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Extracellular Ionic and Volume Changes: The Role in Glia–Neuron Interaction

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ABSTRACT

Activity-related changes in extracellular K^+ concentration ($[K^+]_e$), pH (pH_e) and extracellular volume were studied by means of ion-selective microelectrodes in the adult rat spinal cord *in vivo* and in neonatal rat spinal cords isolated from pups 3–14 days of age (P3–P14). Concomitantly with the ionic changes, the extracellular space (ECS) volume fraction (α), ECS tortuosity (λ) and non-specific uptake (k'), three parameters affecting the diffusion of substances in nervous tissue, were studied in the rat spinal cord gray matter. In adult rats, repetitive electrical nerve stimulation (10–100 Hz) elicited increases in $[K^+]_e$ of about 2.0–3.5 mM, followed by a post-stimulation K^+-undershoot and triphasic alkaline–acid–alkaline changes in pH_e with a dominating acid shift. The ECS volume in the adult rat occupies about 20% of the tissue, $\alpha = 0.20 \pm 0.003$, $\lambda = 1.62 \pm 0.02$ and $k' = 4.6 \pm 0.4 \times 10^{-3}\ s^{-1}$ ($n = 39$). In contrast, in pups at P3–P6, the $[K^+]_e$ increased by as much as 6.5 mM at a stimulation frequency of 10 Hz, i.e. K^+ ceiling level was elevated, and there was a dominating alkaline shift. An increase in $[K^+]_e$ as large as 1.3–2.5 mM accompanied by an alkaline shift was evoked by a single electrical stimulus. The K^+ ceiling level and alkaline shifts decreased with age, while an acid shift, which was preceded by a small initial alkaline shift, appeared in the second postnatal week. In pups at P1–P2, the spinal cord was X-irradiated to block gliogenesis. The typical decrease in $[K^+]_e$ ceiling level and the development of the acid shift in pH_e at P10–P14 were blocked by X-irradiation. Concomitantly, continuous development of glial fibrillary acidic protein positive reaction was disrupted and densely stained astrocytes in gray matter at P10–P14 revealed astrogliosis.

The alkaline, but not the acid, shift was blocked by Mg^{2+} and picrotoxin (10^{-6} M). Acetazolamide enhanced the alkaline but blocked the acid shift. Furthermore, the acid shift was blocked, and the alkaline shift enhanced, by Ba^{2+}, amiloride and SITS. Application of glutamate or gamma-aminobutyric acid evoked an alkaline shift in the pH_e baseline at P3–P14 as well as after X-irradiation. The results suggest that the activity-related acid shifts in pH_e are related to membrane transport processes in mature glia, while the alkaline shifts have a postsynaptic origin and are due to activation of ligand-gated ion channels.

At P4–P6, the ECS volume was almost double that in adult rats, $\alpha = 0.37 \pm 0.01$ ($n = 17$), the ECS tortuosity was significantly higher, $\lambda = 1.78 \pm 0.02$, while the non-specific uptake was not significantly different, $k' = 3.61 \pm 0.56 \times 10^{-3}\ s^{-1}$. The α gradually decreased to about 24% at P12. In adult rats, electrical or adequate stimulation evoked a shrinkage of the extracellular space by 20–50%, while no significant changes in ECS volume were found in P3–P6. We conclude that the $[K^+]_e$ ceiling level, character of the pH_e transients, the size of the ECS volume and the activity-related ECS shrinkage are closely related to gliogenesis.

KEY WORDS: Development Extracellular space volume Glia pH Potassium Spinal cord

INTRODUCTION

In the last decade the expansion of our knowledge about dynamic ionic and volume changes in the extracellular space (ECS) and about glial cell properties led to the new view about neuron–glia interaction. The dynamic changes in ECS composition and volume and electrophysiological and morphological properties of glial cells have been found in a variety of physiological and some pathological states. The ECS functions as the communication channel between neural elements. Accumulation of ions and diffusion of neuroactive substances or metabolites through the ECS significantly influence neuronal and glial cell function. Glial cells control ionic, particularly K^+ and pH,

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homeostasis and ECS diffusion parameters. Recent data suggest that immature glia may not be able to perform its homeostatic function, i.e. K^+, pH and ECS volume homeostasis (Jendelová and Syková, 1991; Syková *et al.*, 1992; Ransom *et al.*, 1985a,b; Lehmenkühler *et al.*, 1993). Equally important is the fact that ECS composition and diffusion parameters significantly influence the complex function of the neurons and glial cells and modify nervous tissue excitability (for review see Syková, 1992). The relationship between neurons, glia and ECS is therefore vitally important for normal mental functioning. Consequently, it is of great interest to find out how the neuron–ECS–glia relationship differs during development of the central nervous system (CNS) and how it might be impaired during various disease states and injury.

Transmembrane ionic shifts resulting from any neuronal activity are accompanied by a redistribution of water, i.e. by a dynamic increase in the volume of active nerve cells and glia and compensatory shrinkage of the extracellular space (Nicholson and Rice, 1988; Svoboda and Syková, 1991). The recent experiments *in vitro* as well as *in vivo* demonstrated that the changes in intra- and extracellular volume result in altered diffusion parameters in ECS, which in turn would affect the activity-related accumulation of ions, neuroactive substances and metabolites in ECS and their movement towards the target cells and access to capillaries. In these studies the real-time iontophoretic method, which employs ion-selective microelectrodes to follow the diffusion of extracellular markers applied by iontophoresis was utilized (Nicholson and Phillips, 1981; Nicholson, 1993). The diffusion in ECS is constrained by two geometrical factors: (1) extracellular volume fraction (α), which is the restricted volume of the tissue available for diffusing particles, and (2) extracellular tortuosity (λ) factor, which represents the increased path length for diffusion between two points resulting from various barriers, e.g. cellular membranes, glycoproteins and macromolecules (Nicholson and Phillips, 1981). Beside that, the diffusion between two points can be effected by non-specific uptake k' (Nicholson, 1985).

This review deals with activity-related ionic and volume changes in the spinal cord of adult rats and during development, during impaired gliogenesis by early postnatal X-irradiation and with transient changes during peripheral stimulation and injury. It focuses on the possible role of glial cells in altered K^+, pH_e and ECS volume homeostasis.

MATERIALS AND METHODS

Animal preparation

All animals were female Wistar rats. In the experiments performed on 3- to 14-day-old rat pups (P3–P14), animals were decapitated and the lumbosacral spinal cord was dissected in a chamber with cold (9–11°C) artificial cerebrospinal solution of the following composition (in mM): NaCl 113.0, KCl 3.5, $CaCl_2$ 2.0, Na_2HPO_4 2.0, $NaHCO_3$ 28, glucose 1 g/l. The isolated cord was placed in a small chamber and the preparation was continuously perfused with the above solution. During 1–2 h the temperature was increased to 21–23°C. The solution was saturated with 95% O_2 and 5% CO_2 (pH 7.3–7.35). Solutions with 20 mM-$MgCl_2$, 5 mM-acetazolamide or other drugs had reciprocally reduced Na^+ concentration.

In the experiments performed on adult rats, the animals were anaesthetized with pentobarbital (40–60 mg/kg). A laminectomy was performed between L_2 and L_6. The animals were artificially ventilated and paralysed with D-tubocurarine. Blood pressure and end-tidal CO_2 were continuously monitored. The arterial pCO_2 was measured in blood samples by ABL4 K and pH/blood gas analyser (Radiometer) and ranged from 35 to 55 mmHg. The animals were mounted in a rigid frame and a pool filled with paraffin oil (37°C) was made around the spinal cord by fixing the skin flaps to a frame. The body temperature was maintained at 37–38°C.

X-irradiation

Rat pups derived from different litters were divided into control and X-irradiated groups. Animals 1 to 2 days old were X-irradiated according to Gilmore (1963a,b). A single dose of about 40.0 Gy was administered with an X-ray therapy unit (Phillips RT 100) under the following parameters: 50 KVP; 10 mA; 10 cm FSD; total dose, 41.1 Gy, dose rate, 7.733 Gy/min; added filtration 0.3 mm aluminium; half-value layer, 0.2 mm aluminium. Prior to irradiation, calibration was made in a cylindrical ionization chamber. The irradiated area was restricted to the spinal cord lumbosacral region demarcated by covering the animals with a protective lead shield, 0.3 mm in thickness containing a slit measuring 5 mm anteroposteriorly by 10 mm transversely.

