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Contributions of the Na⁺/K⁺-ATPase, NKCC1, and Kir4.1 to hippocampal K⁺ clearance and volume responses

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Abstract

Bursts of network activity in the brain are associated with a transient increase in extracellular K⁺ concentration. The excess K⁺ is removed from the extracellular space by mechanisms proposed to involve Kir4.1-mediated spatial buffering, the Na⁺/K⁺/2Cl⁻ cotransporter (NKCC1), and/or Na⁺/K⁺-ATPase activity. Their individual contribution to [K⁺]_o management has been of extended controversy. The present study aimed, by several complementary approaches, to delineate the transport characteristics of Kir4.1, NKCC1, and Na⁺/K⁺-ATPase and to resolve their involvement in clearance of extracellular K⁺ transients.

Primary cultures of rat astrocytes displayed robust NKCC1 activity with [K⁺]_o increases above basal levels. Increased [K⁺]_o produced NKCC1-mediated swelling of cultured astrocytes and NKCC1 could thereby potentially act as a mechanism of K⁺ clearance while concomitantly mediate the associated shrinkage of the extracellular space. In rat hippocampal slices, inhibition of NKCC1 failed to affect the rate of K⁺ removal from the extracellular space while Kir4.1 enacted its spatial buffering only during a local [K⁺]_o increase. In contrast, inhibition of the different isoforms of Na⁺/K⁺-ATPase reduced post-stimulus clearance of K⁺ transients. The glia-specific α2/β2 subunit composition of Na⁺/K⁺-ATPase, when expressed in *Xenopus* oocytes, displayed a K⁺ affinity and voltage-sensitivity that would render this astrocyte-specific subunit composition specifically geared for controlling [K⁺]_o during neuronal activity. In rat hippocampal slices, simultaneous measurements of the extracellular space volume revealed that neither Kir4.1, NKCC1, nor Na⁺/K⁺-ATPase accounted for the stimulus-induced shrinkage of the extracellular space. Thus, NKCC1 plays no role in activity-induced extracellular K⁺ recovery in native hippocampal tissue while Kir4.1 and Na⁺/K⁺-ATPase serve temporally distinct roles.

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INTRODUCTION

Neuronal activity is associated with efflux of K^+ into brain extracellular space, where its accumulation can cause depolarization of neurons and glia and disturbance of neuronal signaling. Therefore, regulation of extracellular K^+ concentration ($[K^+]_o$) is of vital importance for neuronal function. *In vitro* studies have shown that stimulation-induced increases in $[K^+]$ are paralleled by accumulation of K^+ in astrocytes, suggesting an astrocytic contribution to $[K^+]_o$ regulation (Ballanyi et al. 1987; Grafe and Ballanyi 1987). Neuronal activity is, in addition, associated with shrinkage of the extracellular space (Dietzel et al. 1980; Ransom et al. 1985) probably due to astrocytic swelling (MacVicar et al. 2002). The molecular mechanism(s) responsible for clearance of the stimulus-evoked K^+ release and the associated shrinkage of the extracellular space have been intensely debated during several decades, for review, see (Hertz et al. 2013; MacAulay and Zeuthen 2012) and their individual contributions remain to be quantified.

The concept of *spatial buffering* as a mechanism of K^+ clearance was originally coined by Orkand et al. (1966) and is often assigned a prominent role as a mechanism of K^+ clearance (Kofuji and Newman 2004; Walz 2000). The premise is based on influx of K^+ through a K^+ channel, i.e. the astrocytic Kir4.1 (Kofuji and Newman 2004; MacAulay and Zeuthen 2012). The positive charge entering as K^+ spreads electrotonically through the cytoplasm and exits again as K^+ at locations distant from the active neurons (Newman et al. 1984) with no associated overall increase in the intracellular K^+ concentration (Orkand et al. 1966; Walz 2000). Although the existence of spatial buffering of K^+ has been documented (Karwoski et al. 1989; Strohschein et al. 2011), its quantitative contribution to K^+ clearance is uncertain.

Involvement of the Na^+/K^+ -ATPase in clearance of K^+ from the extracellular space following neuronal activity has been demonstrated (D'Ambrosio et al. 2002; Ransom et al. 2000) although the contribution of the different α and β isoforms of the Na^+/K^+ -ATPase remains unresolved.

Kir4.1-dependent influx of K^+ was proposed to functionally couple with aquaporin-4 (AQP4) to mediate the associated flux of water required for astrocytic cell swelling (Amiry-Moghaddam and Ottersen 2003; Nagelhus et al. 2004; Strohschein et al. 2011). This coupling was recently rejected as neither AQP4 nor Kir4.1 appeared to be required for stimulus-induced shrinkage of the extracellular space (Haj-Yasein et al. 2012; Haj-Yasein et al. 2011). The stoichiometry of the Na^+/K^+ -ATPase ($3Na^+:2K^+$) generates an *efflux* of one osmotic particle per turnover and is therefore unlikely to directly provide the osmotic driving force for an accompanying cell swelling. The $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) has been indicated as a mechanism involved in stimulus-evoked astrocytic cell swelling (MacVicar and Hochman 1991; MacVicar et al. 2002; Ransom et al. 1985) and may therefore partake in the clearance of excess K^+ from the extracellular space (MacVicar et al. 2002; Walz 2000). The present study aimed to determine the molecular characteristics of the K^+ transporting proteins in cell-based experimental systems and subsequently quantify their relative contribution to clearance of stimulus-evoked K^+ transients and the associated shrinkage of extracellular space in brain slices.

