophysiology of many neurological diseases, including stroke, migraine, epilepsy, transient global amnesia or head injury, is characterized by a propagating wave of suppressed electrical activity associated with depolarization of neurons and glial cells¹. The typical transient negative

DC shift is accompanied by a rise in extracellular potassium concentration ($[K^+]_e$) to 60-70 mM, whereas sodium, calcium and chloride ions move together with water into cells (Kraig and Nicholson 1978, Phillips and Nicholson 1979). The water influx results in the swelling of neurons and glial cells and in the transient shrinkage of the extracellular space (ECS) (Nicholson and Kraig 1981, van Harreveld and Khattab 1967). ECS shrinkage has been confirmed by measuring changes in tissue resistance

(Marshall 1959); swelling dendrites were visualized using electron microscopy (van Harreveld and Khattab 1967).

The absolute values of ECS volume decrease and concomitant changes in the ECS geometry can be determined by the real-time iontophoretic method introduced by Nicholson and Phillips (1981). This method allows for the determination of the ECS volume fraction (α), the part of the tissue occupied by the ECS ($\alpha = \text{ECS volume}/\text{total tissue volume}$), and the ECS tortuosity (λ), the increase in diffusion path length compared to a free solution ($\lambda^2 = D/\text{ADC}$, where D is the free and ADC the apparent diffusion coefficient). In addition, the nonspecific concentration-dependent uptake k' can also be obtained (for review see Nicholson and Syková 1998).

In adult rat cortex, Hansen and Olesen (1980) estimated a 50 % shrinkage of the ECS during SD *in vivo*. In neocortical slices of 16- to 30-day-old rats, Hablitz and Heinemann (1989) reported a decrease in the ECS volume during SD and a corresponding 40 to 45 % increase in extracellular resistance. These values are only relative and, to our knowledge, no data are available regarding the effects of SD on the absolute values of all three diffusion parameters, α , λ and k' .

The purpose of this study was i) to determine the ECS diffusion parameters α , λ and k' during SD in immature and adult rats *in vivo*; ii) to examine whether immature rats with longer-lasting and more slowly increasing/decreasing SD show different changes in ECS diffusion parameters than adult animals, and iii) to investigate the changes in ECS diffusion parameters after repeated SDs. Such findings would be relevant for determining the susceptibility of the immature brain to SD, ischemia, anoxia or seizures. Persistent changes in ECS diffusion parameters after SD could affect extrasynaptic transmission and functions such as vigilance, sleep, chronic pain, hunger, depression, LTP, LTD, memory formation and other plastic changes in the CNS (Syková and Chvátal 2000, Syková *et al.* 2000).

Methods

Animal preparation and solutions used

Experiments were carried out on 13- to 15-day-old ($n=9$), 21-day-old ($n=10$) and 3-month-old ($n=11$) adult male Wistar rats. The animals were anesthetized with urethane (2.0 g/kg, injected i.p.), or thiopental (100 mg/kg, injected i.p.). The somatosensory cortex was exposed with a 1.5 mm diameter trephination hole and the dura was removed from this area, taking care not to

damage the brain. During measurement, the animals were placed on a heated pad (body temperature was maintained at 37 °C), and breathing was spontaneous. The exposed cortex was superfused with artificial cerebrospinal fluid (ACSF) consisting of 125 mM NaCl, 3.0 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.5 mM NaH₂PO₄, 21 mM NaHCO₃, 2.2 mM urea, 0.3 mM TMA chloride and 3.4 mM glucose. The solution was warmed to 37 °C and equilibrated with 5% CO₂ in O₂, pH was adjusted to 7.4. In immature animals acetate conditioning (75% of the chloride ions in the superfusate were replaced by acetate ions) was used for at least 45 min before the first SD was elicited by needle pricking. After such superfusion, it is possible to elicit SD as early as at P9-10 (Richter *et al.* 1998). Without conditioning, SD can be reliably elicited after P15 (Bureš *et al.* 1964). In all animals SD was elicited by pricking the upper cortical layers with the tip of a 0.5 mm spinal needle.

