Regulation of intracellular sodium in cultured rat hippocampal neurones

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1. We studied regulation of intracellular Na^+ concentration ($[Na^+]_i$) in cultured rat hippocampal neurones using fluorescence ratio imaging of the Na^+ indicator dye SBFI (sodium-binding benzofuran isophthalate).

2. In standard CO_2/HCO_3^--buffered saline with 3 mM K^+, neurones had a baseline $[Na^+]_i$ of 8·9 ± 3·8 mM (mean ± S.D.). Spontaneous, transient $[Na^+]_i$ increases of 5 mM were observed in neurones on 27% of the coverslips studied. These $[Na^+]_i$ increases were often synchronized among nearby neurones and were blocked reversibly by 1 μM tetrodotoxin (TTX) or by saline containing 10 mM Mg^{2+}, suggesting that they were caused by periodic bursting activity of synaptically coupled cells. Opening of voltage-gated Na^+ channels by application of 50 μM veratridine caused a TTX-sensitive $[Na^+]_i$ increase of 25 mM.

3. Removing extracellular Na^+ caused an exponential decline in $[Na^+]_i$ to values close to zero within 10 min. Inhibition of Na^+,K^+-ATPase by removal of extracellular K^+ or ouabain application evoked a $[Na^+]_i$ increase of 5 mM min^{-1}. Baseline $[Na^+]_i$ was similar in the presence or absence of CO_2/HCO_3^-; switching from CO_2/HCO_3^--free to CO_2/HCO_3^--buffered saline, however, increased $[Na^+]_i$ transiently by 3 mM, indicating activation of Na^+-dependent $Cl^- – HCO_3^-$ exchange. Inhibition of $Na^+ – K^+ – 2Cl^-$ cotransport by bumetanide had no effect on $[Na^+]_i$.

4. Brief, small changes in extracellular K^+ concentration ($[K^+]_o$) influenced neuronal $[Na^+]_i$ only weakly. Virtually no change in $[Na^+]_i$ was observed with elevation or reduction of $[K^+]_o$ by 1 mM. Only 30% of cells reacted to 3 min $[K^+]_o$ elevations of up to 5 mM. In contrast, long $[K^+]_o$ alterations (≥ 10 min) to 6 mM or greater slowly changed steady-state $[Na^+]_i$ in the majority of cells.

5. Our results indicate several differences between $[Na^+]_i$ regulation in cultured hippocampal neurones and astrocytes. Baseline $[Na^+]_i$ is lower in neurones compared with astrocytes and is mainly determined by Na^+,K^+-ATPase, whereas Na^+-dependent $Cl^- – HCO_3^-$ exchange, $Na^+ – HCO_3^-$ cotransport or $Na^+ – K^+ – 2Cl^-$ cotransport do not play a significant role. In contrast to glial cells, $[Na^+]_i$ of neurones changes only weakly with small alterations in bath $[K^+]_o$, suggesting that activity-induced $[K^+]_o$ changes in the brain might not significantly influence neuronal Na^+,K^+-ATPase activity.

Regulation of intracellular Na^+ concentration ($[Na^+]_i$) is of vital importance for the nervous system, not only because Na^+ ions are the major current carriers during action potentials in most cells, but also because many other cell functions (e.g. intracellular Ca^{2+} homeostasis, intracellular pH homeostasis, reuptake of transmitters), are directly dependent on the Na^+ gradient (Blaustein, 1988; Chesler, 1990; Nicholls & Attwell, 1990). Until recently, $[Na^+]_i$ was mainly measured using ion-sensitive microelectrodes and, due to the large tip diameter of these electrodes, most data were obtained from invertebrate preparations (Thomas, 1972; Deitmer & Schlue, 1983; Coles & Orkand, 1985; Kaila, Rydqvist, Swerup & Voipio, 1987).

Studies on $[Na^+]_i$ regulation in vertebrate neurones are sparse (Grafe, Rimpel, Reddy & Ten Bruggencate, 1982; Ballanyi, Grafe, Reddy & Ten Bruggencate, 1984; Erecinska, Dagani, Nelson, Deas & Silver, 1991; Jaffe, Johnston, Lasser-Ross, Lisman, Miyakawa & Ross, 1992), and therefore several important questions remain unanswered. Although it is widely accepted that Na^+,K^+-ATPase pumping is responsible for the maintenance of the steep inwardly directed Na^+

gradient in neurones (Sweadner, 1995), the exact value of baseline $[Na^+]_i$ and the 'fine tuning' mechanisms maintaining it, as well as the contribution of other transporters or ion conductances to neuronal $[Na^+]_i$, are poorly understood.

The enzymatic mechanism and molecular characteristics of the Na^+,K^+-ATPase, and its diverse isoforms, respectively, have been studied intensively in a variety of tissues (Sweadner, 1995). Nevertheless it is still controversial whether the isoforms of Na^+,K^+-ATPase expressed in neurones can be stimulated by small increases in extracellular K^+ ($[K^+]_o$; Sweadner, 1995), or if the enzyme is already maximally activated by baseline $[K^+]_o$, which is around 3 mM. A significant alteration of Na^+,K^+-ATPase activity by changes in $[K^+]_o$ would indicate a role for neurones in the clearance of $[K^+]_o$ during neural activity, a function that has been convincingly demonstrated for glial cells (Ransom & Sontheimer, 1992; Newman, 1995).

Using fluorescence ratio imaging with the Na^+ indicator dye SBFI (sodium-binding benzofuran isophthalate; Minta & Tsien, 1989), it was shown recently that small physiologically relevant elevations of $[K^+]_o$ lead to changes in $[Na^+]_i$ in cultured rat hippocampal astrocytes, probably by activating both Na^+,K^+-ATPase and Na^+–K^+–$2Cl^-$ cotransport in these cells (Rose & Ransom, 1996*a*). In the present study, we used SBFI fluorescence imaging to determine basic mechanisms of $[Na^+]_i$ regulation in cultured rat hippocampal neurones. Our study revealed several significant differences in the regulation of baseline $[Na^+]_i$ between cultured hippocampal neurones and glial cells, and indicated that neuronal Na^+,K^+-ATPase, in contrast to the glial enzyme, is not significantly activated by the small elevations in $[K^+]_o$ that are associated with physiological neural activity.

METHODS

Cell cultures

For mixed neurone–glia cultures, rat pups (Sprague–Dawley rats, postnatal day 0) were anaesthetized by CO_2 narcosis and decapitated. Hippocampi were removed and exposed to papain (10 U ml^{-1} for 10 min; Worthington, Freehold, NJ, USA). After trituration the dissociated cells were plated onto poly-L-ornithine/laminin-coated (Sigma) coverslips in DMEM medium (Dulbecco's modified Eagle's medium; JRH Biosciences, Lenexa, KS, USA) mixed 1 : 1 with F12 (Ham's nutrient mixture F12; JRH Biosciences). In addition, the medium contained 10% (v/v) bovine serum (Gibco, Grand Island, NY, USA), 120 μg ml^{-1} streptomycin and 120 U ml^{-1} penicillin (Sigma). Cells were grown at 37 °C in a 5% CO_2 and 95% humid air atmosphere. Twenty-four hours later, cells were transferred into Neurobasal medium (Gibco) with the serum-free supplement B27 (50 : 1; Gibco), half of which was subsequently exchanged every 5–7 days. To inhibit glial cell division, the mitotic inhibitor ARA-C (cytosine-β-D-arabinofuranoside, Sigma; final concentration 5 μM) was added at day 5 in culture.

Cell cultures were obtained from newborn animals by dissecting a region that contained both the hippocampus proper and the *anlage* of the dentate gyrus. Therefore, they probably contained a mixture of neuronal subtypes, including neurones that developed from cells of the proliferation zone of the dentate hilus, as well as a high percentage of pyramidal neurones (cf. Schlessinger, Cowan & Swanson, 1978). Pyramidal cells and neurones from the dentate gyrus can differ in their electrophysiological properties (e.g. Glimm, Ficker & Heinemann, 1996). Basic differences in $[Na^+]_i$ regulation between neurones were not observed in the culture, and therefore cell types were not differentiated.

Measurements were made using cells between 14 and 21 days in culture. Neurones in culture were identified with monoclonal antibodies against neurone-specific enolase (Polysciences, Warrington, PA, USA) and neurofilament (68 kDa, clone NR4; Boehringer Mannheim Biochemica, Indianapolis, IN, USA). During the experiments, neurones were easily identified by their characteristic morphological appearance under the light microscope. Cells that appeared to be neurones with light microscopy were confirmed as such using selective staining techniques.

Solutions

The standard saline used for the experiments contained (mM): 115·75 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1·25 NaH_2PO_4, 23 $NaHCO_3$ and 10 glucose (osmolality, 304 mosmol kg^{-1}). It was continuously bubbled with 5% CO_2 and 95% O_2, resulting in a pH of 7·38. The CO_2/HCO_3^--free saline (316 mosmol kg^{-1}) contained the same amount of KCl, $MgSO_4$, $CaCl_2$, NaH_2PO_4 and glucose, but 126·25 mM NaCl and 25 mM Hepes. It was titrated to a pH of 7·4 with NaOH or HCl. To increase the Mg^{2+} concentration to 10 mM, 8 mM MgCl was added to the saline, while NaCl was reciprocally reduced to maintain constant osmolality. The latter was also true for saline solutions with increased K^+ concentrations.