MATERIALS AND METHODS

Heterologous expression in *Xenopus laevis* oocytes

Rat Na⁺/K⁺-ATPase α 1, α 2, α 3, β 1 and β 2 subunit isoforms and rat NKCC1 were subcloned into the oocyte expression vector pXOOM, linearized downstream from the poly-A segment, and *in vitro* transcribed using T7 mMessage machine according to manufacturer's instructions (Ambion, Austin, TX). cRNA was extracted with MEGAclear (Ambion, Austin, TX) and micro-injected into defolliculated *Xenopus laevis* oocytes: 10-20 ng α 1-3 RNA/oocyte in combination with 3-6 ng β 1 or β 2 RNA/oocyte (10:3 ratio) or 50 ng NKCC1 RNA/oocyte. *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI) or National center for Scientific Research (France). The surgical protocol, by which the oocytes were retrieved, was approved by The Danish National Committee for Animal Studies. The preparation of defolliculated oocytes was carried out as described in (Moeller et al. 2009) and the oocytes were kept in Kulori medium (in mM): 90 NaCl, 1 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.4) for 4-5 days at 19°C prior to experiments.

Primary cultures of rat astrocytes

Astrocytes for radioactive tracer experiments: Cortical rat astrocytes were cultured from dissected cerebral cortices of P7-P8 rat pups (Sprague-Dawley, Taconic, Denmark). The dissected cortices were dissociated mechanically by passing the tissue through an 80 μ m nylon sieve into Dulbecco's modified Eagle's medium (D-5030, Sigma Aldrich) containing an additional 6 mM D-glucose, 2.5 mM L-glutamine, 26.2 mM NaHCO₃, 100,000 IU/L of penicillin and 20% foetal bovine serum (catalog nr 04-007-1a, lot 516714, Biological Industries, Israel). Single cells were generated by trituration with a syringe equipped with a steel cannula and plated in 24-well tissue culture plates (734-2325, VWR, Denmark). The foetal bovine serum concentration was sequentially reduced to 15 % and 10 % on the second and third week of culture. 0.25 mM dB-cAMP (D-0627, Sigma) was added to the culture medium in the third week of culture to obtain morphologically differentiated astrocytes (Su et al. 2000). Immunocytochemical staining for glial fibrillary acidic protein (GFAP), a marker protein for astroglial cells, illustrated that >95% of the cells present in culture were astrocytes (data not shown). The astrocytes were kept at 37°C with an atmosphere of 5% CO₂ and were used for experiments 3-4 weeks after plating.

Astrocytes for volume measurements: Adult male Sprague-Dawley rats (250-300 g) were used for the astrocytic preparation as described in detail in (Langan et al. 1995). These cultures have previously been used for volume measurements in (Assentoft et al. 2013; Ateya et al. 2005). In brief, adult astrocytes were isolated from gelatin-sponge implants left in the animal for three days. Sponges were subsequently removed, minced and triturated followed by trypsinization and passage through a nylon mesh with pore diameter of 20 μ m. The astrocytes were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells between passage 5 and 15 were used and experiments carried out two to five days after passage.

Electrophysiology of oocytes

Conventional two-electrode voltage clamp studies were performed with a DAGAN CA-1B High Performance oocyte clamp (DAGAN, Minneapolis, MN) with Digidata 1322A interface controlled by pCLAMP software, version 9.2 (Molecular Devices, Burlingame, CA). Electrodes were pulled from borosilicate glass capillaries to a resistance of 1-5 M Ω when filled with 1 M KCl. The current traces were obtained by stepping the clamp potential from -50 mV to test potentials ranging from 0 mV to -100 mV in 20 mV increments (200-ms pulses). Recordings were low pass-filtered at 500 Hz and sampled at 2 kHz. The perfusion solution consisted of (in mM): 30 choline chloride (ChCl), 70 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES (pH 7.4), with KCl substituting for ChCl to vary the K⁺ concentration. For each individual oocyte the membrane currents were obtained in a range of test solutions containing increasing concentrations of K⁺ from which the membrane currents obtained at 0 mM KCl were subsequently subtracted to isolate the K⁺-sensitive currents. To obtain the Na⁺/K⁺-ATPase-mediated currents, the experimental series was repeated in the presence of 1 mM ouabain (O-3125, Sigma Aldrich).