Electrophysiological measurements

The extracellular space (ECS) diffusion parameters, ECS volume fraction α , tortuosity λ and nonspecific uptake k' , were measured using the real-time tetramethylammonium (TMA⁺) iontophoretic method of Nicholson and Phillips (Nicholson and Phillips 1981). TMA⁺ (74.1 MW), an ECS marker, was applied using a glass micropipette (containing 0.1 M TMA chloride) and its concentration continually measured at a distance of 40-100 μm from the point of application using a double-barreled TMA⁺-selective microelectrode. The time-dependent rise and fall of the extracellular TMA⁺ concentration during and after an iontophoretic pulse (TMA⁺ diffusion curves) were fitted to a radial diffusion equation modified to account for extracellular volume fraction α , tortuosity λ and nonspecific TMA⁺ uptake k' . TMA⁺-selective microelectrodes were prepared as described elsewhere (Voříšek and Syková, 1997). The tip of the ion-sensitive barrel was filled with Corning 477317 ion-exchanger; the remainder of the barrel was backfilled with 100 mM TMACl. The reference barrel contained 150 mM NaCl. The shank of the iontophoresis micropipette was bent to allow for parallel alignment with the measuring microelectrode. After positioning the tips (each with an outer diameter of 3-6 μm) at a distance of 40 – 100 μm apart, the application micropipette and the TMA⁺-selective microelectrode were glued together using dental cement.

TMA⁺ was applied using a 6 or 40 s iontophoretic current step of +80 nA. To maintain a stable background of the probe ion, a bias current of +20

nA was delivered continuously. Potentials recorded at the reference barrel of the ion-selective microelectrode were subtracted from the ion-selective barrel potentials by means of buffer and subtraction amplifiers. The resulting diffusion curves (20 or 100 s recordings: 2 or 10 s before, 6 or 40 s during and 12 or 50 s after the iontophoretic current step) were analyzed using the program VOLTORO (Nicholson unpublished).

TMA⁺ diffusion curves were initially recorded in a cup filled with 0.3 % agar gel made up of 150 mM NaCl, 3 mM KCl and 0.3 mM TMA chloride. This allowed us to determine the electrode array transport number and TMA⁺ free diffusion coefficient. Following this calibration, a baseline for cortical recordings was established above the cortical surface by recording the voltage in the superfusate containing a known