Stimulation and measurements of ionic changes

Microelectrodes were inserted into the spinal cord from its dorsal surface. Recordings were made from lumbar segments L_4 or L_5. The dorsal root of the same segment or a peripheral nerve was stimulated supramaximally (rectangular pulses of 5 V or less; duration 0.1 ms) with fine bipolar silver electrodes. Transcutaneous electrical nerve stimulation was delivered through a pair of stainless-steel needles inserted through the skin below the plantar surface. Adequate stimuli were applied to the most sensitive area of the receptive field (for details see Syková and Svoboda, 1990).

K^+ activity was recorded with double-barrelled K^+-selective microelectrodes filled with a liquid ion-

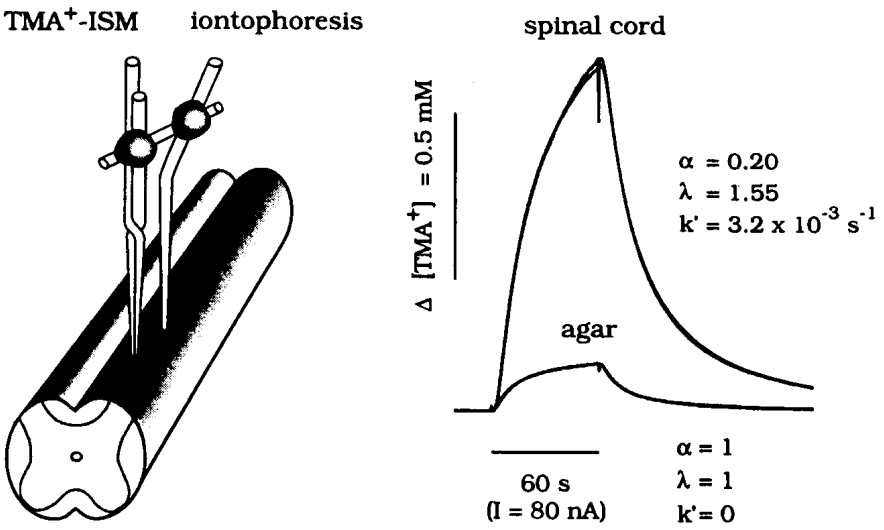

Fig. 1. Method of determining the ECS volume fraction (α), tortuosity (λ) and uptake (k') in spinal cord. Left: schematic drawing of the experimental arrangement for diffusion measurements. Two microelectrodes, the double-barrelled TMA^+-selective microelectrode (TMA^+-ISM) and the micropipette for TMA^+ iontophoresis, were glued together with dental cement at the area of their shanks (not shown) and with a glass tube bridge connecting the two microelectrodes at their upper end to stabilize the intertip distance 80–200 μm. Right: typical diffusion curves obtained in agar gel and in spinal cord of the adult rat. Measurements in agar, where $\alpha = 1$, $\lambda = 1$ and $k' = 0$, enabled the transport number of the iontophoretic electrode to be determined. The iontophoretic current applied for 60 s was 80 nA. When the electrode array was lowered 700 μm into the spinal dorsal horn of an adult rat, and the same main iontophoretic current applied, the resulting increase in concentration was much larger than that in agar due to the restricted volume fraction ($\alpha = 0.20$) and increased tortuosity ($\lambda = 1.55$). The non-specific uptake was $k' = 3.2 \times 10^{-3}\ s^{-1}$. In this figure, and in Fig. 7, the TMA^+ concentration scale is linear and the theoretical diffusion curve (equation 1) is superimposed on each experimental curve.

exchanger (Corning 477317) or with valinomycin ionophore (Fluka 60031) prepared by a procedure described previously (Kříž *et al.*, 1974). K^+-selective microelectrodes were calibrated in solutions containing 3, 4, 5, 6, 8 or 10 mM-KCl in 150 mM-NaCl. Basically, the same procedure was adopted to prepare the double-barrelled pH-sensitive microelectrodes (Chvátal *et al.*, 1988; Syková and Svoboda, 1990). In principle, the reference channel was filled with 0.15 M-NaCl solution while the pH-sensitive channel contained, in a siliconized tip, a 200–1000 μm column of liquid hydrogen ion ionophore II cocktail A (Fluka). The backfilling solution was composed of (mM): KH_2PO_4 40.0, NaOH 23.0, NaCl 15.0 (pH 7.0). Electrode sensitivity was tested in standard solutions, the pH of which was 7.0, 7.2, 7.4, 7.6, 7.8 or 8.0 with a background of 150 mM-NaCl and 3 mM-KCl. The slope of the electrodes was about 57 mV/unit of pH change and the electrodes had a resistance of 700–1200 MΩ. The electrical arrangements were the same as described for K^+-selective microelectrode (Kříž *et al.*, 1974). Each channel of a double-barrel microelectrode was connected to one input of a differential amplifier. Local field potential was recorded with the reference barrel of an ion-selective microelectrode. Microelectrodes were inserted into the spinal cord using two micromanipulators.

Measurements of ECS volume fraction (α), tortuosity (λ) and non-specific uptake (k')

The ECS diffusion parameters were studied by means of the iontophoretic administration of ions that do not cross the cell membranes and therefore remain in the ECS. The ion exchanger for K^+ (Corning 477317) is highly sensitive to TMA^+ ions which, in small concentrations, are not toxic and do not cross cell membranes (Nicholson and Phillips, 1981; Kříž and Syková, 1981). TMA^+ ions were therefore used for testing changes in the size of the ECS. Double-barrelled TMA^+-ISMs were prepared by the same procedure as K^+-ISMs; however, as a backfilling solution, 150 mM-TMACl was used instead of 500 mM-KCl. Reference barrels contained 150 mM-NaCl. TMA^+-ISMs were calibrated in 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 mM TMA^+ with the background of either 3, 10 or 70 mM-KCl and 150, 143 or 80 mM-NaCl to yield electrode slope and interference.

Using a current of 60 to 100 nA, TMA^+ ions were administered for 60–80 s into the ECS with an iontophoretic pipette. In some experiments, 20 nA bias current was used throughout the experiment to obtain a certain TMA^+ base line. The shank of the iontophoretic pipette was bent, before backfilling with 100 mM TMA^+, so that it could be aligned parallel to TMA^+-ISM. Electrode arrays of TMA^+-ISM and iontophoretic pipette were made by gluing both electrodes together with dental cement (Fig. 1). The tip of the TMA^+-ISM was 80–200 μm distance from the tip of the iontophoretic pipette. TMA^+ diffusion curves were at first recorded in 0.3% agar (Difco, USA) dissolved in a solution of 150 mM-NaCl, 3 mM-KCl and 0.3 mM-TMACl. These curves were then analysed by fitting the data to a solution of diffusion equation (Nicholson and Phillips, 1981) to yield the TMA^+ diffusion coefficient (D) and

the iontophoretic electrode transport number (n). Measurement of the concentration change in the agar yields the free-diffusion values for the relevant ion, in which by definition $\alpha = 1$ and $\lambda = 1$. Similar diffusion curves were then recorded in the spinal cord.

The α, λ and k' were determined by a computation procedure developed by Nicholson and Phillips (1981). The results of these studies show that if we incorporate factors α and λ into Fick's law, diffusion in the CNS is described fairly satisfactorily. The micropipette from which TMA^+ was released iontophoretically acts as the source $Q = In/F$, where I is a current increment, F is Faraday's electrochemical equivalent and n is the transport number. The fact that some TMA^+ can also enter vertebrate brain cells could be a source of error and therefore non-specific uptake, k' (s^{-1}), was incorporated into the diffusion equation (Nicholson, 1985). Uptake of TMA^+ is proportional to the extracellular concentration and can be a function not only of average permeability of the membranes to TMA^+ but also of a local volume fraction and the density of membranes in the tissue under study. The possibility that TMA^+ may cross the blood-brain barrier is not justified, since uptake values are similar in both *in vivo* and *in vitro* preparations. Therefore, assuming that loss of TMA^+ from the ECS is a linear concentration-dependent process, this factor can be incorporated into the diffusion equation, where apparent diffusion coefficient $D^* = D/\lambda^2$ (Nicholson, 1985). The appropriate solution of the diffusion equation is:

$$C(r,t) = (Q/(8\pi D^* \alpha r))[\exp(r(k'/D^*)^{1/2}) \operatorname{erfc}(r/(2(D^*t)^{1/2} + (k't)^{1/2}) + \exp(-r(k'/D^*)^{1/2}) \operatorname{erfc}(r/(2(D^*t)^{1/2}) - (k't)^{1/2})] \quad (1)$$

where C is the concentration, r is the distance of ISM from the ion source (iontophoretic pipette), t is the time of the probe ion application and erfc is the complementary error function. This equation enables the non-specific, concentration-dependent uptake k' to be determined, along with the ECS volume fraction (α) and tortuosity (λ). Spinal cord diffusion curves were analysed and diffusion parameters extracted by a non-linear curve-fitting simplex algorithm in the VOLTORO program (Nicholson, unpublished).