The solutions for intracellular calibration of the sensitivity of SBFI to $[Na^+]_i$ (314 mosmol kg^{-1}) contained (mM): 150 ($K^+ + Na^+$), 30 Cl^-, 120 gluconic acid and 10 Hepes; adjusted to pH 7·15 with KOH (see below). Gramicidin (3 μM), monensin (10 μM) and ouabain (0·1 mM) were added for equilibration of extra- and intracellular $[Na^+]$. To test the K^+ sensitivity of intracellular SBFI, cells were perfused with calibration solutions containing (mM): 140, 100 or 60 KCl and 10 NaCl. In saline solutions with reduced KCl, the latter was replaced by equivalent amounts of CsCl ($K^+ + Na^+ + Cs^+ = 150$ mM). Calibration solutions used for testing the pH sensitivity were titrated to pH 6·2, 6·6, 7·0 and 7·4 with KOH.

Drugs and chemicals were obtained from Sigma and (except for tetrodotoxin (TTX)) prepared as 1 M stock solutions in DMSO (dimethyl sulphoxide, Sigma). They were stored in the freezer and added to the saline shortly before use. The fluorescent dye SBFI-AM (acetoxymethylester of sodium-binding benzofuran isophthalate) was purchased from Teflabs (Austin, TX, USA).

As described previously (Rose & Ransom, 1996*a*), bumetanide, a blocker of Na^+–K^+–$2Cl^-$ cotransport, exerted significant autofluorescence when excited with 345 nm (Na^+-insensitive wavelength), while the 385 nm signal was virtually unchanged. This reversible increase in fluorescence was subtracted from the 345 nm signal to allow calculation of a meaningful ratio signal during data analysis.

Intracellular loading and fluorescence ratio imaging of SBFI

Cells were loaded with 20 μM SBFI-AM (Minta & Tsien, 1989) for 90 min in CO_2/HCO_3^--free saline at room temperature (20–22 °C) in the presence of 0·1% (w/v) Pluronic F-127 (BASF, Wyandotte, MI, USA). At the end of the loading period, the cells were transferred to an experimental chamber (volume, ~500 μl) and perfused with CO_2/HCO_3^--buffered solution (standard flow rate, 2 ml min^{-1}) warmed to 32 °C. Cells were equilibrated with the saline for 15–30 min before starting the experiments.

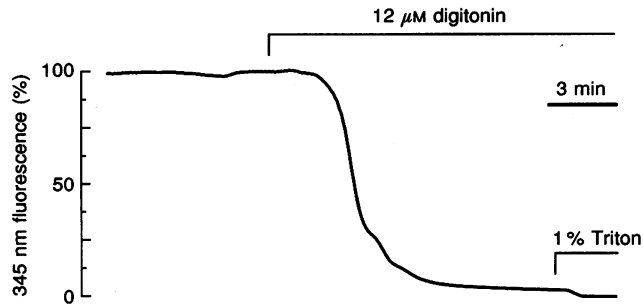

Figure 1. Digitonin releases the majority of intracellular SBFI

To estimate the percentage of dye located in the cytosol, hippocampal neurones were loaded with SBFI and clamped to a $[Na^+]_i$ of 10 mM by perfusion with calibration solution (see Methods). During application of the detergent digitonin (12 μM), the Na^+-insensitive 345 nm fluorescence decreased by an average of 98%, indicating that the majority of SBFI was located in the cytoplasm. Subsequent perfusion with Triton X-100 (1%) released the rest of the dye, presumably from intracellular organelles. In this and all subsequent figures, each experimental record reflects the fluorescence ratio signal from a single neurone.

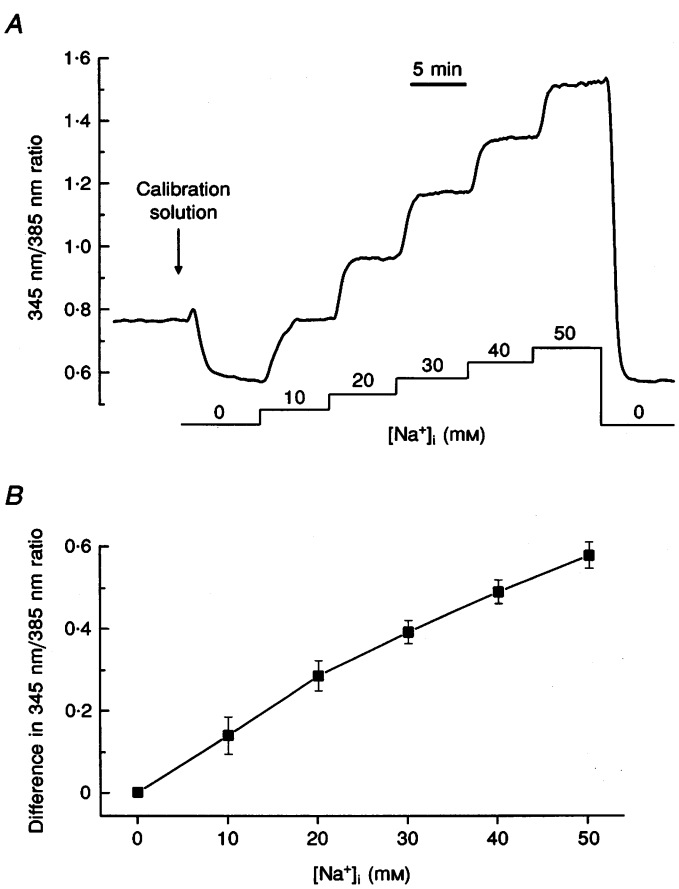

Figure 2. Calibration of SBFI fluorescence ratio signal in single hippocampal neurones

A, after equilibration in standard saline, SBFI-loaded neurones were perfused with calibration solution (indicated by arrow) to equilibrate extra- and intracellular ions (see Methods). Stepwise changes in the $[Na^+]$ of the calibration saline caused stepwise changes in the 345 nm/385 nm fluorescence ratio of the cells, reflecting changes in $[Na^+]_i$. *B*, the 345 nm/385 nm fluorescence ratio signal increased monotonically with changes in $[Na^+]_i$ from 0 to 50 mM. Shown are the mean (± S.D.) ratio signal values from 34 cells calibrated as in *A*. The fluorescence signal at 0 mM $[Na^+]_i$ was set to zero for all cells.

After this loading procedure, the majority of SBFI was located in the cytoplasm. In a calibration solution where $[Na^+]_i$ was clamped to 10 mM, permeabilization of the plasma membrane with digitonin (12 μM) caused a drop of the Na^+-insensitive 345 nm fluorescence signal of SBFI by 98 ± 2% ($n = 23$; Fig. 1). Triton X-100 (1% v/v) released the remainder of the dye, presumably from intracellular organelles (Fig. 1). Therefore, the SBFI ratio signals primarily reflected measurements of cytosolic Na^+ in cultured rat hippocampal neurones.

Fluorescence ratio imaging was performed as described recently (Rose & Ransom, 1996*a*). Briefly, the experimental chamber was mounted on the stage of a Nikon-Diaphot-TMD inverted microscope (Nikon, Japan) equipped with an oil-immersion objective (Nikon Fluor 40/1.30, transmittance to 340 nm ⩾ 50% of transmittance to 546 nm). Cells were excited every 10 s at 345 and 385 nm and emission fluorescence was collected above 510 nm and quantified with a computer program from Georgia Instruments (Roswell, GA, USA), which allowed analysis of single cells. Imaging was restricted to neuronal cell bodies. Autofluorescence (< 1%) and dye bleaching were negligible during the experiments.

Fluorescent dyes detect ion activities, not the absolute concentration of the ion under study. For simplicity, results are given as Na^+ concentrations, obtained by direct comparison with the calibration solutions containing known Na^+ concentrations (cf. Rose & Ransom, 1996*a*). Unless stated otherwise, experiments were repeated on at least four different coverslips, each allowing analysis of three to twelve individual cells. Data are presented as means ± S.D. and were statistically analysed by Student's t test where appropriate (significance level was $P < 0.01$, unless stated otherwise).

RESULTS

Intracellular $[Na^+]$ calibration

Perfusion of the cells with calibration solutions containing different Na^+ concentrations showed that the ratiometric dye signal changed monotonically and stably with changes in $[Na^+]_i$ from 0 to 50 mM (Fig. 2*A*). The ratio of the slope was around 0·13 per 10 mM Na^+ below a $[Na^+]_i$ of 20 mM and around 0·10 per 10 mM Na^+ above 20 mM ($n = 34$; Fig. 2*B*).