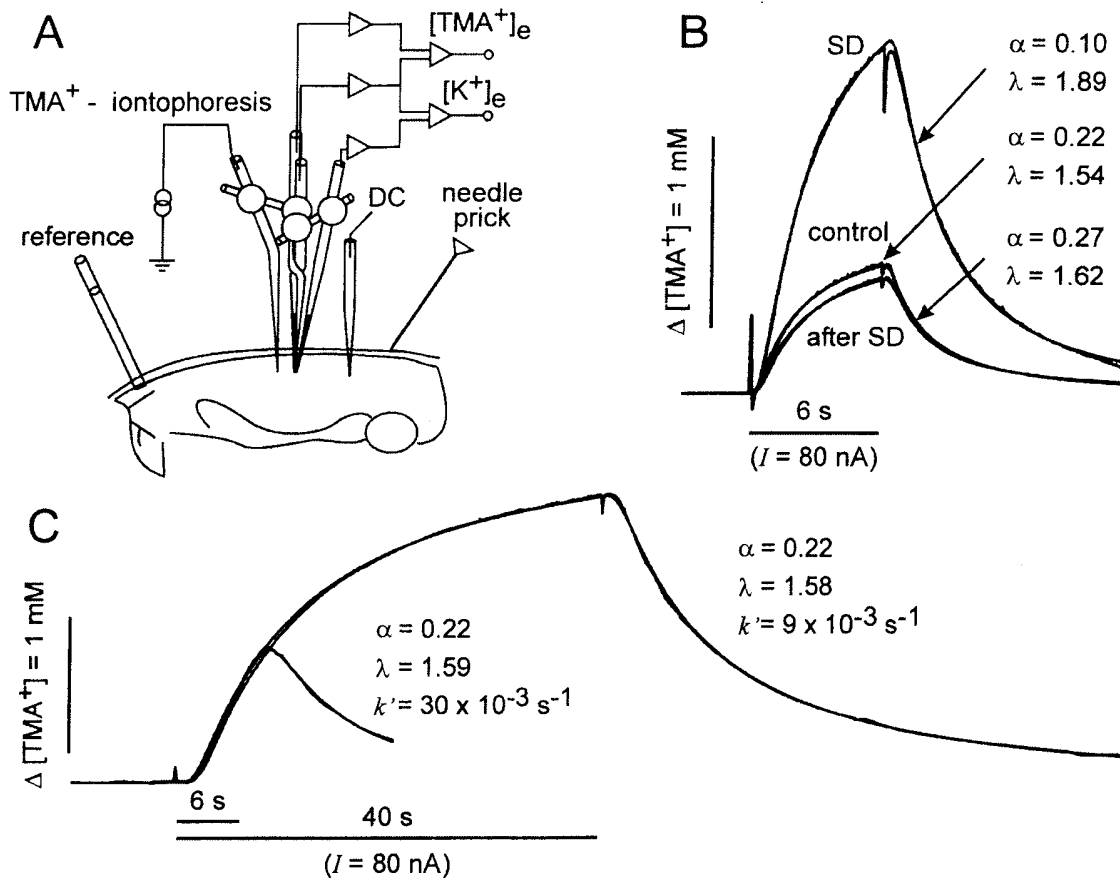


Fig. 1. A: Experimental arrangement for simultaneous DC, [K⁺]_e and [TMA⁺]_e recording. DC was monitored at the reference barrel of either the K⁺-ISM or the TMA⁺-ISMs. Another DC microelectrode (DC_{prick}) was inserted approximately 2 mm caudal to the array to monitor SD propagation. A common DC reference electrode was positioned on the nasal bone. All electrodes were connected by Ag/AgCl wires to a high impedance amplifier. SD was elicited by needle prick. The animals were grounded through an Ag/AgCl electrode inserted below the neck skin. B: Typical TMA⁺ diffusion curves recorded in cortical gray matter at a depth of 800 μm in a P21 rat. The iontophoretic current step of 40 nA was applied for 6 s. The separation between the electrode tips was 46 μm. The control diffusion curve was acquired 4 minutes before the SD ($\alpha=0.22$ and $\lambda=1.54$). During SD, due to shrinkage of the ECS, α decreased to 0.10 and λ increased to 1.89. Ten min after SD, α increased to 0.27 and λ was 1.62. The TMA⁺ concentration scale is linear and the theoretical diffusion curves are superimposed on each experimental curve. C: Comparison of two diffusion curves evoked by a 6 s and a 40 s current step, recorded using the same electrodes in a P21 rat. Calculated diffusion parameters are not different.

concentration of TMA^+ . The electrode array was then lowered into the cortical gray matter to a depth of 700 – 1100 μm that coincides with the known distribution of cortical layers IV and V at various ages (Lehmenkühler *et al.* 1993); the measurements were repeated and analyzed to yield the ECS parameters in the brain.

For simultaneous recording of $[\text{K}^+]_e$, a second ISM with Fluka Ionophore-Cocktail A (Fluka no. 60031), sensitive to K^+ but insensitive to TMA^+ , in its tip and backfilled with 100 mM KCl was glued to this array (Fig. 1). DC was monitored using the reference barrel of either the TMA^+ or K^+ double-barreled ISM. A further DC microelectrode (DC_{prick}) filled with 150 mM NaCl was inserted to a cortical depth of 200 to 400 μm about 2 mm caudally from the array to monitor SD propagation. A common DC reference electrode (outflow electrode with a porous membrane, filled with 2 M KCl) was positioned on the nasal bone (Fig. 1). All electrodes were connected by Ag/AgCl wires to a high impedance amplifier. The animals were grounded through an Ag/AgCl electrode inserted below the neck skin.