RESULTS

K^+ and pH_e changes in adult rat spinal cord

Almost all kinds of stimulation—artificial or adequate—lead to a transient increase in $[K^+]_e$ and alkaline–acid shifts in pH_e in the corresponding spinal cord segments (Svoboda *et al.*, 1988; Syková and Svoboda, 1990). The main source of changes in $[K^+]_e$ are stimulated neurons, unmyelinated fibres and unmyelinated terminals of axons. An increase of 0.1–0.2 mM has been found in the spinal cord after

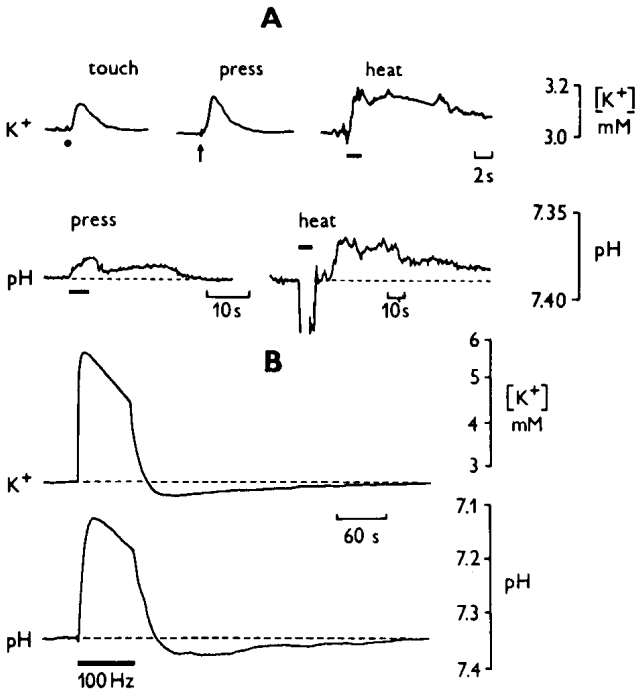

Fig. 2. (A) Transient increase in $[K^+]_e$ and decrease in pH_e in the L_4 spinal segment in response to adequate stimuli applied to the plantar region of the ipsilateral hind paw. The curves represent responses to light touch, strong press and application of about 1 ml of hot water onto the hind limb (heat). Horizontal bars indicate stimulus duration. $(K^+]_e$ and pH_e data are from different experiments. (B) $[K^+]_e$ and pH_e changes recorded simultaneously in dorsal horn of segment L_4 of adult rat spinal cord and evoked by repetitive bipolar electrical stimulation with acupuncture needles (100 Hz, 60 s) in plantar muscles of the ipsilateral hind paw. The horizontal bar indicates stimulus duration. Two K^+ and pH-sensitive microelectrodes were inserted separately from the dorsal spinal surface. All recordings are from a depth of 600–700 μm. (After Svoboda *et al.*, 1988 and Syková and Svoboda, 1990.)

a single electrical stimulus applied to peripheral nerves or after a single adequate stimulus (e.g. light touch or pinch) applied to the skin (Fig. 2A). A long-term increase in $[K^+]_e$ in the spinal dorsal horn of the rat was evoked by chemical and thermal injury of the hind paw (see Fig. 6). Repetitive electrical stimulation or long-term adequate stimulation leads to summation of responses to a certain level, the so-called 'ceiling level', from a baseline ('resting') level of about 3–4 mM to as much as 6–12 mM (Fig. 2B). When stimulation continues, no further changes in $[K^+]_e$ are found because a steady state is established, which is a result of concurrent release and clearance of K^+ (Fig. 2B). This ceiling level (Kříž *et al.*, 1975; Heinemann and Lux, 1975) in the CNS is only broken through by pathological events, e.g. epileptic activity, anoxia, spreading depression, application of convulsive drugs, and is higher in immature nervous tissue, i.e. in situations when K^+ homeostasis is impaired (see below).

Concomitantly with an increase in $[K^+]_e$, any neuronal activity results in a transient change in ECS acid–base balance. In adult animals, the activity-related pH_e changes have a typical time-course: alkaline–acid shift during the stimulation

which is in mammalian spinal cord followed by post-stimulation alkaline undershoot (Syková, 1989; Syková and Svoboda, 1990), while in the frog spinal cord by post-stimulation delayed acid shift (Chvátal *et al.*, 1988). The pH_e resting level recorded in various areas of adult CNS varies between 7.1 and 7.3, i.e. pH_e is slightly alkaline but more acidic than that in blood or cerebrospinal fluid (CSF). In the spinal cord of the rat, regional differences in pH_e were found in unstimulated spinal cords. The pH_e in the lower dorsal horn was about 7.15, i.e. lower by about 0.2 pH units than that measured in the CSF. In the upper dorsal horn and in the ventral horn, the pH_e base line was only slightly more acid (7.25–7.35) than in supraspinal fluid (7.35–7.40). An alkaline shift in pH_e base line of 0.05–0.10 pH units was found after blockade of synaptic activity by applying Mn^{2+} in a concentration of 4–5 mM onto the spinal cord surface. The variations in pH_e base line had a similar depth profile as the variations in $[K^+]_e$ base line in unstimulated spinal cords. It has been demonstrated previously that increases in $[K^+]_e$ base line found in the lower dorsal horn are associated with a high level of spontaneous activity of dorsal horn neurons due to continuous excitatory input from the periphery (Svoboda *et al.*, 1988).

Transient acid shifts in pH_e by 0.01–0.05 pH units were found when acute nociceptive stimuli (pinch, press, heat) were applied to the hind paw (Fig. 2A). Chemical or thermal injury evoked by subcutaneous injection of turpentine or by application of 1–3 ml of hot oil onto the hindpaw produced a long-term decrease in pH_e base line in the lower dorsal horn by about 0.05–0.10 pH units. The decrease in pH_e began 2–10 min after injury and persisted for more than 2 h. Electrical nerve stimulation (10–100 Hz, 20–60 s) elicited triphasic (alkaline–acid–alkaline) changes in pH_e by 0.1–0.2 pH units (Fig. 2B), which had a similar depth profile as the concomitantly recorded increase in $[K^+]_e$.

K^+ and pH_e changes during postnatal development

In unstimulated pups at P3–P14, the $[K^+]_e$ base line was elevated and pH_e was more acidic than in perfusion solution (3.5 mM-K^+, pH 7.3–7.35). The $[K^+]_e$ and pH_e at P3–P6 were 3.91 ± 0.12 mM and 7.19 ± 0.01 ($n = 14$, mean ± S.E.), while at P10–P14 they were 4.35 ± 0.15 mM and 7.11 ± 0.01 ($n = 10$), respectively.

At P3–P6, the $(K^+]_e$ changes evoked in the dorsal horn by a single electrical stimulus were as large as 1.5–2.5 mM. Such changes in $[K^+]_e$ are evoked in the adult rat spinal cord with stimulation at a frequency of 10–30 Hz (Svoboda *et al.*, 1988). The maximal changes by 4.03 ± 0.24 mM ($n = 30$) were found at a stimulation frequency of 10 Hz. In older animals the $[K^+]_e$ changes progressively decreased to 1.95 ± 0.12 mM ($n = 30$). At P3–P8, the stimulation evoked an alkaline shift by 0.05–0.15 pH units, which was followed by a smaller post-stimulation acid shift by 0.03–0.07 pH units (Fig. 3). However, at P10–P14, the stimulation evoked an acid shift by 0.02–0.10, which was preceded by a scarcely discernible alkaline shift as in adult animals (Fig. 3).