Because SBFI has different spectral properties inside cells compared with bulk aqueous solution (Harootunian, Kao, Eckert & Tsien, 1989), the sensitivity of the dye to changes in K^+ and pH within neurones was tested. The K^+ sensitivity of intracellular SBFI was negligible. In neurones where $[Na^+]_i$ was clamped to 10 mM by perfusion with calibration saline (see above), reducing intracellular K^+ from

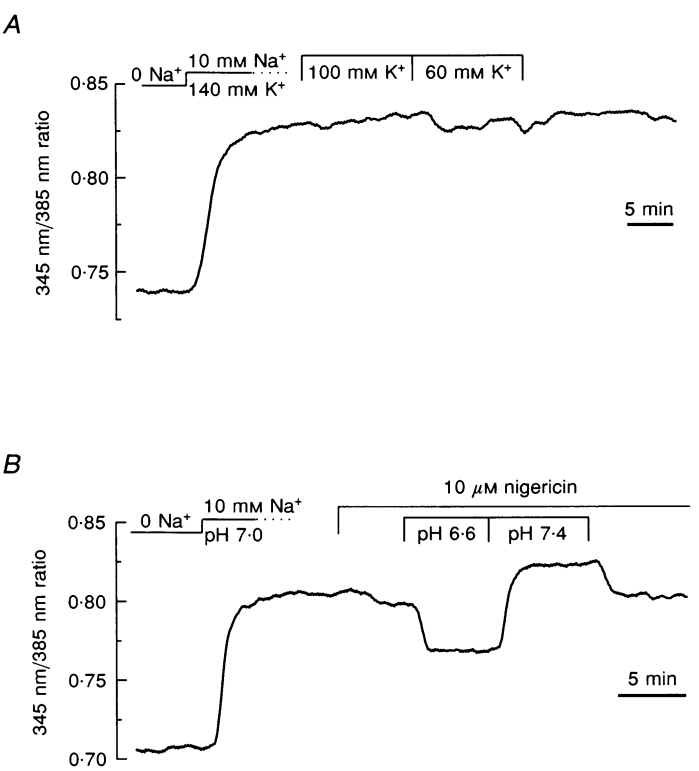

Figure 3. $[K^+]_i$ and pH_i sensitivity of SBFI in neurones

To estimate the sensitivity of SBFI to intracellular changes in $[K^+]$ and pH, neurones were perfused with calibration solution and first stepped from 0 to 10 mM $[Na^+]_i$ to calibrate the $[Na^+]_i$ sensitivity. *A*, while $[Na^+]_i$ was clamped to 10 mM, reducing $[K^+]_i$ from the standard value of 140 to 100 mM had virtually no effect on the 345 nm/385 nm fluorescence ratio of SBFI. Reduction to 60 mM $[K^+]_i$ produced a decrease in the SBFI ratio that corresponded to a $[Na^+]_i$ change of < 2 mM. *B*, the SBFI ratio was slightly dependent on the intracellular pH (pH_i). After addition of nigericin to equilibrate intra- and extracellular pH, decreasing pH_i from 7·0 to 6·6 produced a ratio decrease, whereas subsequent elevation to pH 7·4 caused a ratio increase. pH changes by 0·4 pH units mimicked an apparent change in $[Na^+]_i$ of about 3 mM, with acid shifts mimicking a decrease in $[Na^+]_i$.

140 to 100 mM (KCl substituted by CsCl) did not change the 345 nm/385 nm ratio of SBFI. Reducing intracellular K^+ to 60 mM caused an apparent drop in the 'Na^+ signal' by 1·8 ± 0·9 mM ($n = 21$; Fig. 3*A*).

Changes in intracellular pH (pH_i), in contrast, caused significant changes in the SBFI ratio. In neurones clamped to a $[Na^+]_i$ of 10 mM at pH 7·0, addition of the K^+–H^+ exchanger nigericin (10 μM) did not influence the ratio, indicating that extra- and intracellular pH were already equilibrated by gramicidin and monensin (Fig. 3*B*). Subsequent acidification to pH 6·6 resulted in a decrease (i.e. apparent $[Na^+]_i$ decrease), and alkalinization to pH 7·4 resulted in an increase in the ratio signal. pH changes of 0·4 pH units mimicked apparent $[Na^+]_i$ changes of 3·0 ± 0·6 mM ($n = 15$; Fig. 3*B*).

After each experiment, calibrations of $[Na^+]_i$ were performed within the neurones. For most experiments, two-point calibrations (0 and 20 mM Na^+) were made. When $[Na^+]_i$ increased to more than 20 mM during an experiment, a three-point calibration using 0, 20 and 50 mM Na^+ was performed. These calibration solutions were titrated to a pH of 7·15, the apparent baseline pH_i of these neurones in CO_2/HCO_3^--buffered solutions (see above; Rose & Ransom, 1996*b*). The signal-to-noise ratio in these experiments usually allowed resolution of $[Na^+]_i$ changes as small as 0·5–1 mM.

Baseline $[Na^+]_i$ in hippocampal neurones

Dye calibrations within single cells performed after each experiment (see Methods) showed that baseline $[Na^+]_i$ of cultured hippocampal neurones (14–21 days *in vitro*) was 8·9 ± 3·8 mM ($n = 263$) in standard CO_2/HCO_3^--containing saline with 3 mM K^+. This corresponds to a Na^+ equilibrium potential of +72 mV, indicating that, at the typical negative resting membrane potentials of neurones, $[Na^+]_i$ is maintained against a steep electrochemical gradient.

If no experimental manipulation was performed, baseline $[Na^+]_i$ was usually stable (±1–2 mM) for the entire length of an experiment (up to 2 h) in the majority of neurones. Neurones on 27% of the coverslips investigated ($n = 115$ coverslips in total), however, exhibited spontaneous, transient increases in $[Na^+]_i$ in either all or the majority of cells in the field of view on a particular coverslip (Fig. 4). This observation was not related to a particular set of sister cultures and was independent of the age of the cells (14–21 days in culture). The spontaneous $[Na^+]_i$ increases had a peak amplitude of 4·8 ± 2·8 mM (range, 1–19·8 mM) and a peak-to-peak frequency of 4·6 ± 2·7 min ($n = 69$). The peaks and relative amplitude of the $[Na^+]_i$ increases were synchronized among the cells of one coverslip (Fig. 4).

The spontaneous $[Na^+]_i$ increases were completely and reversibly blocked by addition of 1 μM TTX to the saline ($n = 48$, Fig. 5*A*), or elevation of the extracellular Mg^{2+} concentration to 10 mM ('high Mg^{2+} saline'; $n = 36$, Fig. 5*B*), indicating that they depended on intact Ca^{2+}-mediated synaptic transmission and voltage-gated Na^+ channels. It is probable that Na^+ entry occurred both via Na^+ channels and transmitter-activated ion channels. Neither application of TTX ($n = 44$) nor elevation of Mg^{2+} concentration alone ($n = 58$) influenced baseline $[Na^+]_i$ (not shown). When

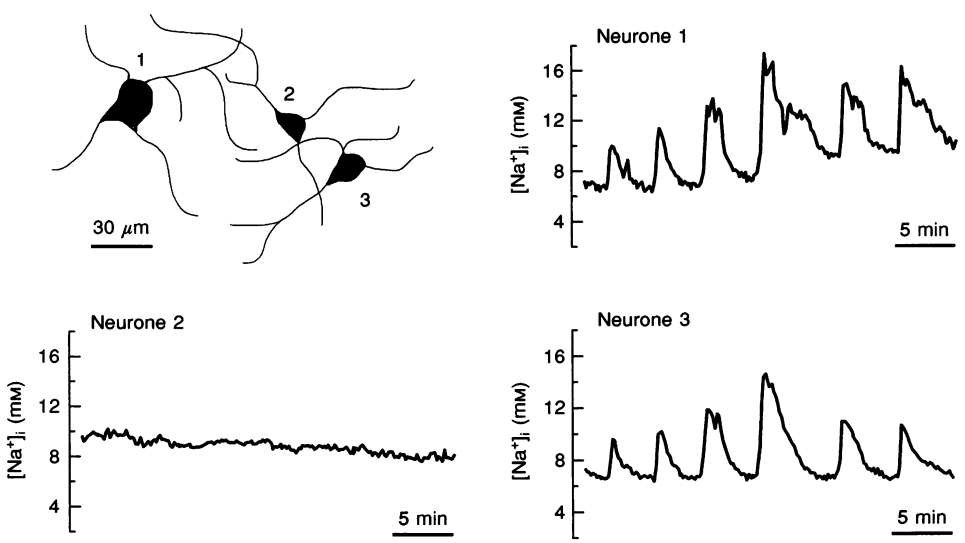

Figure 4. Hippocampal neurones show spontaneous increases in baseline $[Na^+]_i$

Spontaneous increases in neuronal $[Na^+]_i$ were observed in 27% of coverslips investigated. The schematic drawing (upper left) shows the position of cell bodies and larger processes of three neurones studied in a representative experiment. As in all experiments, only the fluorescence signals from the cell bodies were imaged. The data traces show $[Na^+]_i$ measurements in these three cells in standard saline. $[Na^+]_i$ was stable in the middle neurone of this group (neurone 2), but increased spontaneously and transiently by several millimolar in the left and right neurones (neurones 1 and 3). Note that the peaks and relative amplitudes of spontaneous $[Na^+]_i$ increases were synchronized in neurones 1 and 3.