Statistical analysis

The results of the experiments were expressed as the mean values \pm S.E.M. Statistical analysis was evaluated by one way ANOVA (for between group comparisons) or one way repeated measures ANOVA followed by post hoc Dunnett's test (for within group comparisons). Values of $P < 0.05$ were considered significant.

Results

Measurements were performed in layers IV and V of the somatosensory cortex of P13-15 ($n=9$), P21 ($n=10$) and adult ($n=11$) rats.

Time course of SD-related changes in DC, $[\text{K}^+]_e$ and $[\text{TMA}^+]_e$ baseline

DC shifts, $[\text{K}^+]_e$ changes and TMA^+ baseline concentration changes (caused by the continuous application of a 20 nA iontophoretic current expelling TMA^+ ions) were measured simultaneously to compare their time course. The results are summarized in Table 1. At P13-15 the DC shift and $[\text{K}^+]_e$ increase were smaller, lasted longer and exhibited a longer decay phase than in adults. At P21 the DC shift amplitude was smaller, and its decay time as well as the $[\text{K}^+]_e$ decay time were longer than in adults. A typical example of SD with simultaneous recordings of DC, $[\text{K}^+]_e$ and TMA^+ baseline

concentration changes in a P15 rat is shown in Fig. 2. Propagation of the SD is manifested by a delay between the starting points of the SD-related depolarizations (DC_{prick} vs. DC) as the DC_{prick} electrode was inserted closer to the needle-prick site. The negative DC shift and increase in $[\text{K}^+]_e$ reflect typical changes during SD. In addition to the SD-related K^+ changes in the ECS, there was an increase in TMA^+ baseline concentration due to the ECS shrinkage induced by cell swelling.

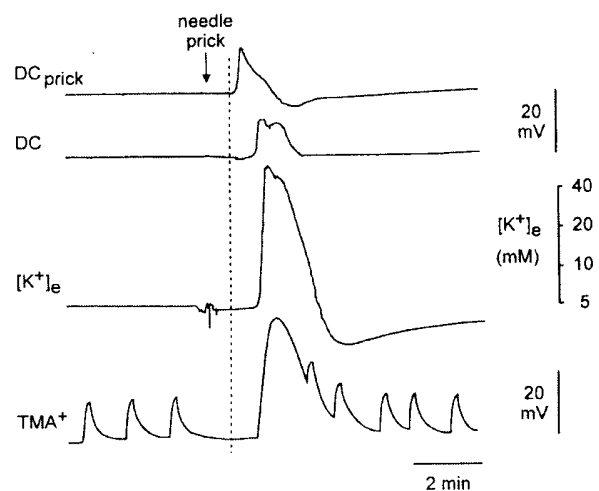


Fig. 2. Typical SD evoked by needle prick in a P15 rat. The needle prick used to induce the SD is indicated by an arrow. The trace at the top, marked as DC_{prick} , shows a DC recording near the site of SD elicitation. The other traces show simultaneous recordings of SD-related DC shift and changes in $[\text{K}^+]_e$ and $[\text{TMA}^+]_e$. TMA^+ diffusion curves during iontophoretic application are superimposed on the baseline. Three TMA^+ diffusion curves were used to obtain control values before SD (left part of the curve, left from dotted line).

Effect of SD on extracellular space (ECS) diffusion parameters

The ECS diffusion parameters were determined before, during and after SD. Recordings were done using a 6 s current step so that we could measure the diffusion parameters even during SD. When using a 40 or 60 s application, as in most TMA^+ studies done so far, the recordings obtained during SD would be distorted by the changing TMA^+ baseline concentration and could not be fitted. In some experiments, both 6 s and 40 s application times were used before eliciting the first SD. No significant differences were found in the volume fraction

or tortuosity obtained using either a 6 s or 40 s current step (Fig. 1). Only the nonspecific TMA⁺ uptake was higher when using a 6 s application.