The activity-related extracellular K^+ and pH_e changes in spinal cord of rats were studied after postnatal X-irradiation (PI)—a procedure which blocks gliogenesis and leads to astrogliosis. PI effects in neonatal rat spinal cord include: inhibition of myelin formation within the spinal cord, yet no extensive change in myelin formation in spinal roots; marked decreases in the number of neuroglial nuclei; hypertrophy of astrocytes, but no widespread alterations in spinal neurons (Gilmore, 1963a,b). Typical development of glial fibrillary acidic protein (GFAP) positive reaction was observed at P3–P14 in non-irradiated (control) pups (Syková *et al.*, 1992). At P3–P6, the membrane limitans was densely stained as well as radial glia from subpial zone inward through the white matter. In gray matter, small densely stained somata with very short processes were seen. At P10–P14, astrocytes in the gray matter were stained more intensively than at P3–P6; they showed larger soma and longer processes. In 10- to 14-day-old pups, PI GFAP staining of radial glia in white matter was disrupted and GFAP staining of astrocytes in gray matter revealed typical astrogliosis; astrocytes had unusually large soma, thick and long processes (Syková *et al.*, 1992).

At P10–P14 PI, the stimulation-evoked $[K^+]_e$ and pH_e changes resembled those found in control animals during the early postnatal days, i.e. at P3–P6. The stimulation at a frequency of 10 Hz increased $[K^+]_e$ by 4.03 ± 0.25 mM ($n = 12$) and there was a typical dominant alkaline shift (Fig. 4). Furthermore, the measurements of the K^+ and pH_e base lines in unstimulated pups revealed similar values as in control animals at P3–P6. These results show that postnatal X-irradiation impairs normal development of extracellular K^+ and pH_e homeostasis.

Mechanisms of the extracellular K^+ and pH homeostasis

Active neurons lose K^+ and induce a change in the ionic composition of the ECS. This may be an important way in which neurons interact and integrate their activity; however, stability of nervous tissue function requires fast renewal of the ECS ionic composition. While the pH_e homeostasis is more complicated (see below), K^+ homeostasis in CNS is ensured by two main mechanisms: (1) activation of the Na/K pump in neurons and glia and (2) glial cell K^+ uptake or buffering of extracellular rise in $[K^+]_e$ (for reviews see Syková, 1983; Walz, 1989; Syková, 1992). The conclusion that K^+ is cleared by means of active transport is strongly supported, since there is a post-stimulation decrease below resting level, the so-called 'K^+-undershoot' (Figs 2 and 4), which can be blocked by inhibitors of

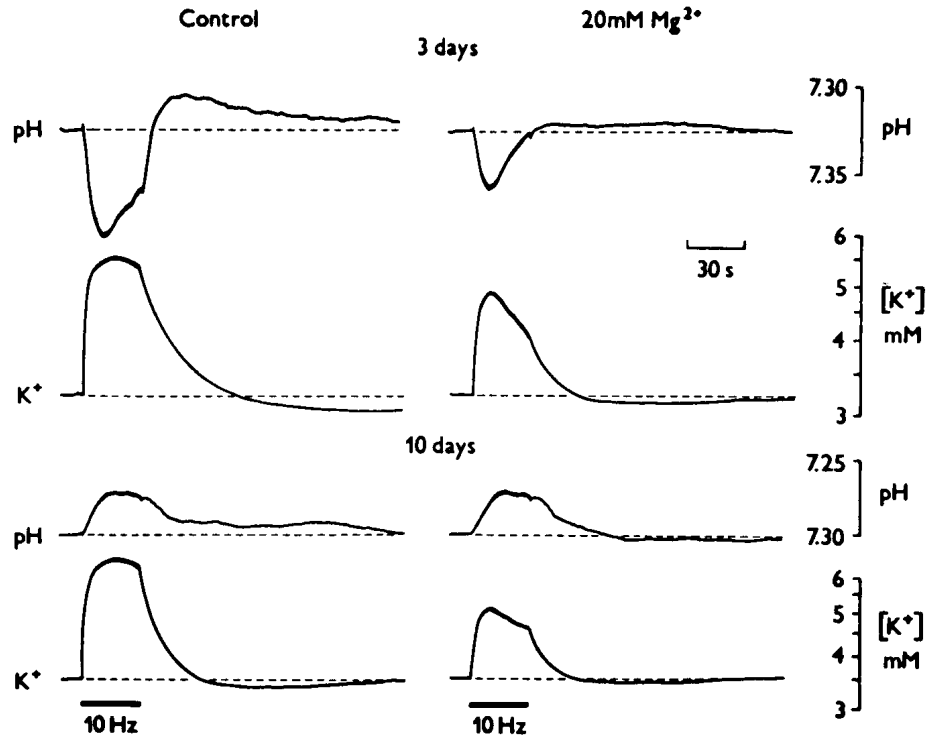

Fig. 3. Left: stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of rats 3 and 10 days old. Note that the stimulation of the dorsal root at a frequency of 10 Hz evoked an alkaline shift in the 3-day-old pup, which was accompanied by an increase in $[K^+]_e$; when stimulation was discontinued, the post-stimulation acid shift of smaller amplitude appeared, which was accompanied by a K^+-undershoot. In the 10-day-old rat, the $[K^+]_e$ increase was smaller; there was a slight initial alkaline shift, which was followed by an extracellular acid shift. Right: effect of 20 mM-Mg^{2+} on pH_e and $[K^+]_e$ in the spinal dorsal horn of rats 3 and 10 days old. Note decrease of the alkaline shift, decrease of the post-stimulation acid shifts, and decrease of $[K^+]_e$ changes. Only the acid shift that occurred during stimulation in the 10-day-old rat remained unaffected. (From Jendelová and Syková, 1991, with permission.)

the Na/K pump by e.g. ouabain, or by anoxia, ischaemia or anaesthetic drugs. Glial cells, however, also contribute significantly to activity-related clearance of K^+ increase in ECS, preventing long-term and widespread $[K^+]_e$ shifts. There is evidence that K^+ clearance by glia includes uptake of K^+ driven by the Na/K pump (Hertz, 1965), KCl uptake and K^+ spatial buffering (Orkand *et al.*, 1966). Our results show that mature glial cells significantly contribute to K^+ and pH homeostasis, since K^+ as well as pH_e homeostasis is impaired in the first postnatal week, when many glial cells are still in the precursor stage (Syková *et al.*, 1992; Chvátal *et al.*, 1993) as well as in pups PI, when gliogenesis is impaired and astrocytes proliferate (Syková *et al.*, 1992).

Effects of Mg^{2+} and acetazolamide

Application of 20 mM-$MgCl_2$ reversibly blocked the alkaline shifts at P3–P6 (Fig. 3) as well as in animals PI. Since Mg^{2+} is known to block Ca^{2+}-dependent presynaptic transmitter release, these results show that the alkaline shifts are of synaptic origin. The application of Mg^{2+} also lowered the $[K^+]_e$ changes by 60–70%, suggesting that about 30–40% of stimulation-evoked K^+ release could be presynaptic, i.e. from activated primary afferent fibres (Fig. 3). These findings suggest that during development a greater portion of K^+ comes from primary afferent fibres themselves than in adult animals, where the majority of K^+ ions are released postsynaptically (Syková and Vyklický, 1977).

In immature rats and in animals PI, the alkaline shift was enhanced by the carbonic anhydrase (CA) inhibitor acetazolamide (0.05–0.5 mM (by $26.4 \pm 15.9\%$ ($n=6$). The acetazolamide blocked the post-stimulation acid shift in rats at P3–P6 and decreased its amplitude at P10–P14 (Jendelová and Syková, 1991; Syková *et al.*, 1992). The enzyme CA, which catalyses the conversion of CO_2 and water to HCO_3^- and H^+, is mainly located in glia and myelin, but its presence in the ECS has also been demonstrated (Chen and Chesler, 1992; Kaila *et al.*, 1992). Our results suggest that there is little or no extracellular CA present during the first postnatal week and in rats PI.