Radioactive uptake

Transport activity of NKCC1 and the Na⁺/K⁺-ATPase was determined as the bumetanide-sensitive (NKCC1) or ouabain-sensitive (Na⁺/K⁺-ATPase) influx of the radioactive K⁺ congener ⁸⁶Rb⁺ (NEZ072001MC, Perkin Elmer, Germany). Oocytes were pre-incubated at room temperature in (in mM): 30 ChCl, 70 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES (at pH 7.4) for 30 min, 10 oocytes per condition. The oocytes were subsequently transferred to isosmotic solutions in which KCl substituted for ChCl to obtain different [K⁺]_o with 2 μ Ci/ml ⁸⁶Rb⁺ added. 10 μ M bumetanide (B3023, Sigma) or 1 μ L/ml EtOH (vehicle control) was included in the uptake solutions. The uptake experiment was carried out at room temperature for 5 min which we previously demonstrated was at a time point in which time-linearity was maintained (Zeuthen and MacAulay 2012). The assay was terminated with three brief washes in ice-cold media containing 30 mM KCl prior to individual solubilization in scintillation vials with 200 μ L 10% sodium dodecyl sulfate (SDS). The radioactivity present was determined by liquid scintillation counting with Opti Fluor scintillation liquid (6013199, Perkin Elmer) in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard). The obtained counts were corrected for the varying amount of K⁺ present in the uptake solution.

Primary cultures of rat astrocytes grown in 24-well plates were allowed to equilibrate at 37°C for 5 min in solution containing (in mM): 30 ChCl, 104 NaCl, 1.2 CaCl₂, 1 MgCl₂, 5 D-glucose, 20 HEPES (pH 7.4). ⁸⁶Rb⁺ tracer uptake took place at 37°C for a period of 20 sec in an isosmotic solution in which KCl substituted for ChCl to obtain the required [K⁺]_o, with 2 μ Ci/ml ⁸⁶Rb⁺ present in the given solution. For each K⁺ concentration, a control uptake experiment was carried out in triplicate in parallel with experiments with bumetanide (10 μ M) or ouabain (1 mM), with all solutions containing 1 μ L/mL EtOH as a vehicle control for bumetanide. The experiment was terminated by aspiration of the ⁸⁶Rb⁺-containing medium followed by three rapid rinses with a solution containing 30 mM KCl, 10 μ M bumetanide, 1 mM ouabain and 50 μ M BaCl₂ to minimize leakage of ⁸⁶Rb⁺. The cells were lysed in 250 μ L 1 M NaOH, 100 μ L transferred to scintillation tubes, while the rest was

used for protein content determination (BioRad DC Protein Assay cat. no. 500-0116, BioRad). The radioactive content was determined by liquid scintillation counting with UltimaGold™ XR (6013119, Perkin Elmer) in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard). The obtained counts were corrected for the varying amount of K^+ present in the uptake solution.

Microfluidic cell volume sensor

A microfluidic cell volume sensor was used to study volume changes in astrocytes, as previously described (Ateya et al. 2005). Astrocytes were grown on a microscope slide that was inverted over the sensor chip thereby creating a flow chamber with a fixed volume of 60 nl. A sinusoidal current of 50 Hz, 1 μA was provided to the two outer electrodes in the chamber and the voltage was measured between the two inner electrodes. An increase in cell volume will increase the chamber resistance and thus an increase in voltage serves as the functional read-out of cell swelling. The solutions containing the different K^+ concentrations were created from two isosmolar stock solutions: a 0 mM K^+ stock consisting of (in mM): 129 NaCl, 30 ChCl, 1 $MgCl_2$, 1.2 $CaCl_2$, 5 glucose, 20 HEPES, 24.15 mannitol, and a 30 mM K^+ stock consisting of (in mM): 114 NaCl, 30 KCl, 1 $MgCl_2$, 1.2 $CaCl_2$, 5 glucose, 20 HEPES, 36.2 mannitol. The concentrations of NaCl and mannitol in the two solutions were adjusted to obtain identical conductivity and osmolarity (verified with a cryoscopic osmometer (Gonotec, Berlin, Germany) and a conductivity meter (Thermo Scientific)). The flow rate of the solution (~30 $\mu l/min$) was controlled by adjusting hydraulic pressure at the inlet.

Brain slices and solutions

Experiments were performed on male Wistar rats at P21-P30. Rats were anaesthetized using gaseous 2-Bromo-2-Chloro-1,1,1-Trifluoroethane (B-4388, Sigma). Following decapitation, the brain was quickly removed and placed into ice-cold cutting solution containing (in mM): 87 NaCl, 70 sucrose, 2.5 KCl, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4 , 7 $MgSO_4$, and 25 D-glucose, equilibrated with 95% O_2 , 5% CO_2 . Oblique sagittal (transverse) hippocampal slices (400 μm) were cut with a Campden Vibrating Microtome (7000SMZ, Campden Instruments, UK). Slices were transferred to the standard solution containing (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4 , 2 $MgSO_4$ and 10 D-glucose, and equilibrated with 95% O_2 , 5% CO_2 (pH 7.4 at the experimental temperature of 33-34°C) and left to recover at 34°C for 30 min and then kept at room temperature. In experiments involving $BaCl_2$, NaH_2PO_4 was replaced with NaCl and $MgSO_4$ replaced with $MgCl_2$ in order to prevent precipitation. All experiments were approved by the Ethics Committee for Animal Research at the University of Helsinki and complied with the policy of the Society for Neuroscience (USA).

Ion-sensitive microelectrodes and electrophysiological recordings in slices

Electrophysiological recordings were carried out in a custom-made submerged-type recording chamber (volume 800 μl , 33-34°C) with continuous superfusion of both sides of the slice at a flow rate of 2 ml/min. Recordings were performed within stratum radiatum of the CA1 region. High-frequency stimulation (HFS) was delivered by a bipolar electrode inserted into the stratum radiatum in the vicinity (< 500 μm) of the recording site.