At least three almost identical control diffusion curves were obtained in succession before the first SD was elicited. Measurements were then repeated at the plateau of the SD, at the end of the repolarizing phase and during the subsequent 60 min at 3 to 5 min intervals (Fig. 3). Sixty minutes after SD, the experiment was

either terminated or another SD was evoked with the same follow-up. The results are summarized in Table 1. Table 1 shows that in agreement with our previous studies (Lehmenkühler *et al.* 1993; Voříšek and Syková 1997), the mean value of ECS volume fraction α at P13-15, P21 and in adults varied between 0.21 and 0.23, ECS tortuosity between 1.55 and 1.65, and nonspecific TMA⁺ uptake between 9 and 21 $\times 10^{-3}$ /s. These differences were not statistically significant before SD was elicited.

Table 1. Spreading depression (SD) time course and extracellular space diffusion parameters (α , λ and k') before, during and after a cortical SD at different ages. Data are given as mean values \pm S.E.M.; n is the number of animals. Statistical analysis was made by one way ANOVA or one way repeated measures ANOVA followed by post hoc Dunnett's test as appropriate. Significant differences between the ECS diffusion parameters before SD (1-15 min before SD) and during and after SD are marked by *. Significant differences compared to adults are marked by # ($p < 0.05$). Significant differences between the time course of $[K^+]_e$ and $[TMA^+]_e$ and the time course of DC are marked by \$. In all cases, one symbol indicates $p < 0.05$, two symbols $p < 0.01$, three symbols $p < 0.001$.

		P13-15 (n=5)	P21 (n=5)	adult (n=6)
SD duration (DC) [s]		69.9 \pm 9.8 #	44.9 \pm 4.2	45.6 \pm 1.1
SD duration ($[K^+]_e$) [s]		110.3 \pm 19.7 #	63.8 \pm 3.0 \$\$	56.6 \pm 2.7 \$\$
SD duration ($[TMA^+]_e$) [s]		218.6 \pm 8.4 ###\$	72.0 \pm 2.9 \$\$\$	69.6 \pm 7.4 \$
DC rise time [s]		2.52 \pm 0.27	1.91 \pm 0.24	1.78 \pm 0.28
$[K^+]_e$ rise time [s]		4.52 \pm 1.32 \$	1.61 \pm 0.21	1.60 \pm 0.32
DC decay time [s]		11.2 \pm 1.2 #	8.8 \pm 1.1 #	5.2 \pm 0.9
$[K^+]_e$ decay time [s]		21.2 \pm 1.3 #	17.9 \pm 1.2 #	12.5 \pm 1.6
DC amplitude [mV]		13.6 \pm 1.2 #	15.9 \pm 1.2 #	21.2 \pm 1.9
$[K^+]_e$ increase [mM]		49.6 \pm 8.3 #	77.1 \pm 3.5	70.3 \pm 4.8
control	α	0.23 \pm 0.02	0.22 \pm 0.02	0.21 \pm 0.01
	λ	1.55 \pm 0.05	1.63 \pm 0.04	1.65 \pm 0.03
	k'	9 \pm 2 $\times 10^{-3}$ s ⁻¹	15 \pm 5 $\times 10^{-3}$ s ⁻¹	21 \pm 7 $\times 10^{-3}$ s ⁻¹
SD plateau	α	0.05 \pm 0.01 ***#	0.07 \pm 0.02 ***	0.09 \pm 0.01 ***
	λ	1.95 \pm 0.07 ***	2.07 \pm 0.07 **	1.99 \pm 0.05 **
	k'	42 \pm 20 $\times 10^{-3}$ s ⁻¹	35 \pm 18 $\times 10^{-3}$ s ⁻¹	49 \pm 20 $\times 10^{-3}$ s ⁻¹
5-30 min after SD	α	0.24 \pm 0.02 *	0.24 \pm 0.02 *	0.25 \pm 0.01 *
	λ	1.62 \pm 0.05 *	1.68 \pm 0.03 **	1.70 \pm 0.04
	k'	11 \pm 2 $\times 10^{-3}$ s ⁻¹	16 \pm 5 $\times 10^{-3}$ s ⁻¹	21 \pm 4 $\times 10^{-3}$ s ⁻¹
30-60 min after SD	α	0.24 \pm 0.02 *	0.24 \pm 0.02	0.24 \pm 0.02 *
	λ	1.64 \pm 0.08 *	1.70 \pm 0.03 **	1.67 \pm 0.02
	k'	9 \pm 2 $\times 10^{-3}$ s ⁻¹	18 \pm 5 $\times 10^{-3}$ s ⁻¹	22 \pm 5 $\times 10^{-3}$ s ⁻¹