Effects of amiloride, SITS and Ba^{2+}

It has been shown that acid extrusion in CNS neurons and glia involves amiloride-sensitive Na^+/H^+ exchange, SITS-sensitive Cl^-/HCO_3^- or $Na^+/Cl^-/H^+/HCO_3^-$ cotransport (for reviews see Walz, 1989; Chesler, 1990; Syková, 1992). In the presence of Ba^{2+}, the sensitivity of the glial membrane to changes in K^+ is reduced, because of competition between K^+ and Ba^{2+} for K^+ channels. We studied

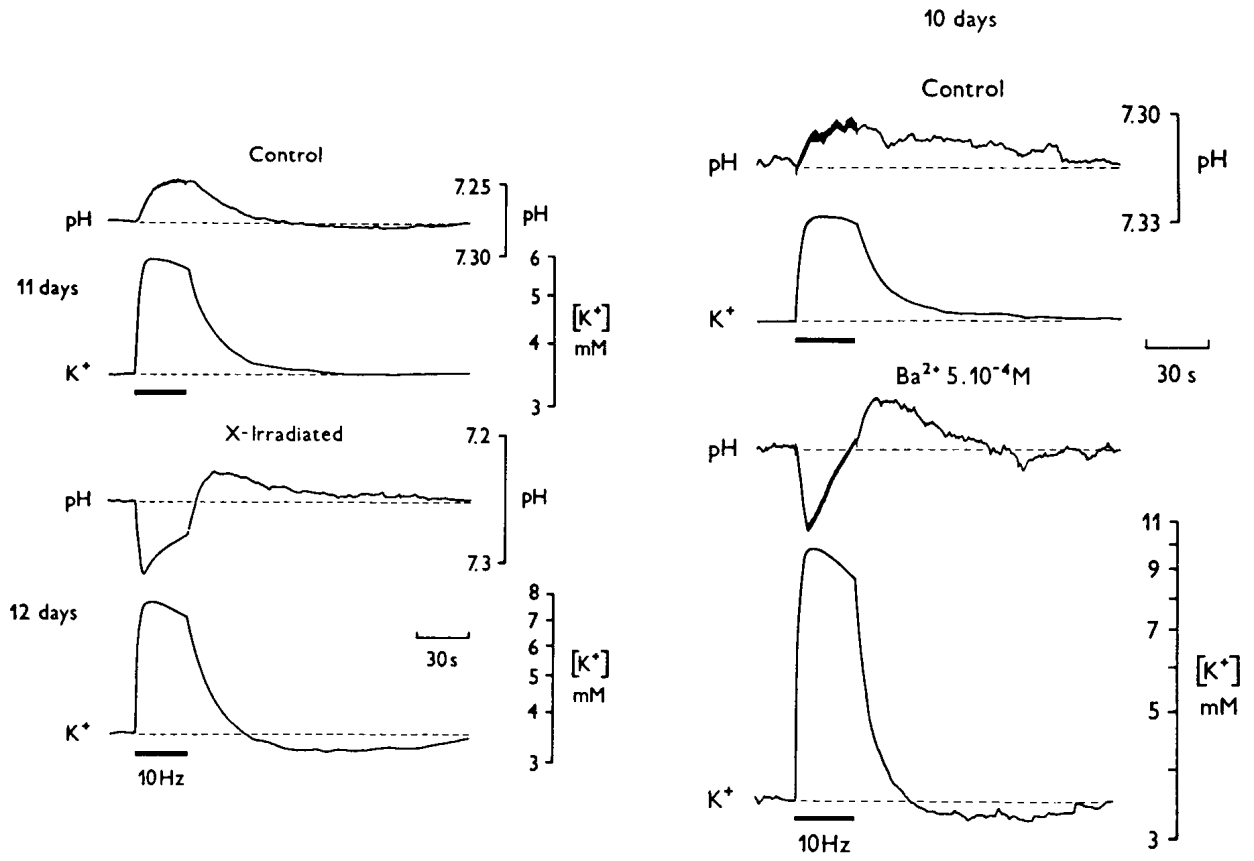

Fig. 4. Left: stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of non-irradiated 11-day-old rat and in 12-day-old rat PI. Note that the stimulation of the dorsal root at a frequency of 10 Hz evoked an acid shift in the 11-day-old pup. The alkaline shift which was accompanied by an increase in the $[K^+]_e$ ceiling level was found in 12-day-old animals PI. Right: effect of Ba^{2+} (5×10^{-4} M) on pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 10-day-old rats. Note the reversal of acid shift into alkaline shift. Ba^{2+} enhanced the $[K^+]_e$ ceiling level.

effects of these inhibitors of membrane transport processes on the activity-related alkaline shift, presumably of a neuronal origin, and on the acid shift, which was found to be related to gliogenesis.

In adult rats, P3–P14 rats and in animals PI, the application of amiloride in concentrations of 3 mM enhanced the alkaline and blocked the acid shifts evoked by stimulation. Application of SITS in concentrations of 0.5 mM had a similar effect. In applied concentrations, amiloride and SITS did not significantly affect changes in $[K^+]_e$, showing that the observed effects were specific. In control rats at P10–P14, the addition of 0.5 mM-Ba^{2+} resulted in dramatic enhancement of the K^+ ceiling level to about 8–10 mM, apparently because the K^+ buffering by glia was blocked (Fig. 4). The addition of Ba^{2+} had no effect on the already higher K^+ ceiling level at P3–P6 rats PI, apparently due to incomplete gliogenesis. Since glial cells appear to extrude acid in response to membrane depolarization due to activity-related rises in $[K^+]_e$ (Chesler and Kraig, 1989; Syková *et al.*, 1992), application of Ba^{2+} enhanced the stimulation-evoked alkaline shifts or even reversed the acid shift to alkaline (Fig. 4). In control rats PI, Ba^{2+} had no effect on alkaline shifts. These results suggest that the stimulation-evoked acid shifts in pH_e are related to depolarization of glial cell membranes by elevated K^+. Ba^{2+} prevents the activity-related depolarization of glia, eliminates its intracellular alkalinization and thus results in the removal of a rapid acid source.

Effects of gamma-aminobutyric acid (GABA) and glutamate

A number of channels could mediate alkaline shifts in pH_e. It has been shown that the GABA-gated anion channel has a significant permeability to HCO_3^- (Bormann *et al.*, 1987; Kaila *et al.*, 1989; Kaila and Voipio, 1987). Since the electrochemical gradient for HCO_3^- is outward, release of GABA can evoke a HCO_3^--dependent fall in intracellular pH of neurons and a concomitant rise in pH_e. Superfusion of spinal cords of P3–P14 rats as well as of PI rats, with GABA (10^{-3} M), elicited extracellular alkaline shift in the pH_e baseline (Fig. 5A), which was blocked by picrotoxin. The alkaline shifts evoked in our experiments by superfusion of the spinal cord with GABA were accompanied by an increase in $[K^+]_e$ (Fig. 5A). However, the superfusion of spinal cord with Ringer solution containing elevated K^+ produced an acid shift in the pH_e baseline (Fig. 5B). Picrotoxin (10^{-7}–10^{-6} M) had no effect on $[K^+]_e$ and the pH_e baseline; however, it depressed the stimulation-evoked alkaline shifts in

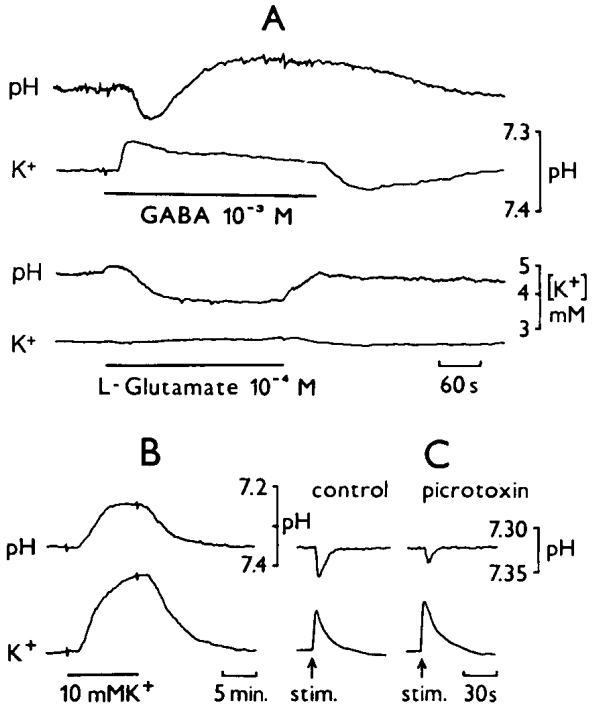

Fig. 5. (A) Effect of GABA (1 mM) and glutamate (0.1 mM) on pH_e and $[K^+]_e$ base line in the isolated spinal cord of 4-day-old rat. Note the alkaline shift and increase in $[K^+]_e$ during application of GABA. (B) Simultaneous pH_e and $[K^+]_e$ records in the dorsal horn during perfusion of the spinal cord with a solution containing 10 mM-K^+. (C) Stimulation of the dorsal root with a single electrical pulse evoked an alkaline shift in 5-day-old rat, which was accompanied by an increase in $[K^+]_e$. Application of picrotoxin (10^{-6} M) decreased the alkaline shift.

pH_e by about 40–60% (Fig. 5C). In nominally HCO_3^--free solution, buffered with HEPES, GABA evoked no alkaline shift. These experiments suggest that gating of GABA channels in immature rats as well as in rats with blocked gliogenesis can give rise to an HCO_3^- efflux which can partially (about 50%) explain the pH_e alkaline shifts observed in the rat dorsal horn during a stimulation of an afferent input.