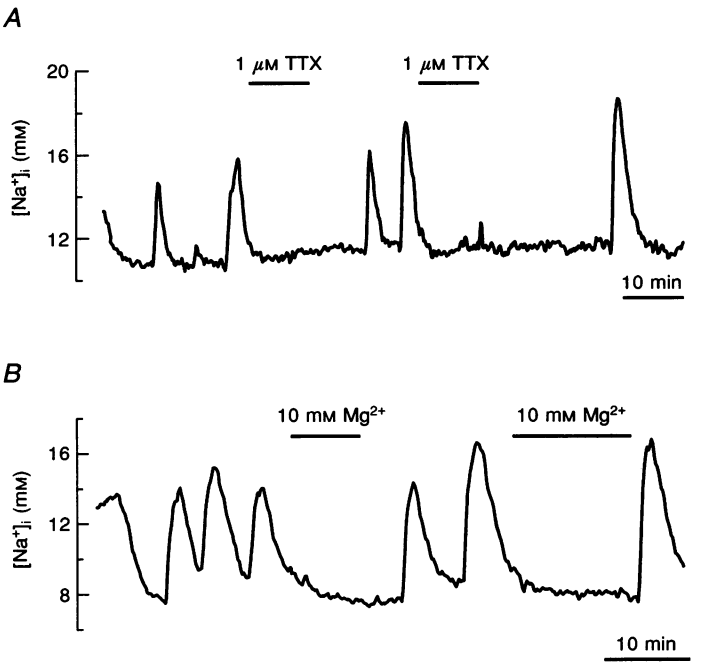

Figure 5. Effects of TTX and high Mg^{2+} on spontaneous neuronal $[Na^+]_i$ increases

Spontaneous, synchronized increases in $[Na^+]_i$ were completely and reversibly blocked by perfusion with 1 μM TTX (*A*), or by increasing the extracellular Mg^{2+} concentration to 10 mM (*B*), suggesting that they were due to Na^+ influx via voltage-gated channels and were dependent on Ca^{2+}-dependent synaptic transmission between neurones.

switching back from high Mg^{2+} saline to standard saline, transient increases in $[Na^+]_i$ of 2–3 mM were observed in about 30% of cells (cf. Fig. 7*B*, asterisk).

Effects of veratridine on $[Na^+]_i$

Voltage-dependent, TTX-sensitive Na^+ channels mediate the influx of Na^+ from the extracellular space during action potential firing. At normal resting membrane potential, these channels are generally closed; consequently, application of TTX did not change baseline neuronal $[Na^+]_i$ (see above).

To study the influence of an opening of voltage-gated Na^+ channels on neuronal $[Na^+]_i$, we used the lipophilic toxin veratridine, which binds to channel sites buried in the lipid bilayer of the plasma membrane. Veratridine binding causes Na^+ channels to remain open at rest by impairing channel inactivation (Strichartz, Rando & Wang, 1987). Application of 50 μM veratridine for 3 min caused a rapid $[Na^+]_i$ increase of 24·7 ± 8·6 mM ($n = 32$, Fig. 6). $[Na^+]_i$ continued to rise slowly even when the drug was removed, and recovered to baseline only when 1 μM TTX was added to the saline

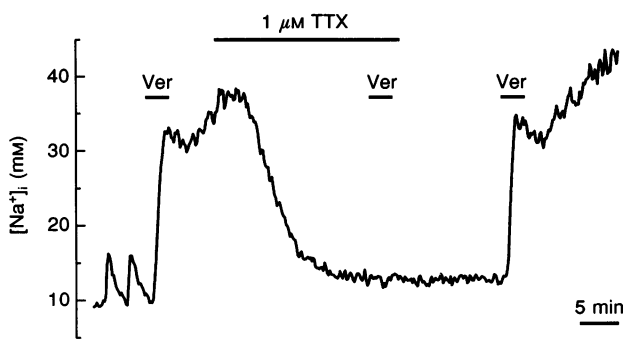

Figure 6. Veratridine increases $[Na^+]_i$ in hippocampal neurones

Application of 50 μM veratridine (Ver) for 3 min to open voltage-gated Na^+ channels rapidly increased $[Na^+]_i$ in hippocampal neurones. The effect of veratridine was only reversible by perfusion of the cells with 1 μM TTX. TTX also reversibly blocked the veratridine-induced $[Na^+]_i$ increase. Note the spontaneous increases in $[Na^+]_i$ occurring at the beginning of the experiment in standard saline.

($n = 32$, Fig. 6). With TTX present, veratridine application did not alter $[Na^+]_i$, indicating that the $[Na^+]_i$ increase was solely due to influx of Na^+ via voltage-gated Na^+ channels; this effect was fully reversible (Fig. 6).

In contrast to the remarkable effect of veratridine, elevation of $[K^+]_o$ from 3 to 20 mM for 2 min to depolarize the neurones caused $[Na^+]_i$ to increase by only $3{\cdot}0 \pm 1{\cdot}9$ mM ($n = 14$, not shown). Elevation of $[K^+]_o$ to 40 mM for 2 min resulted in a $[Na^+]_i$ increase of $4{\cdot}7 \pm 3{\cdot}1$ mM ($n = 75$, Fig. 7). In four neurones, this increase was followed by a transient decrease in $[Na^+]_i$ of $1{\cdot}8 \pm 1{\cdot}4$ mM. The K^+-induced $[Na^+]_i$ increase was reversibly blocked by TTX ($n = 38$, Fig. 7*A*) and significantly reduced by 63% in high Mg^{2+} saline ($n = 53$, Fig. 7*B*), suggesting that it strongly depended on voltage-gated Na^+ channels and, at least partly, depended on Ca^{2+}-mediated synaptic transmission.

In 41% of neurones investigated, perfusion with 10 mM Mg^{2+} or TTX unmasked a small K^+-induced decrease in $[Na^+]_i$ ($1{\cdot}2 \pm 0{\cdot}9$ mM; Fig. 7*C*). This indicates that the large increase in $[K^+]_o$ probably promoted, at least in some cells, both Na^+ influx via Na^+ channels and activation of Na^+,K^+-ATPase, which was independent of a $[Na^+]_i$ increase. Alternatively, the application of high K^+ may have induced neuronal swelling with a dilutional decrease in $[Na^+]_i$.

Effects of removal of extracellular Na^+ and inhibition of Na^+,K^+-ATPase

Maintenance of baseline neuronal $[Na^+]_i$ was strongly dependent on the presence of extracellular Na^+. Following complete replacement of extracellular Na^+ by 23 mM choline and 117 mM NMDG (*N*-methyl-D-glucamine), neuronal $[Na^+]_i$ decreased exponentially with a time constant of $2{\cdot}7 \pm 1{\cdot}1$ min (τ, time until $[Na^+]_i$ had dropped to 36·7% of its original value; Fig. 8*A*), reaching values close to zero after about 10 min ($n = 34$, Fig. 8*A*). The maximal efflux rate was $4{\cdot}1 \pm 1{\cdot}5$ mM min^{-1}. Restoring $[Na^+]_o$ rapidly restored baseline $[Na^+]_i$ to its original level with a maximal Na^+ influx rate of $7{\cdot}3 \pm 2{\cdot}8$ mM min^{-1} ($n = 34$, Fig. 8*A*).

To estimate passive and transport-mediated influx rates of Na^+ into the neurones under resting conditions with standard $[Na^+]_o$ (140 mM), we blocked Na^+,K^+-ATPase activity by either removal of $[K^+]_o$ or application of the cardiac glycoside ouabain. Removing $[K^+]_o$ caused an increase in $[Na^+]_i$ of $5{\cdot}0 \pm 1{\cdot}7$ mM min^{-1} (maximum slope as determined during the first 3 min in 0 $[K^+]_o$), leading to $[Na^+]_i$ values of $49{\cdot}7 \pm 17{\cdot}1$ mM after a 10 min exposure to the K^+-free saline ($n = 15$, Fig. 8*B*, continuous trace). After return to normal $[K^+]_o$, eight of fifteen neurones were able to recover partly from this Na^+ load within 20 min (Fig. 8*B*).

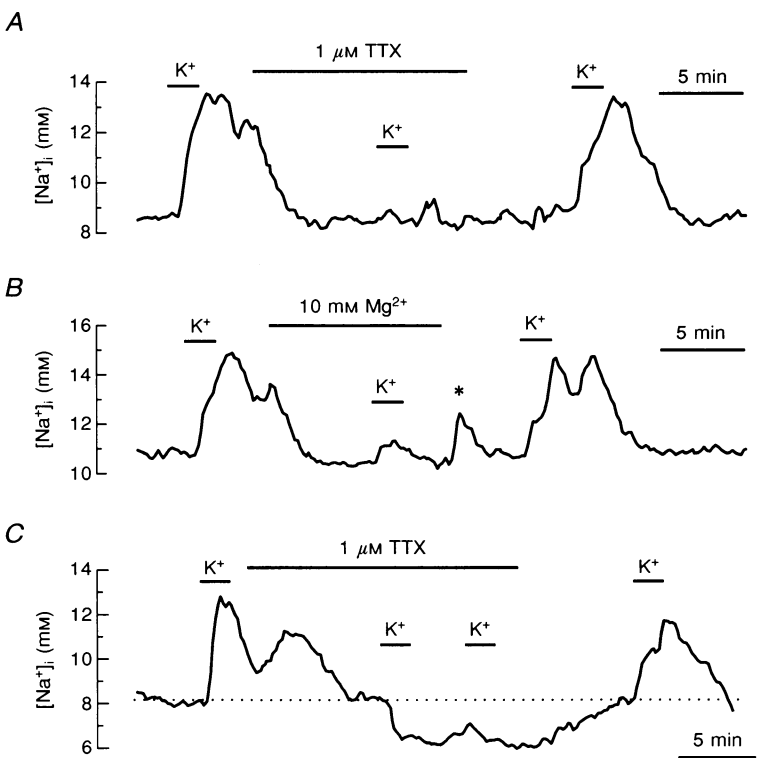

Figure 7. $[Na^+]_i$ increases induced by elevation of $[K^+]_o$ to 40 mM

Elevation of $[K^+]_o$ from 3 to 40 mM for 2 min to depolarize the neurones increased $[Na^+]_i$ by several millimolar. The K^+-induced $[Na^+]_i$ increases were completely and reversibly blocked by 1 μM TTX (*A*) and markedly reduced by elevation of the extracellular Mg^{2+} concentration to 10 mM (*B*). *C*, in some neurones, elevation of $[K^+]_o$ to 40 mM during application of TTX unmasked a K^+-induced decrease in neuronal $[Na^+]_i$. In *B*, note the transient, spontaneous increase in $[Na^+]_i$ when switching back from 10 mM Mg^{2+} saline to standard saline (*).