Stimulation trains (10-20 V, 60-80 μ s pulses at 20 Hz for 10 sec) were delivered at 5 min intervals. The resulting extracellular field potentials were recorded with glass capillary microelectrodes pulled to resistances of 1-10 M Ω when filled with the standard solution (see above). This electrode served as reference signal for the ion-sensitive microelectrodes. Ion-sensitive microelectrodes were prepared from thin walled non-filamented glass capillaries (GC150T-7.5, Harvard Apparatus) pulled to obtain a tip diameter in the range of 1-2 μ m (Voipio et al. 1994). The capillaries were then silanized with gaseous N,N-dimethyltrimethylsilylamine (41716, Sigma Aldrich) at 200°C for 20 min prior to being filled with the selected backfilling solution and a short column of liquid membrane solution. Potassium-sensitive microelectrodes were made with a valinomycin-based membrane solution (99373, Potassium Ionophore I Cocktail B, Sigma) and backfilled with 150 mM NaCl, 3 mM KCl. The tetramethylammonium (TMA⁺)-sensitive microelectrodes were generated with a membrane solution consisting of 50 mg/ml potassium tetrakis (4-chlorophenyl) borate (60591, Sigma-Aldrich) in 1,2-dimethyl-3-nitrobenzene (40870, Sigma) and backfilled with 150 mM TMA-Cl (Nicholson and Phillips 1981; Voipio et al. 1994). For recordings of extracellular volume changes by means of these TMA⁺-sensitive electrodes, 1.5 mM TMA-Cl (stock solution, 5M, Sigma) was added to the recording solution. For simultaneous recordings of [K⁺]_o and extracellular volume changes, the valinomycin-containing electrodes were employed to monitor [K⁺]_o while TMA⁺-sensitive electrodes monitored changes in extracellular space volume. For simultaneous recordings with a valinomycin-based K⁺-sensitive microelectrode and a TMA⁺-sensitive microelectrode, their tips were placed within 50 μ m from each other in the core of the slice. In experiments designed to exclusively monitor [K⁺]_o, TMA⁺-sensitive electrodes were employed. These electrodes act as K⁺-sensors in the absence of TMA⁺ in the recording solution (Voipio et al. 1994). The ion-sensitive microelectrodes were calibrated at the end of the experiments and the recorded signals were converted off line to obtain the K⁺ concentration and % change in extracellular space (Voipio et al. 1994). Focal application of K⁺ by means of iontophoresis (the release of substances through the application of current) was carried out with a glass capillary microelectrode, similar to the one recording extracellular field potentials, filled with 1 M KCl. To prevent leakage, a continuous backing current of -1 nA was applied. K⁺ was delivered to the slice by application of a 0-100 nA current for 5 sec at 10 min intervals. In the iontophoresis experiments, the recording solution contained a cocktail of inhibitors to prevent spiking and activation of glutamate and GABA receptors: DL-2-amino-5-phosphonopentanoic acid (AP-5, 40 μ M), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX, 10 μ M), picrotoxin (PiTX, 100 μ M), (2S)-3-[[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid (CGP-55845, 1 μ M), tetrodotoxin (TTX, 1 μ M), all from Tocris Cookson (Bristol, UK). For all experiments, three consecutive control responses were recorded at intervals of 5 min (iontophoresis; 10 min) prior to bath application of drug. Wash-in of TMA⁺ was estimated to be completed after ~6 min. All drug traces used for illustration and quantification were recorded ~7 min after bath application of the drug with exception of 1) the iontophoretic experiments where the slice was exposed to Ba²⁺ for ~15 min prior to recording the response and 2) the 50 μ M ouabain, with which the traces were recorded after ~3 min to limit slice damage observed during extended exposure (at this time point extracellular [K⁺]_o had stabilized, indicating that ouabain had reached its

full effect). With exception of 50 μM ouabain, a second response to drug was recorded 5 minutes later (10 min for iontophoretic experiments) to ensure that the drug had reached its full effect. All recorded signals were anti-alias filtered, sampled at 1 kHz and stored for off-line analysis with WinEDR (courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK) and GraphPad Prism 5.0 (GraphPad Software).

Quantification of K^+ clearance rate

The maximum rate of K^+ clearance activated by an experimentally evoked transient rise in $[\text{K}^+]_o$ is proportional to the rate of fall in $[\text{K}^+]_o$ that is seen immediately after termination of stimulation-induced or iontophoretic release of K^+ into the extracellular space. In our experiments, the fall in $[\text{K}^+]_o$ during the first one (iontophoresis) or two (stimulus) seconds of K^+ clearance was linear, and it occurred in individual experiments at overlapping levels of $[\text{K}^+]_o$ in the presence and absence of inhibitors except when using ouabain. Therefore, we quantified the initial post-stimulus rate of fall in $[\text{K}^+]_o$ (in mM/sec) and used it as a direct measure of K^+ clearance in the initial post-stimulus phase. The extracellular space volume changes during this interval were small, and little affected by the applied inhibitors, and therefore did not affect the quantification. We did not use the time constant or half-time of $[\text{K}^+]_o$ recovery, since addition of inhibitors of K^+ translocating mechanisms may alter the kinetics of the recovery phase by e.g. increasing peak $[\text{K}^+]_o$ and/or generating a post-clearance undershoot in $[\text{K}^+]_o$, as has been reported for the Kir4.1 inhibitor Ba^{2+} (Bay and Butt 2012; Jauch et al. 2002; Oakley II et al. 1992). These additional effects of inhibitors may indirectly affect the *time constant* of the clearance while not interfering with the rate of K^+ clearance (in mM/sec).