Fig. 5A shows the typical alkaline shift in spinal cord of P3–P14 rats which was evoked by glutamate. Glutamate at concentrations of 0.01–0.1 mM evoked alkaline shifts which were blocked by Mn^{2+} or Mg^{2+}. The alkaline shifts evoked by glutamate, however, persisted in HCO_3^--free solution and were insensitive to picrotoxin, indicating that the observed alkaline shifts are not due to efflux of HCO_3^- through $GABA_A$ receptor channels. The alkaline shifts elicited by excitatory amino acids (EAA) therefore have a completely different mechanism. The mechanism is apparently similar to the alkaline shifts described at the molecular layer of the cerebellum, which were blocked by the EAA antagonist kynuretic acid (Chester and Chan, 1988; Chesler and Rice, 1991), or to the alkaline shifts in hippocampal slices blocked by CNQX but not APV, and therefore generated postsynaptically by AMPA/kainate receptors (Chen and Chesler, 1992). The extracellular alkaline shifts are apparently

Table 1. ECS diffusion parameters (mean ± S.E.) in the rat spinal cord as a function of age, α is ECS volume fraction, λ is ECS tortuosity, k' is non-specific uptake, n is number of experiments. For further details see Methods

Age (days)	α	λ	k' (10^{-3} s^{-1})	n
4–6	0.37±0.01	1.78±0.02	3.61±0.56	17
10–14	0.24±0.003	1.74±0.01	4.7±1.2	3
90–120	0.20±0.003	1.62±0.02	4.6±0.4	39

associated with activation of a number of channels not only in the adult animals, but also during development.

ECS volume and diffusion parameters in spinal cord of adult rat

After the TMA^+ diffusion coefficient (D) and the transport number for the iontophoresis pipette (n) had been determined in 0.3% agar (for details see Methods), the microelectrode array was inserted from the dorsal spinal surface into the spinal dorsal horn at depths between 500 and 900 μm, where diffusion measurements were made. A typical TMA^+ diffusion curve recorded in agar and a spinal cord of an adult rat is illustrated in Fig. 1. Superimposed on each experimental curve is the theoretical curve, derived from equation 1, using the extracted parameters α, λ and k', together with the other parameters defining the experiment. A good correlation between the experimental and theoretical curve is the test of the quality of the curve-fitting procedure and of the extracted diffusion parameters. In adult rats, diffusion parameters in the dorsal horn gray matter at a depth of 500–900 μm were: $\alpha = 0.20 \pm 0.003$; $\lambda = 1.62 \pm 0.02$, $k' = 4.6 \pm 0.4 \times 10^{-3}$ s^{-1}, $n = 39$ (Table 1). No significant differences in α, λ and k' have been found at the depth from 500 to 900 μm.

It has been recognized that during depolarization and neuronal activity, the ECS can reversibly decrease due to the activity-related cell swelling. Experimental elevation of $[K^+]_e$ by KCl application leads to membrane depolarization of various brain cells, to an increase in the volume of the cells, and to a compensatory ECS volume decrease due to cellular accumulation of ions (particularly K^+, Na^+, Cl^-, HCO_3^-) and of water. K^+-induced swelling is mostly attributed to glia, particularly to astrocytes (for review see Kimelberg and Ransom, 1986), but swelling of neuronal elements has also been shown (Ritchie *et al.*, 1981; Trubatch *et al.*, 1977; Tasaki and Iwasa, 1982). These previous studies, however, did not furnish any information on the size of the ECS volume changes or on their dynamics. Repetitive electrical stimulation of peripheral nerves at 30–100 Hz increased ECS volume fraction in Rexed laminae III–V by 15.8±2.7% ($n = 5$), i.e. during the electrical stimulation ECS volume increased

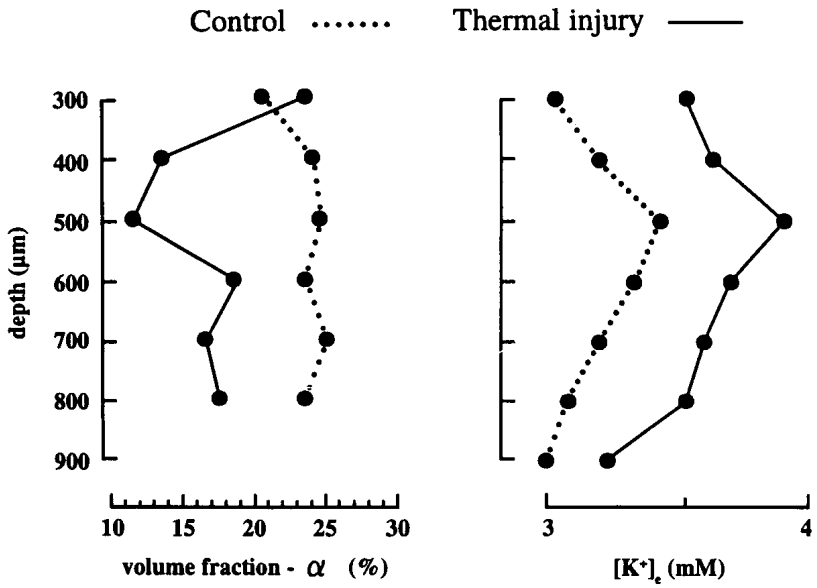

Fig. 6. Effect of thermal injury of the hind paw on the ECS volume fraction (α) at various depths of the spinal dorsal horn. Left: typical ECS volume fraction at various depths of the spinal dorsal horn in the non-stimulated animal before injury (control) and the ECS volume fraction decrease at a depth of 400–700 μm (injury). The maximal ECS volume fraction decrease from about 24% to about 12% was found at a depth of 500 μm 10–25 min after thermal injury. Right: $[K^+]_e$ base line at various depths of the spinal dorsal horn in the control rat and in the rat after injury.

apparently due to shrinkage of some neural elements. However, after the end of stimulation, when the $[K^+]_e$ decreased below the original baseline (during K^+-undershoot), the ECS volume decreased by 20–40%, i.e. to α about 0.14–0.17 (Svoboda and Syková, 1991). The magnitude and duration of the ECS volume decrease were positively related to the stimulation frequency and duration. The ECS volume decrease was maximal at 2–10 min after the stimulation had been discontinued, and it returned to the prestimulation value in 15–40 min. The ECS volume decreased by 20–50% (but no more than $\alpha = 0.12$) after injury of the ipsilateral hind paw evoked either by subcutaneous injection of turpentine or by thermal injury (Fig. 6). The maximal changes were found in dorsal horn Rexed laminae III–V, 5–10 min after injection of turpentine and 10–25 min after thermal injury, and persisted for more than 120 and 30 min, respectively. The ECS tortuosity and non-specific uptake were not significantly altered by stimulation or injury. These measurements indicate that the normal volume of the ECS in adult rats occupies 1/5–1/4 of the total CNS volume and the significant dynamic changes in the ECS volume accompany transmembrane ionic shifts resulting from neural activity.

ECS diffusion parameters during postnatal development

The ECS volume fraction, tortuosity and non-specific cellular uptake were examined in isolated spinal cords of rats at P3–P14, i.e. during the period of extensive gliogenesis. We demonstrated recently, that similarly in cortical gray and in corpus callosum, the ECS is larger in immature rats, and its size decreases with the animal's age (Lehmenkühler *et al.*, 1993).