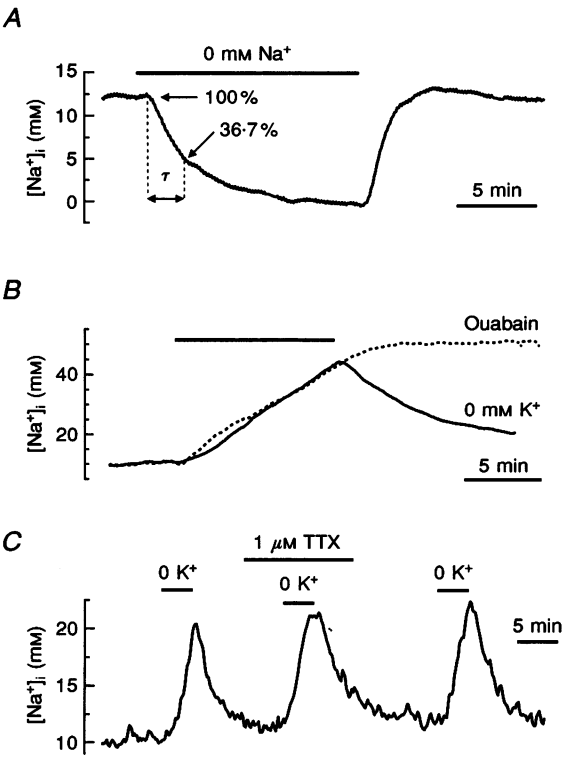

Figure 8. Effects of $[Na^+]_o$ removal or inhibition of Na^+,K^+-ATPase on neuronal $[Na^+]_i$

A, removal of $[Na^+]_o$ (0 mM Na^+, indicated by bar) led to an exponential decay in $[Na^+]_i$ with a time constant of 2·7 min (τ; time until $[Na^+]_i$ had dropped from its initial value, 100%, to 36·7%). Reintroduction of standard saline rapidly restored $[Na^+]_i$ to its original level. *B*, the effects of inhibition of Na^+,K^+-ATPase activity by removal of $[K^+]_o$ (continuous trace) or application of 0·5 mM ouabain (dashed trace) on $[Na^+]_i$ are shown (the period of removal of $[K^+]_o$ or application of ouabain is indicated by the bar). Both protocols caused a comparable increase in $[Na^+]_i$, but only the effects of $[K^+]_o$ were reversible. *C*, the increase in $[Na^+]_i$ caused by removal of $[K^+]_o$ (4 min) was not influenced by application of 1 μM TTX.

The $[Na^+]_i$ increase following inhibition of Na^+,K^+-ATPase activity by ouabain (0·5 mM) was comparable to that induced by $[K^+]_o$ removal; during ouabain perfusion, $[Na^+]_i$ increased by $4·7 \pm 2·7$ mM min^{-1} ($n = 13$, Fig. 8*B*, dashed trace). In contrast to the effects caused by removal of $[K^+]_o$, however, the effects of ouabain were always irreversible (Fig. 8*B*).

The increase in $[Na^+]_i$ caused by Na^+,K^+-ATPase inhibition was not influenced by application of 1 μM TTX ($n = 41$, Fig. 8*C*). During TTX perfusion, neither the slope nor the absolute amplitude of the $[Na^+]_i$ increase caused by removal of $[K^+]_o$ were significantly altered. This indicated that Na^+ influx via voltage-gated Na^+ channels did not significantly contribute to the observed steep increase in $[Na^+]_i$.

Influence of CO_2/HCO_3^- and effects of bumetanide

The major acid extrusion mechanism of hippocampal neurones is a Na^+-dependent Cl^-–HCO_3^- exchanger (Schwiening & Boron, 1994). Constant inward transport of

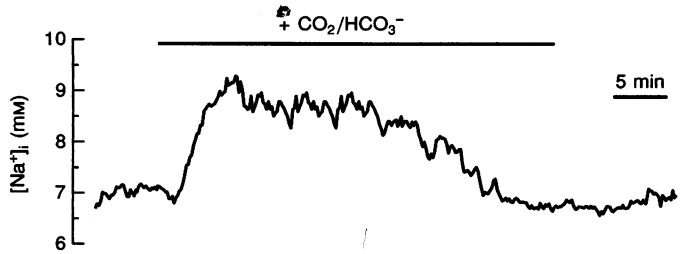

Figure 9. Effects of addition of CO_2/HCO_3^- on neuronal $[Na^+]_i$

When cells were exposed to CO_2/HCO_3^--free, Hepes-buffered saline for a period of 30 min, switching to standard saline containing CO_2/HCO_3^- caused a transient $[Na^+]_i$ increase, which recovered after 20–30 min while still in CO_2/HCO_3^--buffered saline.

HCO_3^- via this transporter is responsible for a more alkaline baseline intracellular pH in CO_2/HCO_3^--buffered compared with CO_2/HCO_3^--free saline (Schwiening & Boron, 1994). We investigated whether this transporter might also influence baseline $[Na^+]_i$ in hippocampal neurones.

To activate Na^+-dependent Cl^-–HCO_3^- exchange activity, cells were perfused with CO_2/HCO_3^--free, Hepes-buffered saline for 30 min following dye loading, and then exposed to CO_2/HCO_3^--buffered saline. For this series of experiments, the pH of the calibration solutions was titrated to 6·9, to compensate for the more acidic intracellular pH of hippocampal neurones in the absence of CO_2/HCO_3^- (Schwiening & Boron, 1994; Rose & Ransom, 1996*b*; see Methods).

In contrast to intracellular pH (see above), neuronal baseline $[Na^+]_i$ was not significantly different in Hepes-buffered compared with CO_2/HCO_3^--buffered saline (9·7 ± 4·7 mM, $n = 72$). Switching from CO_2/HCO_3^--free to CO_2/HCO_3^--containing saline transiently increased $[Na^+]_i$ by 3·3 ± 2·6 mM, indicating activation of Na^+-dependent Cl^-–HCO_3^- exchange ($n = 57$; Fig. 9). After 20–30 min in CO_2/HCO_3^--containing saline, $[Na^+]_i$ recovered to its initial value and switching back to CO_2/HCO_3^--free saline did not alter $[Na^+]_i$ (Fig. 9).

These results indicate that strong activation of Na^+-dependent Cl^-–HCO_3^- exchange can transiently increase $[Na^+]_i$, but that the steady-state activity of this transporter does not significantly influence baseline $[Na^+]_i$ in hippocampal neurones. Stilbenes such as DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid), which block Na^+-dependent Cl^-–HCO_3^- exchange (Schwiening & Boron, 1994), could not be applied to investigate this question further, because they showed strong autofluorescence at the excitation wavelengths used for SBFI ratio imaging.

In cultured rat hippocampal astrocytes, blocking Na^+–K^+–$2Cl^-$ cotransport by bumetanide reversibly decreased $[Na^+]_i$ by about 2 mM, indicating that this transporter caused a steady Na^+ influx into the astrocytes under control conditions (Rose & Ransom, 1996*a*). In contrast to this, application of 50 μM bumetanide did not significantly alter $[Na^+]_i$ in the present study ($n = 27$, not shown). This result suggests that Na^+–K^+–$2Cl^-$ cotransport does not significantly contribute to baseline $[Na^+]_i$ in hippocampal neurones.

Effects of small alterations in $[K^+]_o$

Due to the loss of K^+ ions from neurones during the repolarization phase of action potentials, neuronal activity

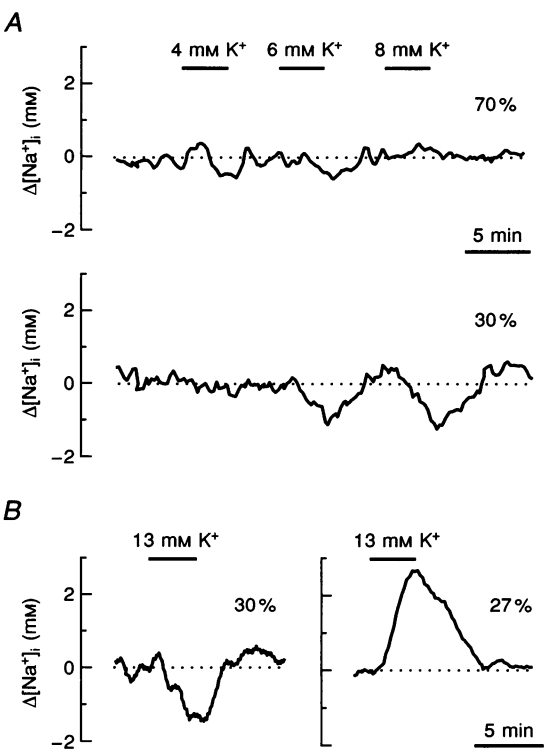

Figure 10. Effects of small, 3 min increases in $[K^+]_o$ on neuronal $[Na^+]_i$

The effects of small increases in $[K^+]_o$ for 3 min on $[Na^+]_i$ are shown. These pulses mimic intense neural activity and were too short to result in a new steady-state $[Na^+]_i$ (cf. Fig. 11). *A*, increasing $[K^+]_o$ from 3 mM to 4, 6, or 8 mM for 3 min did not cause visible changes in $[Na^+]_i$ in 70% of the neurones investigated (upper trace). In 30% of cells (lower trace), increasing $[K^+]_o$ to 6 or 8 mM for 3 min slowly decreased $[Na^+]_i$ by about 1 mM. *B*, 30% of cells reacted with a $[Na^+]_i$ decrease to elevation in $[K^+]_o$ to 13 mM (left-hand trace), and 27% reacted with an increase (right-hand trace). The rest (43%) showed no response (not illustrated).

can increase $[K^+]_o$ in the brain by 2–3 mM (Heinemann & Lux, 1977). The excess $[K^+]_o$ may be reduced by several mechanisms including active K^+ uptake into both neurones and glial cells (Ransom & Sontheimer, 1992; Newman, 1995). In cultured hippocampal astrocytes it was recently shown that small, physiologically relevant increases in $[K^+]_o$ cause a decrease in glial $[Na^+]_i$, indicating activation of glial Na^+,K^+-ATPase. This implied that the glial isoform of Na^+,K^+-ATPase was well suited to play a significant role in the clearance of $[K^+]_o$ (Rose & Ransom, 1996*a*).