Statistics

All data are given as mean $\pm$ SEM. Statistical significance was tested with either Student's t-test or one-way ANOVA with Dunnett's or Tukey's multiple comparison *post hoc* test, as indicated in figure legends. P values < 0.05 were considered statistically significant. Data on astrocytes and oocytes were obtained from at least three different animal preparations.

RESULTS

K^+ transport properties of NKCC1 and Na^+/K^+ -ATPase

For any molecular mechanism involved in the clearance of K^+ from the brain extracellular space, a certain prerequisite should be met: The transport protein must increase its activity in response to an increase in $[\text{K}^+]_o$ above the basal level of $\sim 3 \text{ mM}$ $[\text{K}^+]_o$. Primary cultures of rat astrocytes were employed to determine the K^+ -activation properties of NKCC1 and the Na^+/K^+ -ATPase. The transport rates were determined by uptake experiments with $^{86}\text{Rb}^+$, a radioactive congener of K^+ , as the bumetanide-sensitive (NKCC1) or ouabain-sensitive (Na^+/K^+ -ATPase) uptake. Time-dependent saturation occurred after approximately 30 sec of transport activity (Fig. 1A insert). The subsequent uptake measurements were therefore carried out for 20 sec to ensure that time-dependent saturation would not obscure the factual transport limit and thus skew the apparent $K_{0.5}$ obtained for the transport proteins (all listed $K_{0.5}$ values are apparent affinities). NKCC1 displayed increased turnover velocity as a function of $[\text{K}^+]_o$ (1-30 mM), with a $K_{0.5} = 21.3 \pm 2.7 \text{ mM}$, $n = 4$ (Fig. 1A). Earlier studies

carried out with $^{86}\text{Rb}^+$ -uptake periods outside the linear range, arrived at lower $K_{0.5(K^+)}$ for NKCC1 (Tas et al. 1987). For illustrative purposes we therefore allowed a 10 min long uptake period in an otherwise identical experiment and obtained a $K_{0.5}$ for K^+ of 1.9 ± 0.2 mM, $n = 3$ (data not shown), similar to that obtained by Tas et al. (1987).

The Na^+/K^+ -ATPase activity of cultured astrocytes is likely to consist of a mix of α - and β isoforms, most likely $\alpha 1$ and $\alpha 2$ (Juhaszova and Blaustein 1997) in combination with the accessory β subunit, either $\beta 1$ or $\beta 2$. The apparent affinity of the Na^+/K^+ -ATPase for K^+ ($K_{0.5} = 2.0 \pm 0.7$ mM, $n = 4$) was higher than that of NKCC1. Although saturation was approached already at 5 mM $[\text{K}^+]_o$, full saturation was not reached until an extracellular concentration of 10 mM K^+ (Fig. 1B). Thus K^+ transport into cultured astrocytes at low to basal extracellular concentrations of K^+ (i.e. 1-5 mM) was predominantly carried out by the Na^+/K^+ -ATPase. The contribution of the Na^+/K^+ -ATPase was roughly equal to that of NKCC1 at 10 mM $[\text{K}^+]$, above which NKCC1 became the primary mediator of the inwardly-directed K^+ transport, Fig. 1C. Notably, a contribution of spatial buffering through Kir4.1 to K^+ uptake cannot be determined in a culture bathed in extracellular solution containing K^+ , since the requirements for spatial buffering are not fulfilled in this experimental setting.

In addition, we determined the K^+ -dependency of NKCC1 (bumetanide-sensitive fraction of the $^{86}\text{Rb}^+$ uptake) upon heterologous expression of rat NKCC1 in *Xenopus laevis* oocytes (Fig. 1D). While the $K_{0.5}$ was lower (7.3 ± 1.3 mM, $n = 3$) than that obtained in astrocytes, the data support the conclusion from above; NKCC1 increases its transport activity along with K^+ concentrations that may be reached in the perisynaptic area of active neurons.