During SD, typical changes of ECS diffusion parameters were found (Fig. 1). The mean values of ECS volume fraction α declined from 0.23 to 0.05 in P13-15, from 0.22 to 0.07 in P21 and from 0.21 to 0.09 in adult

animals. This corresponds to a relative shrinkage of the ECS of approximately 78 % in P13-15, 68 % in P21 and 57 % in adult animals. After SD termination the volume

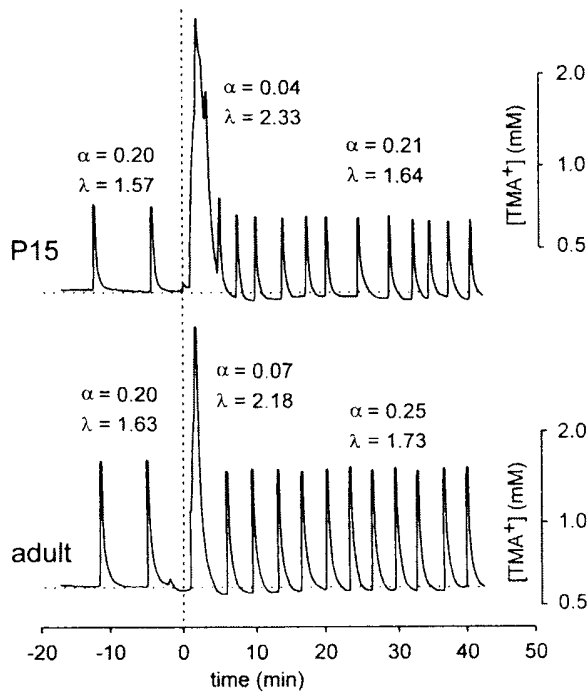


Fig. 3. Comparison of the time courses of TMA^+ baseline changes evoked by SD in a P15 and an adult rat. Duration of SD at P15 is longer. Note the decrease in the TMA^+ baseline after the SD, due to an increase in ECS volume fraction.

fraction rapidly recovered and even exceeded the initial values (Fig. 1 and 3).

The SD-related increase in ECS tortuosity λ had a similar time course as the changes in α (Fig. 3). The increase in λ during SD was followed by a rapid but incomplete recovery after SD. Thirty and even sixty minutes after SD, the tortuosity at P13-15 and P21 was still significantly higher than before SD (Fig. 1, Fig. 3, Table 1). The changes in nonspecific TMA uptake k' were not significant.

Figure 4 shows typical changes in ECS volume fraction and tortuosity during and between three subsequent SDs elicited at 60 min intervals in P13-15, P21 and adult rats. ECS volume fraction α decreased and tortuosity increased during the second and third SD to values that did not differ significantly from those during the first SD. Also, the time courses of the α and λ changes, DC shifts, K^+ rise and TMA^+ baseline changes after the 2nd and 3rd SD were not longer than those after the 1st SD. Recovery of α and λ after the 2nd and 3rd SD was, however, incomplete, thus leading to a gradual increase in both α and λ (Fig. 4).