To investigate the possible role of neuronal Na^+,K^+-ATPase activity in $[K^+]_o$ homeostasis, the response of neuronal $[Na^+]_i$ to alterations in $[K^+]_o$ was studied in two sets of experiments. In the first set, $[K^+]_o$ was increased from the standard value of 3 mM to 4, 5, 6, 7, 8 or 13 mM for 3 min. These 3 min applications should mimic $[K^+]_o$ changes that might occur during variably intense neuronal activity; they would be too brief, however, to result in a new steady-state $[Na^+]_i$ value (Fig. 10). In the second set of experiments, $[K^+]_o$ was increased or decreased for periods greater than 10 min to study the influence of relatively long-term $[K^+]_o$ alterations on steady-state neuronal $[Na^+]_i$ (Fig. 11). The results obtained from both experimental designs were independent of the order in which $[K^+]_o$ changes were made.

Increasing $[K^+]_o$ from 3 to 4 mM for a 3 min period ($n = 48$) did not lead to visible changes in neuronal $[Na^+]_i$ (Fig. 10*A*). Likewise, in the majority of instances (~70% of cells), increases to 5, 6, 7 or 8 mM $[K^+]_o$ did not change $[Na^+]_i$ ($n \geqslant 25$; the noise level was usually around 0·5–1 mM, see Methods; Fig. 10*A*). In 30% of the neurones exposed to these $[K^+]_o$ concentrations (5–8 mM), however, $[Na^+]_i$ decreased by about 1 mM (decrease not significant; $P \leqslant 0{\cdot}1$). A 3 min elevation of $[K^+]_o$ to 13 mM ($n = 32$) resulted in a $[Na^+]_i$ decrease of 1·3 ± 0·7 mM in 30% of cells ($P \leqslant 0{\cdot}1$), whereas $[Na^+]_i$ increased by 2·6 ± 1·5 mM in 27% of cells ($P \leqslant 0{\cdot}025$), and was unchanged in the rest of the cells (43%; Fig. 10*B*).

Long periods of $[K^+]_o$ increase to 6, 8 or 13 mM ($n = 39$) caused either a decrease in $[Na^+]_i$ to a new steady-state value, or a transient increase in $[Na^+]_i$, or had no effect (Fig. 11*A*). These different response types were sometimes found in a single cell, e.g. cells that did not react to elevations in $[K^+]_o$ to 6 mM could show an increase or decrease in $[Na^+]_i$ with $[K^+]_o$ elevations to 8 or 13 mM (Fig. 11*A*). In summary, prolonged $[K^+]_o$ elevations decreased $[Na^+]_i$ in a total of 63% of cells investigated, whereas $[Na^+]_i$ increased in 26% of cells, and was unchanged in 11% of cells (Fig. 11*A*). The mean amplitudes of $[Na^+]_i$ changes of the neurones in response to different $[K^+]_o$ increases are graphically summarized in Fig. 12.

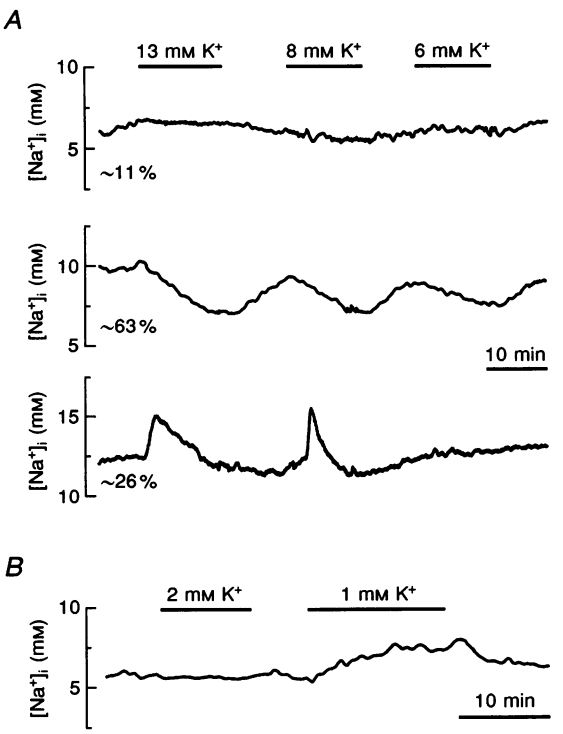

Figure 11. Effects of small, prolonged changes in $[K^+]_o$ on neuronal $[Na^+]_i$

The effects of prolonged (⩾ 10 min) changes in $[K^+]_o$ (indicated by bars) on $[Na^+]_i$ are shown. *A*, long duration increases to 13, 8 or 6 mM $[K^+]_o$ had no effect on $[Na^+]_i$ in 11% of cells (upper trace), slowly decreased steady-state $[Na^+]_i$ in 63% of cells (middle trace), and increased $[Na^+]_i$ transiently in 26% of cells (lower trace; see Results). *B*, reduction in $[K^+]_o$ from 3 to 2 mM did not change steady-state $[Na^+]_i$ in the majority of hippocampal neurones, whereas reduction to 1 mM $[K^+]_o$ caused a reversible increase in $[Na^+]_i$ by about 5 mM in 69% of cells.

The change in steady-state $[Na^+]_i$ of hippocampal neurones during prolonged decreases in $[K^+]_o$ is shown in Fig. 11*B*. Reducing $[K^+]_o$ from 3 to 2 mM changed $[Na^+]_i$ in only two out of twenty-two cells (12%); in these two cells $[Na^+]_i$ increased by an average of 2·3 mM. In contrast, when $[K^+]_o$ was reduced to 1 mM, $[Na^+]_i$ increased significantly by 5·1 mM in 69% of cells (Fig. 12).

These results show that very small or relatively brief alterations in $[K^+]_o$ had very little effect on $[Na^+]_i$ of cultured hippocampal neurones. Virtually no $[Na^+]_i$ changes could be detected with elevation or reduction in $[K^+]_o$ by 1 mM. Most cells (70%) did not react to $[K^+]_o$ increases up to 8 mM, when the duration of this increase was restricted to 3 min. Prolonged periods of $[K^+]_o$ alteration, by greater than 2 mM, were necessary to influence $[Na^+]_i$ significantly, leading either to a transient increase in $[Na^+]_i$ or, in the majority of cells, to a slow decrease in steady-state $[Na^+]_i$.

DISCUSSION

Baseline $[Na^+]_i$ and activity-correlated $[Na^+]_i$ changes in rat hippocampal neurones

Our *in vivo* signal calibrations of SBFI fluorescence revealed a baseline $[Na^+]_i$ of about 9 mM in cultured rat hippocampal neurones in standard, CO_2/HCO_3^--buffered saline containing 3 mM K^+. This value is in good agreement with an earlier study, which reported a baseline $[Na^+]_i$ of 8·2 mM for cultured hippocampal neurones using SBFI imaging (Pinelis, Segal, Greenberger & Khodorov, 1994). Somewhat lower values were obtained for cultured cerebellar neurones using SBFI (4·0 mM, Kiedrowski, Brooker, Costa & Wroblewski, 1994; 7·5 mM, Cousin, Nicholls & Pocock, 1995). These and other studies on $[Na^+]_i$ regulation of vertebrate and invertebrate neurones (Thomas, 1972; Grafe *et al.* 1982; Deitmer & Schlue, 1983; Ballanyi *et al.* 1984; Coles & Orkand, 1985; Kaila *et al.* 1987; Erecinska *et al.* 1991; Friedman & Haddad, 1994) illustrate the general phenomenon of a very steep electrochemical gradient for Na^+ in neurones.

The $[Na^+]_i$ of hippocampal neurones was somewhat lower than the $[Na^+]_i$ of cultured hippocampal astrocytes (15 mM) measured recently with the same technique in our laboratory (Rose & Ransom, 1996*a*). It has been speculated that the higher astrocytic $[Na^+]_i$, compared with neuronal $[Na^+]_i$, counters the tendency for $[Na^+]_i$ to drop in these cells when their Na^+,K^+-ATPase activity increases during periods of elevated extracellular K^+ (Sweadner, 1995). Our studies indicated that glial $[Na^+]_i$ is rapidly stabilized by other Na^+-dependent transport mechanisms that come into play under these conditions (Rose & Ransom, 1996*a*). The reason, therefore, for baseline differences in astrocyte and neurone $[Na^+]_i$ remain unclear.