NKCC1-mediated cellular swelling in astrocytes

The robust contribution of NKCC1 to the overall K^+ uptake in cultured astrocytes and its associated K^+ -activation profile promote NKCC1 as a possible contributor to the clearance of stimulus-evoked K^+ in the central nervous system. NKCC1 may, possibly aided by its ability to cotransport water along with its substrate (Zeuthen and MacAulay 2012), thus serve as a molecular mechanism responsible for removal of K^+ and simultaneously induce stimulus-evoked astrocytic cell swelling. To determine the ability of NKCC1 to directly mediate astrocytic cell swelling upon an increase in extracellular K^+ , we employed a microfluidic cell volume sensor (Fig. 2C). This experimental device is based on cells placed in a small, closed chamber with application of a constant current through the chamber. Upon cell swelling, the free volume of the sensing chamber is reduced and the electric resistance therefore increased. The associated change in voltage is thus a sensitive and robust read-out of cell volume changes and is stable over the entire duration of the experiment (Ateya et al. 2005). With a transition from a K^+ -free solution (equimolar replacement with choline) to an isosmotic solution containing 15 mM K^+ , a rapid increase in cell volume was recorded (Fig. 2A, left panel). The K^+ -induced volume increase could be repeated following a 15-min return to perfusion with the K^+ -free control solution (Fig. 2A, middle panel). Inhibition of NKCC1 with bumetanide (10 μM) reduced the K^+ -mediated increase in cell volume (Fig. 2A, right panel) to 3 ± 1 % of control, $n = 9$. With a transition from an external solution containing 3 mM K^+ to an isosmolar solution containing 7 mM K^+ , the cells still presented

with a robust rise in volume (Fig. 2B). Bumetanide reduced the astrocytic cell swelling to $1 \pm 1\%$ of control, $n = 7$. When situated in primary cultures of rat astrocytes, it is thus evident that NKCC1 is capable of removing extracellular K^+ and in the process cause rapid uptake of water as well.

Inhibition of NKCC1 does not affect the rate of K^+ clearance nor extracellular space shrinkage in hippocampal slices

The contribution of NKCC1 to clearance of stimulus-evoked K^+ and the associated shrinkage of the extracellular space was determined in hippocampal slices of the rat. We employed ion-sensitive microelectrodes to simultaneously record the concentration of extracellular K^+ (with a valinomycin-based liquid-membrane solution) and the relative size of the extracellular space (with a liquid-membrane solution sensitive to the probe cation TMA^+) in the same local area. The tips of the two ion-sensitive electrodes and a field potential electrode were placed in CA1 stratum radiatum in the core of the slice (see Fig. 3A insert for a schematic drawing of the experimental design). 1.5 mM TMA^+ was added to the superfusion solution and allowed to equilibrate into the slice prior to recording of the responses. TMA^+ , being practically membrane-impermeable, increases its concentration upon shrinkage of the extracellular space and thus serves as a read-out for changes in extracellular space volume (Nicholson and Phillips 1981; Voipio et al. 1994). Local electrical stimulation (20 Hz, 10 sec) in the CA1 region of hippocampus gave rise to $[K^+]_o$ transients to 5–10 mM ($n = 4$, representative trace illustrated in Fig. 3A) that were paralleled by negative shifts in the extracellular field potential signal (not illustrated) and shrinkage of the extracellular space to 91.6 – 97.8 % of control, $n = 4$ (representative trace illustrated in Fig. 3B). Both the $[K^+]_o$ and volume responses kept on rising until the end of the stimulation train and their recovery started promptly upon cessation of electrical stimulation. Inhibition of NKCC1 by bumetanide (10 μ M) had no effect on the rate of K^+ clearance (see representative traces in Fig. 3A). For quantification of the post-stimulus K^+ clearance we used its initial rate (see Materials and Methods for details). During the 2-sec quantification interval, marked by dashed lines in Fig. 3A, $[K^+]_o$ was a linear function of time and was cleared at a rate of 0.67 ± 0.16 mM/sec, $n = 4$. The rate of K^+ clearance in the presence of bumetanide was normalized to that of the control trace and data are summarized in Fig. 3C, left panel, $n = 4$. Inhibition of NKCC1 had no effect on the shrinkage of the extracellular space either (grey line in Fig. 3B, data normalized and summarized in Fig. 3C, right panel, $n = 4$). Taken together, NKCC1 did not partake in post-stimulus K^+ -recovery or the associated shrinkage of the extracellular space in hippocampus, despite the obvious capabilities thereof inherent in the molecular mechanism (Figs. 1 and 2).

Kir4.1-dependent spatial buffering as a contributor to K^+ clearance

The experimental design above was applied to resolve the role for Kir4.1 in stimulus-induced K^+ clearance and associated shrinkage of extracellular space in hippocampal slices. In this set of experiments, the stimulation parameters (20 Hz, 10 sec) generated increases in the extracellular K^+ concentration to levels of 4–10 mM, $n = 5$. No statistically significant reduction of the post-stimulus K^+ decay rate was observed upon block of Kir4.1 with 100 μ M $BaCl_2$ (Ransom and Sontheimer 1995) (see representative traces in Fig. 4A, left panel). The decay rate (0.61 ± 0.15 mM/sec, $n = 5$) was obtained from the initial 2 sec of K^+

clearance, where the K^+ removal was a linear function of time. The rate of clearance obtained in the presence of $BaCl_2$ was normalized to that of the control trace and data are summarized in Fig. 4A, right panel, $n = 5$. Inhibition of Kir4.1 did not affect the extracellular space volume changes during high-frequency stimulation (see representative traces in Fig. 4B, left panel, and summarized data in the right panel, $n = 3$).