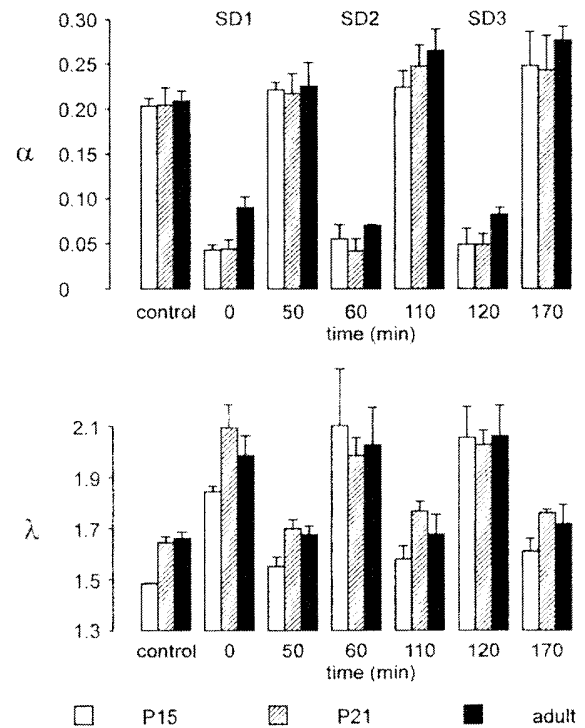


Fig. 4. Time course of ECS volume fraction and tortuosity changes during and after 3 subsequent episodes of SD. SDs were elicited at 1 hour intervals and were characterized by a marked decrease in volume fraction and an increase in tortuosity. After SD, both parameters were increased as compared to pre-SD values; the increases persisted even 50 min after the SD. After the second and third SDs, further increases were observed.

Discussion

In previous studies, only relative changes in ECS volume during SD have been investigated with pulsed applications of TMA or TEA (Phillips and Nicholson 1979, Dietzel *et al.* 1980, Hablitz and Heinemann 1989, Jing *et al.* 1994). The present study shows that short TMA iontophoresis times of only 6 s duration and microelectrode intertip distances of 50-100 μm are sufficient for a stable and accurate evaluation of ECS diffusion parameters. Only with such short applications we were able to record and fit diffusion curves even during an SD wave that only lasts about 1 min in adults. The values of ECS volume fraction and tortuosity calculated from these diffusion curves did not differ from those obtained using longer application times and larger

intertip distances (Lehmenkühler *et al.* 1993, Voříšek and Syková 1997).

SD, from the first age when it could be elicited in these experiments, was associated with ECS shrinkage and increased diffusion path length (λ). To some extent, the mechanisms of ionic and volume changes in SD are similar to those during anoxic depolarization (AD) (Lauritzen and Hansen 1992). Similarly as the substantial shortening with age of phase I-II of anoxic depolarization (Mareš *et al.* 1976, Voříšek and Syková 1997), the SD in immature animals was slower (Bureš 1957). As in AD, the duration of the DC negative shift, the $[K^+]_e$ and $[TMA^+]_e$ increases, and the decay phase was significantly longer at early postnatal days than in adults. This may be explained by immature cellular transport mechanisms responsible for ion and water redistribution, immature astrocytes and oligodendrocytes, and inefficient glial coupling (Lehmenkühler *et al.* 1993, Voříšek and Syková 1997, Martins-Ferreira *et al.* 1999). SD is accompanied by the entry of Ca^{2+} into cells and fails when gap junctions are blocked (for rev. see Martins-Ferreira *et al.* 1999). Astrocytic Ca^{2+} waves are probably involved in the initiation of SD, while other factors, including K^+ and both glutamate and purinergic receptors, are necessary for sustained propagation (Bureš *et al.* 1974, Basarsky *et al.* 1999). Mature astrocytes and oligodendrocytes, which play a major role in ionic and volume homeostasis (Syková 1997, Syková and Chvátal 2000), therefore play an important role in SD propagation. This is supported by the fact that in all age groups, the $[K^+]_e$ and $[TMA^+]_e$ increases, which are affected by the maturation of glia, lasted significantly longer than the negative DC shift.