Baseline neuronal $[Na^+]_i$ was stable within a range of ±1 mM in most experiments, but on 27% of the coverslips studied, spontaneous transient increases in $[Na^+]_i$ (by about 5 mM) were observed, which were synchronized between cells. Since they were blocked by both TTX and high Mg^{2+} saline, they probably reflected periodic bursting behaviour of synaptically coupled neurones, as described in cultured

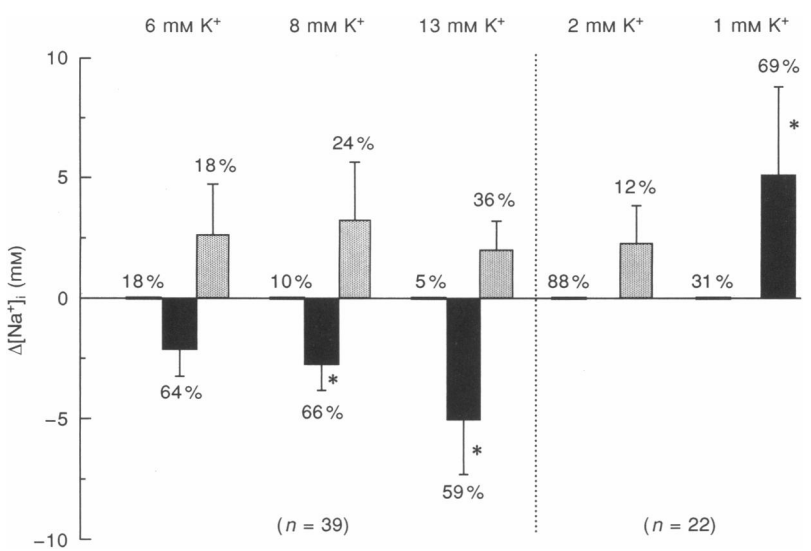

Figure 12. Graphic summary of the effects of prolonged $[K^+]_o$ changes on $[Na^+]_i$

This histogram summarizes changes in $[Na^+]_i$ ($\Delta[Na^+]_i$) of hippocampal neurones, induced by changes in $[K^+]_o$ for longer than 10 min. Mean values, standard deviations, and the number of cells exposed to increased (6, 8 and 13 mM) or decreased (2 and 1 mM) $[K^+]_o$, are shown. For each $[K^+]_o$ change, $[Na^+]_i$ showed two or three types of response: no change, transient increase, or decrease in steady-state value. The percentage of cells exhibiting each of these types of response is indicated above or below the single columns. The predominant $[Na^+]_i$ response for each of the different $[K^+]_o$ changes is indicated by the filled columns; * significantly different from steady-state $[Na^+]_i$ in standard saline.

neurones from rat cortex (Kamioka, Maeda, Jimbo, Robinson & Kawana, 1996). Their amplitude corresponded well with $[Na^+]_i$ increases induced by repetitive electrical stimulation in crayfish stretch receptors or frog spinal motoneurones (Grafe *et al.* 1982; Kaila *et al.* 1987). Synchronized bursting patterns between neurones are not restricted to neurones in cell cultures. Bursting neurones have been described in the developing mammalian retina. In the retina, synchronized neuronal bursting together with synchronized oscillations in intracellular $[Ca^{2+}]$ play an important role in the establishment of synaptic connections (Wong, Chernjavsky, Smith & Shatz, 1995).

The synchronized $[Na^+]_i$ increases measured in the present study can be explained reasonably well by Na^+ influx via voltage-dependent Na^+ channels during bursts of action potentials. Assuming a cell diameter of 25 μm, the measured increase in $[Na^+]_i$ of 5 mM during one burst translates into a Na^+ increase of $4{\cdot}1 \times 10^{-17}$ M per cell. The Na^+ entry into one hippocampal neurone per action potential can be calculated to be $9{\cdot}8 \times 10^{-19}$ M (based on a Na^+ influx of 5×10^{-12} M cm^{-2} impulse^{-1}; Hodgkin & Keynes, 1955). Therefore, about forty action potentials would cause the observed $[Na^+]_i$ increase. Because outward transport of Na^+ via the Na^+,K^+-ATPase counteracts the Na^+ influx, the number of action potentials per burst would probably need to be higher than that. Another source for the $[Na^+]_i$ increase could be Na^+ influx via transmitter-activated ion channels (Rose & Ransom, 1996*b*).

Transmembrane Na^+ movements

Na^+ influx via TTX-sensitive Na^+ channels. Hippocampal neurones possess voltage-dependent Na^+ currents that can generate action potentials, leading to an increase in $[Na^+]_i$ (Jaffe *et al.* 1992). In this study, opening of these channels by veratridine caused a fast, TTX-sensitive increase in $[Na^+]_i$, suggesting that slow, TTX-insensitive Na^+ currents (Hoehn, Watson & MacVicar, 1993) were not significantly involved in the veratridine-induced $[Na^+]_i$ increase. As expected (Strichartz *et al.* 1987), the veratridine binding to the channels reversed slowly, and $[Na^+]_i$ only recovered after closing the channels with TTX.

Compared with the veratridine-induced increase in $[Na^+]_i$, the $[Na^+]_i$ increase induced by elevation of $[K^+]_o$ to 40 mM was relatively moderate. This could be due to the fact that Na^+ channels were largely inactivated during the sustained K^+-induced depolarization. The K^+-induced decreases in $[Na^+]_i$ uncovered during TTX perfusion or in high Mg^{2+} saline in some neurones indicated that the increase in $[K^+]_o$ might additionally have stimulated Na^+,K^+-ATPase activity in these cells. Alternatively, the exposure to high $[K^+]_o$ may have triggered an increase in neurone volume with a dilutional decrease in $[Na^+]_i$.

Na^+ fluxes during removal of $[Na^+]_o$ and inhibition of Na^+,K^+-ATPase. As a consequence of the reversal of the Na^+ gradient, hippocampal neurones rapidly lost Na^+ ions when $[Na^+]_o$ was removed. The efflux routes of Na^+ could include Na^+ movement through channels or Na^+-dependent transporters operating in the reverse mode. The cells were depleted of $[Na^+]_i$ within about 10 min, which was about twice as fast as that seen in hippocampal astrocytes (Rose & Ransom, 1996*a*). This discrepancy might be due to the smaller volume of the neurones compared with astrocytes (but see below).

During inhibition of Na^+,K^+-ATPase, $[Na^+]_i$ increased by 5 mM min^{-1} ($8{\cdot}3 \times 10^{-5}$ M s^{-1}) in hippocampal neurones. Assuming a spherical shape of the cell body and a cell diameter of 25 μm (see above), the absolute Na^+ influx was $16{\cdot}2 \times 10^{-12}$ M cm^{-2} s^{-1}. This value is close to Na^+ influx rates found for squid axons (32×10^{-12} M cm^{-2} s^{-1}; Hodgkin & Keynes, 1955) and snail neurones ($\sim 25 \times 10^{-12}$ M cm^{-2} s^{-1}; Thomas, 1972), and reflects the high Na^+ permeability of the plasma membrane. In the face of this high rate of Na^+ influx, the need for Na^+,K^+-ATPase activity to maintain low baseline $[Na^+]$ is apparent.

In cultured hippocampal astrocytes, inhibition of Na^+,K^+-ATPase activity increased $[Na^+]_i$ by 2–5 mM min^{-1} (Rose & Ransom, 1996*a*). Comparison of these values with those obtained for neurones, however, is difficult, since the morphology of astrocytes is diverse and does not allow a simple calculation of surface area. Nevertheless it can be safely assumed that the membrane surface of typical cultured hippocampal astrocytes is much larger than those of hippocampal neurones, and consequently that the astrocytic Na^+ influx per square centimetre of membrane surface was significantly lower than for neurones.

In contrast to hippocampal astrocytes (Rose & Ransom, 1996*a*), where $[Na^+]_i$ completely recovered after application of 1 mM ouabain, ouabain effects on hippocampal neurones were irreversible. This confirms the notion that distinct α-subunit isoforms are expressed in these two cell types, with high ouabain-affinity α-subunits ($\alpha 2$, $\alpha 3$) present primarily in neurones and low affinity α-subunits ($\alpha 1$) present primarily in astrocytes (Sweadner, 1995).

Role of CO_2/HCO_3^- and Na^+–K^+–$2Cl^-$ cotransport. Due to the activity of Na^+-dependent Cl^-–HCO_3^- exchange, hippocampal neurones have a more alkaline intracellular pH in CO_2/HCO_3^--buffered saline solutions compared with CO_2/HCO_3^--free saline solutions (Schwiening & Boron, 1994). Switching from CO_2/HCO_3^--free to CO_2/HCO_3^--buffered saline caused a transient increase in $[Na^+]_i$. A transient rise in $[Na^+]_i$ upon exposure to CO_2/HCO_3^-, probably due to activation of Na^+-dependent acid extrusion mechanisms, was also seen in snail neurones (Thomas, 1977). Apart from this transient $[Na^+]_i$ increase, inward transport of Na^+ via Na^+-dependent Cl^-–HCO_3^- exchange does not seem to influence baseline $[Na^+]_i$ in hippocampal neurones, since steady-state $[Na^+]_i$ was the same in both solutions.