In 4- to 6-day-old rat pups, the diffusion parameters in the dorsal horn gray matter at depth 300 μm were: $\alpha = 0.37 \pm 0.01$, $\lambda = 1.78 \pm 0.02$, $k' = 3.61 \pm 0.56 \times 10^{-3}$ s^{-1} ($n = 17$) (Table 1), i.e. α was significantly higher, λ was significantly larger, while the k' was not significantly different from adult rat spinal cord values *in vivo*. The α and λ gradually decreased with age, at P10–P14 $\alpha = 0.24 \pm 0.003$, $\lambda = 1.74 \pm 0.01$ and $k' 4.7 \pm 1.20 \times 10^{-3}$ s^{-1} ($n = 3$), i.e. α was still significantly higher than in adult animals (Table 1). Fig. 7 shows the typical diffusion curves recorded in isolated rat spinal cord at postnatal day 5 and 12. It can be seen that α decreased from 0.38 to 0.24, respectively. The ECS volume fraction and tortuosity at P5–P12 were therefore significantly higher than in adult rats (see also Fig. 1 and Table 1). It is therefore evident that the different diffusion parameters of the ECS during development could significantly alter the movement of ions, neurotransmitters and metabolites in the microenvironment of neuronal and glial cells during physiological as well as pathological events.

DISCUSSION AND CONCLUSIONS

Extracellular K^+ homeostasis

Elevations in $[K^+]_e$ are associated with neural activity evoked by various kinds of stimulation. The main sources of K^+ are stimulated neurons, unmyelinated terminals and fibres. The reason for the increase is obvious: as K^+ is released with each impulse, ongoing activity results in substantial K^+ accumulation in ECS. The mechanisms of K^+

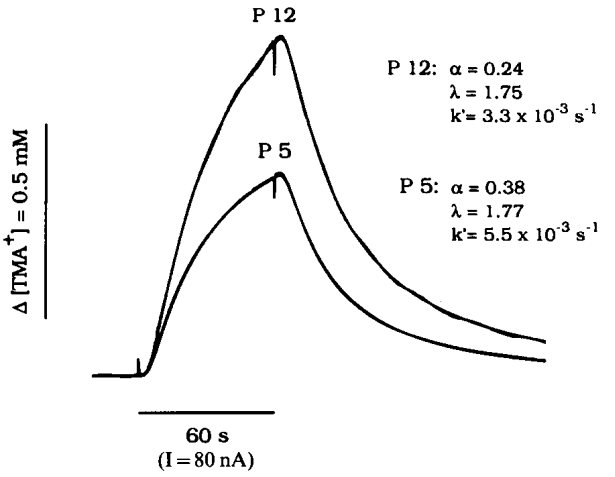

Fig. 7. TMA^+ diffusion curves obtained in the isolated rat spinal cord in the dorsal horn of segment L_4 at P5 and P12. Diffusion parameters: volume fraction (α), tortuosity (λ) and non-specific cellular uptake (k') were determined with the use of the microelectrode array with spacing between the TMA^+-sensitive microelectrode and iontophoresis pipette of 140 μm and 150 μm, iontophoresis transport number $n = 0.417$ and $n = 0.485$, respectively. The main iontophoretic current was 80 nA (bias current was 20 nA). Note that the increase in diffusion curve amplitude reflects the declining volume fraction with age. The values of α, λ and k' are shown with each record.

accumulation, homeostasis and physiological significance have been reviewed in several papers (Syková, 1983; Walz, 1989; Syková, 1992) and will therefore not be discussed in detail.

In 3- to 6-day-old rats and in rats PI, K^+ and pH_e homeostasis is impaired (Jendelová and Syková, 1991), but there are no apparent changes in neuronal Na/K pump activity (see Figs 3–5, normal K^+-undershoot) and the increase in $[K^+]_e$ is as fast as in adult rats. Similarly, larger stimulation-evoked changes in $[K^+]_e$ were found in immature optic nerve (Connors *et al.*, 1982; Ransom *et al.*, 1985b) and in cerebral cortex (Hablitz and Heinemann, 1987; Mutani *et al.*, 1974). The changes are larger despite the fact that the size of the ECS in immature nervous tissues is bigger (Fig. 7, Lehmenkühler *et al.*, 1993) and that the stimulation of the afferent input does not cause ECS shrinkage, which can aggravate the ionic changes (Ransom *et al.*, 1985a). The K^+ ceiling level gradually decreased with the animal's age, and in 12- to 14-day-old pups was comparable with the K^+ ceiling level of adult rats. A large increase in the K^+ ceiling level in 10- to 14-day-old control animals was found after blockade of glial cells' K^+ channel permeability by Ba^{2+} (see Fig. 4), while there was no further increase in ceiling level with Ba^{2+} in immature rats and in rats PI (Syková *et al.*, 1992). The maturation of astrocytes, oligodendrocytes and myelinization (Connors *et al.*, 1982; Gilmore, 1971; Sims *et al.*, 1985), as well as CA activity (Davis *et al.*, 1987) occurs in the first 8–10 days postnatally. Although the rate of K^+ release from neurons as well as the properties, distribution and density of K^+ and Cl^- channels can also change during development (for further discussion see Jendelová and Syková, 1991; Walton and Chesler, 1988; Walz, 1989), the observed differences in K^+ ceiling level give strong evidence that immature glia in the first 8–10 days postnatally and proliferative glia after PI are not able to control K^+ homeostasis.

Mechanisms of alkaline shifts in pH_e

Alkaline shifts related to neuronal activity have been described in many studies (for reviews see Chesler, 1990; Syková, 1992). It has been suggested that alkaline shifts are due to (1) channel-mediated flux of acid equivalents, i.e. efflux of HCO_3^- through GABA-gated Cl^- channels, or (2) bicarbonate-insensitive flux of hydrogen ions through cationic channels, activated by glutamate or aspartate. The Ca^{2+} channel blockers (Mn^{2+}, Mg^{2+}) diminished the alkaline shifts, suggesting that the activity-related alkaline shifts are related to voltage-activated Ca^{2+} channels. However, since the kynurenate similarly blocked the alkaline shifts in cerebellum (Chesler and Chan, 1988) it is evident that the effect of divalent cations was due rather to a block of synaptic transmission. The alkaline shifts can also be evoked by directly iontophoresing glutamate or aspartate in turtle cerebellum *in vitro*, even in solution containing divalent cations (Chesler and Rice, 1991), indicating that the gating of voltage-sensitive Ca^{2+} channels was not directly involved in the generation of the alkaline shifts. It is clear that inhibition of the alkaline shifts by a specific antagonist can so far only indicate which channel is triggering the response, but not that alkaline shifts are due to a channel-mediated flux of acid equivalents (see also Chesler and Rice, 1991). The stimulation-evoked alkaline shifts were enhanced in HEPES-buffered saline, and when activity of CA was blocked by acetazolamide (Syková, 1989). These findings suggest that bicarbonates play an important role in the mechanism of the initial alkaline shifts. Their enhancement is most likely attributed to the decrease in extracellular buffering power.

In our experiments on rat spinal cord at P3–P14, the superfusion of spinal cord with GABA (10^{-3} M) in the presence of HCO_3^- elicited an extracellular alkaline shift in control 3- to 14-day-old pups as well as in pups PI (Fig. 5), and this response was blocked by picrotoxin in concentrations 10^{-7}–10^{-6} M (in this concentration picrotoxin had no effect on K^+ and pH_e resting levels). This suggests that Cl^- channels opened by GABA in spinal cord during development give rise to an HCO_3^- efflux which, as is the case in the brain (Chen and Chesler, 1990, 1991; Kaila *et al.*, 1992) and in muscle fibres (Kaila *et al.*, 1989), increases the pH_e. In contrast, glutamate-evoked alkaline shifts persisted in HCO_3^--free solution and were not affected by picrotoxin. The mechanisms of alkaline shifts evoked by excitatory amino acids were discussed above and still remain to

be clarified. However, both GABA- and glutamate-evoked alkaline shift are not related to gliogenesis, since they are present in the early postnatal period and in rats PI.

The contribution of the stimulation-enhanced blood flow and washout of CO_2 to the alkaline shift was suggested by Urbanics *et al.* (1978). However, *in vitro* measurements in isolated turtle cerebellum (Chesler and Chan, 1988), isolated spinal cord of the frog (Chvátal *et al.*, 1988) and of the rat (Jendelová and Syková, 1991) revealed that alkaline shift could occur in the absence of blood flow. Finally, the recently described stimulation-related ECS volume shrinkage in spinal cord of adult rats (Svoboda and Syková, 1991), which, however, has not been found during development (Ransom *et al.*, 1985a,b), may play a role in pH_e shifts.