The values of ECS volume fraction and tortuosity, measured before the first SD was elicited, did not differ from those found in the cortex without acetate conditioning (Lehmenkühler *et al.* 1993, Voříšek and Syková 1997). Therefore, it is proposed that acetate conditioning does not have a major effect on ECS parameters. In agreement with previous studies (Lehmenkühler *et al.* 1993, Voříšek and Syková 1997), immature animals exhibited a tendency to show a higher α and lower λ . The nonspecific TMA^+ uptake was higher than previously observed. This was probably caused by the use of shorter diffusion times, since in other experiments done without acetate conditioning and with such short diffusion times we obtained similarly increased values (Mazel and Syková, unpublished data). We also noticed a weak negative correlation between the electrode array spacing (distance between the tips of the

iontophoresis and the measuring electrode) and the nonspecific uptake.

During SD α and λ reached values similar to those observed during anoxia at the same postnatal ages (Voříšek and Syková 1997) with α in the range of 0.05 – 0.09 and λ between 1.95 and 2.07. The extent of ECS shrinkage during SD was larger at P13-15 than at P21 or in adult animals. This somewhat contrasts with the smaller DC amplitude and $[K^+]_e$ increase found at P13-15. This might be explained by the longer SD time course at P13-15, allowing more time for $[K^+]_e$ -induced glial swelling before the ECS size decreases to its minimum (Syková 1997, Nicholson and Syková, 1998).

After SD, both α and λ increased, with the maximal increase in the ECS volume fraction occurring 5-20 min after SD, within the interval that is considered the relative refractory phase (Bureš *et al.* 1974). Then α slowly decreased towards the initial values (30-60 min after SD). The tortuosity time course differed with a slow continuous increase throughout the entire one hour observation period. After three SDs, both the volume fraction and tortuosity were often increased (Fig. 3). The progressive increase in ECS tortuosity and volume fraction after repeated SDs suggests that the effect increases with repetition. A reason for this diffusion hindrance could be astrogliosis caused by the increased $[K^+]_e$. In immature spinal cord, exposure to 50 mM K^+ for 20-45 min produces a decrease in ECS volume fraction and an increase in tortuosity to 2.0. When the concentration of K^+ in the bathing solution returns to the initial 3 mM, the increase in tortuosity persists and the ECS volume fraction often remains increased (Syková *et al.* 1999). Histological observations have confirmed the presence of astrogliosis (Syková *et al.* 1999), which can produce additional diffusion barriers even when the ECS volume fraction is increased (Syková *et al.* 1999, Roitbak and Syková 1999).

The present study shows that the absolute values of the extracellular space diffusion parameters can be measured during cortical SD *in vivo*. The extent of ECS shrinkage during SD at P13-15 was larger than in adult animals. Sixty minutes after SD, the ECS volume fraction and tortuosity remained increased above the values before SD. If SD was repeated, both the volume fraction and tortuosity gradually increased. Such an increase in ECS diffusion barriers in the CNS can affect the neurotoxicity of various substances, impair extrasynaptic "volume" transmission and affect behaviour and learning (Syková *et al.* 2000).

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Appendix

Eva Syková first had the pleasure of working in the laboratory of Dr. Bureš as a 16-year-old laboratory technician, after being denied the opportunity to attend university because of my family's persecution by the communist regime. The first research project in which I participated in the lab of Dr. Bureš and his wife Olga was a study of potassium-induced spreading depression.

It is not an exaggeration to say that my stay in Dr Bureš' laboratory determined the future direction of my professional life. I became determined to study brain function, and it was Dr. Bureš who gave me the

recommendation I needed to begin my studies at the Medical Faculty of Charles University. Later, he accepted me as a medical student in his laboratory and taught me how to conduct scientific research not just at a national level, but at an international level. Dr. Bureš always encouraged his students and colleagues to pursue new ideas. He never judged people on the basis of nationality or gender; even during the sixties and seventies, his laboratory was home to visiting scientists from both East and West. He was kind and friendly to all, but at the same time displayed a strong drive to accomplish significant scientific work. He provided support and encouragement to his students' efforts to establish independent research groups, to secure their own grants, to gain membership in international scientific organizations.

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