The paradigm of spatial buffering relies on K^+ moving from distinct areas of high K^+ loads to more distant compartments where $[K^+]_o$ remains unchanged. It can be argued that high-frequency stimulation, such as the one employed in the present experimental design, results in an area of increased $[K^+]_o$ which is simply too large to allow for spatial buffering to be observed at the site of recording. In essence, within the stimulated part of hippocampus, an area in which $[K^+]_o$ remains at its basal level may not occur and the prerequisite for spatial buffering will thus not be fulfilled. In the subsequent set of experiments, we therefore relied on a focal application of K^+ into the brain slice through iontophoresis via a glass capillary micropipette. In this way, we generated a constant point source of K^+ within the slice. To prevent spontaneous neuronal activity, a series of inhibitors were added to the artificial cerebrospinal fluid to block the action of the voltage-gated Na^+ channel (TTX), GABA- (PiTX, CGP-55845), and glutamate receptors (NBQX, AP-5). A 5 sec pulse generated K^+ rises in the extracellular space in the range 6-15 mM, $n = 4$. The iontophoretic pulse generated comparable rises in $[K^+]_o$ during three consecutive control pulses (Fig. 4C, insert). Iontophoretic pulses applied following bath application of $BaCl_2$ consistently generated an increase in peak $[K^+]_o$, as illustrated by the representative traces in Fig. 4C, left panel and summarized in the right panel. Addition of $BaCl_2$ increased the peak $[K^+]_o$ to 122.1 ± 5.1 % of control, $n = 4$, $p < 0.01$. The ensuing rate of post-stimulus K^+ clearance rate was similar in the absence and presence of 100 μM $BaCl_2$ (see representative traces in Fig. 4C). The rate of clearance (3.81 ± 1.24 mM/sec, $n=4$) was quantified during the initial 1 sec following the pulse, marked with dashed lines, where the falling of $[K^+]_o$ was a linear function of time (normalized data summarized in Fig. 4A, right panel, $n = 4$). To exclude that the recovery rate was affected by the higher peak K^+ , a parallel quantification was carried out at comparable $[K^+]_o$ concentrations (from the peak of the control trace, lasting 0.5 sec during which both traces were in their linear phase). No Ba^{2+} -mediated change in the recovery rate of $[K^+]_o$ was detected with this mode of quantification (data not shown). It would thus appear that Kir4.1-dependent spatial buffering does not account for a significant contribution to the *post-stimulus* recovery of $[K^+]_o$ or to the associated shrinkage of extracellular space. Kir4.1, however, partakes in the K^+ clearance taking place *during* the application of the iontophoretically applied $[K^+]_o$ increase.

Block of the Na^+/K^+ -ATPase compromises extracellular K^+ removal in a dose-dependent manner related to the subunit isoforms

With no evident involvement of neither NKCC1 nor Kir4.1 in post-stimulus $[K^+]_o$ recovery, we sought to determine the contribution of the various isoforms of the Na^+/K^+ -ATPase. In this set of experiments, the high-frequency stimulation of the hippocampal slices (20 Hz, 10 sec) generated, in control solution, increases in extracellular K^+ concentration in the range 4-13 mM, with a decay-rate of 0.73 ± 0.18 mM/sec, $n = 7$. In rodents, the $\alpha 1$ isoform of the Na^+/K^+ -ATPase α -subunit displays a low sensitivity to the Na^+/K^+ -ATPase inhibitor

ouabain (around 1 mM ouabain is required to obtain a full block of $\alpha 1$ activity, whereas the $\alpha 2$ and $\alpha 3$ isoforms are fully inhibited at 5 μ M (Blanco et al. 1993)). This difference allowed us to test the contribution of $\alpha 2/\alpha 3$ separately from that of $\alpha 1$. It should be noted, however, that complete block of Na^+/K^+ -ATPase activity compromised the slice viability and we therefore opted for partial inhibition of $\alpha 1$ with an ouabain concentration of 50 μ M, which roughly constitutes the IC_{50} for rat $\alpha 1$ (Jewell and Lingrel 1991). The basal level of K^+ in the extracellular space increased upon application of ouabain, as seen in the representative traces in Fig. 5A: 5 μ M ouabain lead to a basal $[\text{K}^+]_o$ of 3.9 ± 0.4 mM, $n = 7$ and the 50 μ M ouabain to a $[\text{K}^+]_o$ of 4.8 ± 0.8 mM, $n = 5$. The post-stimulus $[\text{K}^+]_o$ decay rate displayed a marked decrease following block of the $\alpha 2/\alpha 3$ -subunits of the Na^+/K^+ -ATPase (see representative trace, dark grey, in Fig. 5A). Additional application of ouabain (to 50 μ M) led to a severely compromised K^+ clearance (light grey trace in Fig. 5A). The ouabain-dependent decrease in the rate of post-stimulus $[\text{K}^+]_o$ recovery was normalized to the control trace and summarized in Fig. 5C, left panel. The rate of decay was significantly reduced at both 5 μ M (69.3 ± 9.3 %, $n = 7$, $p < 0.05$) and 50 μ M (31.5 ± 7.8 %, $n = 5$, $p < 0.001$) compared to control. Inhibition of Na^+/K^+ -ATPase with 5 μ M ouabain (block of $\alpha 2/\alpha 3$) did not affect the extracellular space volume changes during high-frequency stimulation (see representative traces in Fig. 5B, and summarized data in Fig. 5C, right panel, $n = 3$). Additional inhibition of Na^+/K^+ -ATPase activity with 50 μ M ouabain caused a tendency towards *increased* stimulus-induced extracellular space shrinkage, i.e. the astrocytes swelled faster and more when the Na^+/K^+ -ATPase was less operational. These data demonstrate that the Na^+/K^+ -ATPase-mediated K^+ removal does not, in itself, generate stimulus-induced astrocytic cell swelling (see representative trace in Fig. 5B insert and summarized data in Fig. 5C, right panel). It would thus appear that the combined actions of different Na^+/K^+ -ATPase isoforms is the key mechanism underlying uptake of neuronally released K^+ following electrical stimulation albeit without causing astrocytic cell swelling in the process.