Mechanisms of the ECS acid shifts

The acid shifts observed in our experiments could be a result of: (1) classic acid extrusion membrane transport systems (Na^+/H^+ or $Na^+/HCO_3^-/Cl^-/H^+$); (2) Na^+-HCO_3^- cotransport into glial cells; and (3) efflux of lactic acid from metabolically active cells. Both the neurons and glial cells (oligodendrocytes as well as astrocytes) have been shown to possess several membrane transport systems which transport ions, including H^+ and HCO_3^- (for reviews see Walz, 1989; Chesler, 1990). Mammalian astrocytes actively regulate their pH_i by acid extrusion membrane transport processes which are dependent on Na^+, Cl^- and HCO_3^-. Similar to adult rats, the acid shift in immature rats was blocked by amiloride and SITS and therefore has a complex mechanism which may include Na^+/H^+ exchange, $Na^+/Cl^-/HCO_3^-/H^+$ exchange and Na^+-HCO_3^- cotransport. Glial cells in tissue culture also extrude lactic acid but only in response to the massive depolarization by large elevations of bath $[K^+]_e$ (Walz, 1989). However, superfusion of isolated frog spinal cord with NaF, which blocks the glycolytic portion of metabolic processes and production of lactate, had no effect on stimulation-evoked changes in pH_e (Syková, 1992). It is, therefore reasonable to assume that glial cells regulate pH_e homeostasis at the expense of their intracellular pH. Our experiments revealed that activity-related acid shifts in pH_e mask the alkaline shifts resulting from neuronal activity. The acid shifts are closely related to gliogenesis and were absent in the glial cell-depleted spinal cord PI.

In the spinal cord at P3–P14, Ba^{2+}, which blocks K^+ channel permeability in glial cells, blocked the stimulation-evoked acid shift, enhanced the alkaline shift and the K^+ ceiling level (Fig. 4B). With superfusion of the rat cortex with Ba^{2+}, the initial alkaline shift was also enhanced (Chesler and Kraig, 1989). If the HCO_3^- is a counterion to K^+ during glial cell depolarization, the block of K^+ channels with Ba^{2+} would result in a block of the pH_e acid shift. Similarly, Ba^{2+} induced hyperpolarization of glia in *Necturus* optic nerve and increased Na^+-HCO_3^- cotransport into glial cells (Astion *et al.*, 1987, 1989). Increase in Na^+-HCO_3^- cotransport in glia can, therefore, contribute to the observed stimulation-evoked acid shifts. Blocking any acid extrusion system in glial cells would consequently result in enhancement of any extracellular alkaline shift, as has been found in our experiments after application of Ba^{2+}, amiloride or SITS.

ECS diffusion parameters

The mechanisms of cellular shrinkage and swelling during neuronal activity is discussed in several papers (for reviews see Kempski, 1986; Kimelberg and Ransom, 1986; Svoboda and Syková, 1991). The mechanisms include: (1) extracellular ionic changes (H^+ and K^+) accompanied by uptake of Na^+, Cl^- and water, (2) increase in intraneuronal osmolarity and/or local extracellular decrease in osmolarity, (3) glial spatial buffer mechanism—glial swelling due to increase in intracellular particles accompanied by water, (4) glial swelling due to metabolic alterations such as acid–base balance, (5) increase in intraneuronal particles, produced by enhanced metabolism during excessive neuronal activity. It is evident that the glial cells, and particularly astrocytic swelling, play an important role in activity-related changes. However, there is no direct evidence for astrocytes swelling in mammalian CNS *in vivo*.

We performed experiments on rat spinal cord and cortex during development, i.e. during the period of extensive gliogenesis. As in cortex (Lehmenkühler *et al.*, 1993), the ECS in spinal cord at P4–P14 is larger, and its size decreases with the animal's age. The diffusion will be governed by the value of α and λ, with the apparent diffusion coefficient given by D/λ^2, where D is the free diffusion coefficient. It has long been known that developing nervous tissue has a larger ECS and greater abundance of extracellular glycosaminoglycans and hyaluronate than adult tissue (Margolis *et al.*, 1986), which might affect the movement of charged molecules. Our results show that tortuosity is significantly higher in the developing rat spinal cord, which is not the case in developing cortex (Lehmenkühler *et al.*, 1993). It remains to be shown whether these diffusion parameters differ under pathological conditions in the developing brain.

Under physiological conditions, a decrease in the ECS volume fraction would lead to a larger rise in the interstitial concentration of transmitters, such as glutamate, and thus could lead to larger tonic activation of N-methyl-D-aspartate receptors. It may also lead to greater transient ionic changes, particularly enhanced $[K^+]_e$ during cell firing, which affects neuronal excitability and synchronization. In contrast, the large activity-related ionic changes which occur during pathological events such as

anoxia, seizures or spreading depression would be reduced, e.g. excessive accumulation of EAA, inhibitory transmitters and metabolic substrates. Low ECS volume fraction is a prerequisite for a more marked change (shrinkage) during activity and elevation in $[K^+]_e$. This could be a reason for the increased vulnerability of the adult brain to cell damage after ischaemia or hypoxia, epileptic seizures and spreading depression, which cause a rise in $[K^+]_e$, acidosis and ECS volume shrinkage (Lehmenkühler *et al.*, 1985; Phillips and Nicholson, 1979; Rice and Nicholson, 1991; Syková *et al.*, 1992). Indeed, the relative susceptibility of the immature brain to anoxia, ischaemia and seizures is smaller than in adult brain.

Physiological significance of the ionic and volume changes

The physiological significance of K^+, pH_e and ECS volume changes has been of great interest. It is evident that transient ionic changes in the nerve cell microenvironment might be a powerful mechanism in the regulation and integration of CNS function. Changes in $[K^+]_e$ (and also in $[Ca^{2+}]_e$) might serve as a signal. K^+ and Ca^{2+} can influence neuronal excitability, transmitter release, intercellular interaction and glial cell function (for reviews see Somjen, 1979; Nicholson, 1980; Syková, 1983). It is assumed that the increase in $[K^+]_e$ that is associated with repetitive neuronal activity reduces transmitter release from presynaptic terminals by curtailing the presynaptic spike amplitude by presynaptic depolarization (Kříž *et al.*, 1975; Czeh *et al.*, 1981). Moreover, this effect of increased $[K^+]_e$ could be enhanced by a concomitant decrease in $[Ca^{2+}]_e$. Instead of its presynaptic action, the increase in $[K^+]_e$ influences the impulse transmission postsynaptically, by depolarization of neurons and glia. K^+-mediated depolarization of postsynaptic membranes enhances neuronal excitability, which under some circumstances can dominate over its presynaptic inhibitory effect. K^+ accumulation in ECS therefore could act as a negative–positive feedback control system. The $[K^+]_e$ changes, determined by neuronal release, clearance and by glial cell (particularly astrocytes) redistribution, constitute another way by which neurons and astrocytes can communicate. Astrocytes exhibit a potassium-induced, non-calcium-dependent release of glutamate, which can exert an action on neuronal glutamate receptors, and they exhibit higher activity of the enzymes glutamine synthetase and pyruvate carboxylase (Norenberg *et al.*, 1988). An existence of a glutamate–glutamine cycle between neurons and astrocytes may serve as neuronal–astrocytic interaction (for review see Hertz, 1990). The role of K^+ accumulation and altered K^+ homeostasis during CNS development, in the modulation of neuronal activity and glial cell function, in states of CNS hypo- and hyperexcitability, in learning and memory formation and during pathological states is far from clear and is presumably related to the role of glia.

Activity-related dynamic changes in pH_e can be another important modulating and controlling factor in the CNS. Changes in pH_e of only a few tenths of a pH unit have been shown to evoke marked effects on neuronal ionic channels (Iljima *et al*; Konnerth *et al.*, 1987), to affect neuronal excitability (Gruol *et al.*, 1980; Balestrino and Somjen, 1988), cell to cell coupling (Roos and Boron, 1981) and glial cell membrane properties (for reviews see Walz, 1989; Chesler, 1990).

To conclude, significant changes in ECS volume which occur in the CNS during physiological conditions, development and in pathological states, could in addition to ionic changes in the ECS, influence neuronal excitability, properties of ionic channels, and glial cell function. Indeed, changes in the shape and swelling of dendritic spines have been found to occur in high K^+ or after extensive stimulation and can be the basis of long-term potentiation (Van Harreveld and Fifkova, 1975). An increase in synaptic efficiency due to the ECS shrinkage produced by swelling of glial cells may also be one of the mechanisms leading to long-term excitability increase, plastic neuronal changes, influence protheosynthesis and memory formation.

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