The same was true for Na^+–K^+–$2Cl^-$ cotransport since its inhibition by bumetanide did not change $[Na^+]_i$. Electroneutral Na^+–K^+–$2Cl^-$ cotransport has been identified in a wide variety of cell types (Haas, 1994), and is likely to play

a role in reuptake of K^+ into neurones and glial cells, following neural activity-induced loss of this ion (Ballanyi *et al.* 1984; Kimelberg & Frangakis, 1985; Rose & Ransom, 1996*a*). Our studies showing no effect of this transporter on baseline neuronal $[Na^+]_i$ suggest that it is not highly active at rest.

These results imply further differences between regulation of $[Na^+]_i$ in cultured hippocampal neurones and astrocytes. In contrast to neurones, $[Na^+]_i$ of hippocampal astrocytes decreased during removal of CO_2/HCO_3^- or application of bumetanide, indicating that inwardly directed Na^+–HCO_3^- cotransport (which is not present in neurones; Schwiening & Boron, 1994) and Na^+–K^+–$2Cl^-$ cotransport, respectively, contribute to baseline $[Na^+]_i$ under control conditions (Rose & Ransom, 1996*a*). The apparent difference in the behaviour of Na^+–K^+–$2Cl^-$ cotransport in astrocytes and neurones raises the possibility that multiple forms of this transporter exist and have different functional characteristics (Haas, 1994).

Effects of small changes in $[K^+]_o$ on $[Na^+]_i$: implications for extracellular K^+ homeostasis

Active neurones release K^+ into the extracellular space of the brain, and removal and uptake mechanisms have evolved to limit the extent of $[K^+]_o$ increase. Glial cells exhibit several uptake mechanisms (Ransom & Sontheimer, 1992; Newman, 1995). Recent work from this laboratory indicates that both Na^+,K^+-ATPase and Na^+–K^+–$2Cl^-$ cotransport of hippocampal astrocytes are quickly activated by small elevations in $[K^+]_o$, making them ideally suited to help buffer small increases in $[K^+]_o$ accompanying neuronal activity (Rose & Ransom, 1996*a*).

The role of neurones in K^+ uptake is more controversial. Early studies (e.g. Thomas, 1972) showed that changing $[K^+]_o$ had little effect on neuronal $[Na^+]_i$, whereas others indicated active K^+ uptake by neurones due to Na^+,K^+-ATPase pumping and Na^+–K^+–$2Cl^-$ cotransport (Ballanyi *et al.* 1984). It is unclear, however, if neuronal Na^+,K^+-ATPase activity is influenced by small fluctuations in $[K^+]_o$, like those seen with physiological activity (Heinemann & Lux, 1977). Neurones seem to express predominantly Na^+,K^+-ATPase α-subunits with a high affinity for $[K^+]_o$, and, therefore, are less likely to be significantly stimulated by elevations in $[K^+]_o$ beyond 3 mM (Sweadner, 1995), whereas ouabain-sensitive uptake of K^+ into cultured astrocytes increases up to a $[K^+]_o$ of 12 mM (Grisar, Frere & Franck, 1979; Walz & Hertz, 1982). This scheme was also supported by a recent neurochemical study on cultured cerebral astrocytes and neurones. It was found that Na^+,K^+-ATPase isolated from astrocytes had a significantly lower K^+ affinity than the neuronal enzyme, and was activated, therefore, by increasing $[K^+]_o$ above the control level (Hajek, Subbarao & Hertz, 1996).

The findings in this study agree with this model. $[Na^+]_i$ in the majority of hippocampal neurones, in contrast to $[Na^+]_i$ in hippocampal astrocytes (Rose & Ransom, 1996*a*; see above), was not significantly influenced by small, brief changes in $[K^+]_o$, suggesting a dominant expression in these neurones of α-subunits of Na^+,K^+-ATPase with a high K^+ affinity. Another explanation for the lack of a measurable effect of small $[K^+]_o$ increases on $[Na^+]_i$ would be a perfect balance between outward transport of Na^+, due to enhanced Na^+,K^+-ATPase activity, and inward Na^+ transport, by

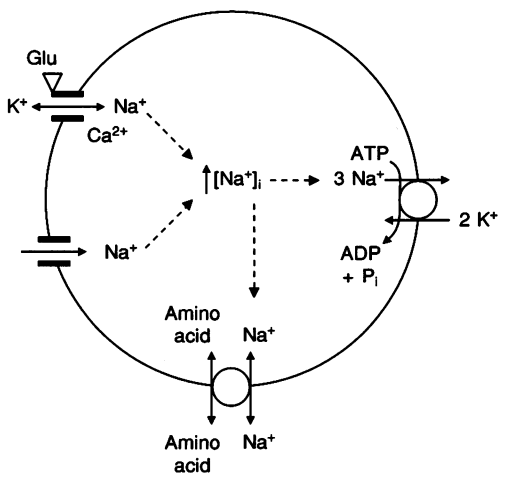

Figure 13. Main pathways for Na^+ fluxes in hippocampal neurones

This diagram shows the main pathways for transmembrane Na^+ fluxes in active hippocampal neurones. Na^+ entry via voltage-gated and/or transmitter-dependent ion channels can lead to increases in $[Na^+]_i$ of several millimolar during bursting behaviour. Na^+ export via Na^+,K^+-ATPase maintains low baseline $[Na^+]_i$ in the face of a steep electrochemical gradient. Another pathway that could mediate transmembrane Na^+ movement in these neurones is Na^+-dependent amino acid transport. This important pathway would be influenced by changes in $[Na^+]_i$, as suggested by the dashed line.

Na^+–K^+–$2Cl^-$ cotransport. Influx of Na^+ ions via voltage- or transmitter-gated Na^+ channels could also counteract a possible $[Na^+]_i$ loss following Na^+,K^+-ATPase activation, as indicated by the overall increase in $[Na^+]_i$ in about 26% of neurones during longer periods of K^+ elevation to 13 mM. However, the brief $[K^+]_o$ increases by 1 or 3 mM performed in this study were probably not high enough to alter neuronal membrane potential significantly.

Rausche and co-workers reported a depression of synaptic transmission in hippocampus slices with increases in $[K^+]_o$ of 2 mM (Rausche, Igelmund & Heinemann, 1990). In the cultured neurones of this study, however, depression of synaptic transmission was unlikely to be responsible for the $[K^+]_o$-induced effects on $[Na^+]_i$, because inhibition of synaptic transmission with high Mg^{2+} saline, or blocking Na^+ channels with TTX, did not alter baseline $[Na^+]_i$. It seems more likely that neuronal Na^+,K^+-ATPase is only weakly activated by small increases in $[K^+]_o$, so that changes in $[Na^+]_i$ were only visible during longer exposure to elevated $[K^+]_o$ in the majority of neurones. This interpretation is consistent with the prevailing view that K^+ uptake by previously active neurones is a slow process and does not contribute strongly to rapid management of activity-induced $[K^+]_o$ increases (see Newman, 1995).

Conclusions

The results of this study suggest several differences between $[Na^+]_i$ regulation of cultured rat hippocampal neurones and astrocytes (cf. Rose & Ransom, 1996*a*). In contrast to astrocytes, $[Na^+]_i$ of hippocampal neurones was mainly determined by Na^+,K^+-ATPase, whereas Na^+-dependent Cl^-–HCO_3^- exchange, Na^+–HCO_3^- cotransport or Na^+–K^+–$2Cl^-$ cotransport did not play a significant role (Fig. 13). As with astrocytes, Na^+,K^+-ATPase activity held $[Na^+]_i$ at a very stable value in the majority of cultured neurones if no other experimental manipulation was performed. In some neurones, however, spontaneous synchronized Na^+ bursts were observed, indicating that despite the powerful mechanisms to maintain a low baseline $[Na^+]_i$, synaptic activity and action potentials might be accompanied by significant $[Na^+]_i$ fluctuations that could influence the efficiency of a variety of other transporters dependent on the inwardly directed Na^+ gradient (e.g. Na^+-coupled uptake of transmitters; Fig. 13).

In contrast to cultured astrocytes, small, brief changes in bath $[K^+]_o$ had only modest effects on $[Na^+]_i$ of cultured hippocampal neurones. Therefore, our data suggest that activity-induced $[K^+]_o$ alterations alone might not induce significant changes in neuronal Na^+,K^+-ATPase activity in the intact hippocampus. This picture might be somewhat different for other regions of the nervous system, since Na^+,K^+-ATPase isozymes are not uniformly distributed in the brain (Sweadner, 1995). It should be kept in mind that the cultured neurones of this study differ from those *in vivo*; among other things, they lack a restricted extracellular space, and have different morphology and spatial arrangement. The results of the present study, however, correspond well to findings from invertebrate preparations where neuronal Na^+ regulation was investigated in a relatively intact environment (Thomas, 1972, 1977), supporting the notion that the basic properties of intracellular Na^+ regulation were preserved in the cultured cells.

It has been shown that both neuronal activity and various transmitters lead to large increases in neuronal $[Na^+]_i$ (Grafe *et al.* 1982; Coles & Orkand, 1985; Lasser-Ross & Ross, 1992; Rose & Ransom, 1996*b*), and to stimulation of Na^+,K^+-ATPase activity (e.g. Ransom, Barker & Nelson, 1975). Therefore, in intact tissue, stimulation of neuronal Na^+,K^+-ATPase activity by increased neuronal $[Na^+]_i$, and not by increased $[K^+]_o$, might be the signal initiating reuptake of $[K^+]_o$ into the hippocampal neurones. This scheme would have the advantage of ensuring that active neurones, with increased $[Na^+]_i$ and decreased $[K^+]_i$, would be the ones reaccumulating K^+.

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