The $\alpha 2\beta 2$ Na^+/K^+ -ATPase subunit composition appears geared for K^+ clearance

Astrocytes express $\alpha 1$ and $\alpha 2$ as well as the accessory subunit, $\beta 1$ and $\beta 2$ whereas neurons express $\alpha 1$, $\alpha 3$, and possibly $\alpha 2$, all in combination with $\beta 1$ (Cameron et al. 1994; Cholet et al. 2002; Juhaszova and Blaustein 1997; Li et al. 2013; McGrail et al. 1991). It remains unknown what fractions of the different α isoforms are expressed at the cell membranes and to which β subunit the glial α isoforms associate. To determine the molecular characteristics of the different combinations of these subunits and isoforms, we expressed the various combinations of the rat Na^+/K^+ -ATPase heterologously in *Xenopus laevis* oocytes. The advantage thereof is the low background of *Xenopus* α and β subunits, compared to those heterologously expressed: The ouabain-sensitive currents of the uninjected oocytes amounted to < 10 % of those obtained in oocytes heterologously expressing the $\alpha 1$ and $\alpha 2$ isoforms of the rat Na^+/K^+ -ATPase and < 25 % of those expressing the $\alpha 3$ isoform (data not shown). This expression system thereby provided us with the ability to determine the individual properties of the different combinations of the isoforms. The K^+ -activation profile for each combination of Na^+/K^+ -ATPase was determined by two-electrode voltage clamp with the I/V relationship established at each applied K^+ concentration. The experimental protocol was subsequently repeated in the presence of ouabain (1 mM) in order to extract the ouabain-sensitive current representing Na^+/K^+ -ATPase activity. Representative current

traces, obtained with 5 mM K^+ in the extracellular solution, for an $\alpha 2\beta 2$ -expressing oocyte are depicted in Fig. 6A in the absence (upper panel) and presence (lower panel) of ouabain (1 mM). The $\alpha 1$ isoform displayed a $K_{0.5}$ for K^+ of 0.25 ± 0.06 mM, $n = 7$ when co-expressed with the $\beta 1$ accessory subunit and 0.67 ± 0.20 mM, $n = 7$ when co-expressed with $\beta 2$. Both subunit compositions approached full saturation at an extracellular K^+ concentration around 2 mM, Fig. 6B. The $K_{0.5}$ for K^+ of the $\alpha 3$ isoform was similar to that of $\alpha 1$, whether co-expressed with $\beta 1$ (0.55 ± 0.13 mM, $n = 4$) or $\beta 2$ (0.40 ± 0.08 mM, $n = 4$). The molecular combination of $\alpha 2$ with $\beta 1$ yielded a $K_{0.5}$ for K^+ of 0.91 ± 0.08 mM, $n = 7$, which was comparable to those obtained with the $\alpha 1$ and $\alpha 3$ isoform. In contrast, the $\alpha 2\beta 2$ combination displayed a significantly higher $K_{0.5}$ for K^+ of 3.6 ± 0.5 mM, $n = 5$ ($p < 0.001$) and only approached saturation with extracellular K^+ concentrations of 10 mM. The low K^+ affinity of $\alpha 2\beta 2$ thus provides this constellation of the Na^+/K^+ -ATPase with the aforementioned requirement of increased transport activity upon a stimulus-induced rise in $[K^+]_o$ level in the brain. On the other hand, the remaining subunit isoform combinations will respond only slightly to increments above the basal 3 mM $[K^+]_o$.

An increase in $[K^+]_o$ will promote a depolarization of the astrocytic and neuronal cell membranes. We therefore determined the $K_{0.5}$ for K^+ as a function of the membrane potential, Fig. 6C. The $K_{0.5}$ for K^+ of the $\alpha 3$ isoform combinations was not affected by changes in membrane potential whereas $K_{0.5}$ for K^+ decreased for all $\alpha 1$ and $\alpha 2$ isoform combinations as the membrane depolarized ($p < 0.05$). The effect was most prominently observed for $\alpha 2\beta 2$. In addition, all isoform constellations displayed increased activity rate upon depolarization of the cell membrane in test solutions containing 5 mM K^+ , $n = 6-8$ of each isoform combination, Fig. 6D. The voltage-dependence of $\alpha 2\beta 2$ was, however, significantly more pronounced than that of $\alpha 1\beta 1$ (in $10^{-3} I_{rel}/mV$; 18.2 ± 0.8 for $\alpha 2\beta 2$ vs. 11.4 ± 2.1 for $\alpha 1\beta 1$, $n = 6$, $p < 0.05$) and the $\alpha 2\beta 2$ subunit composition will thus increase its activity prominently as the astrocytic cell membrane depolarizes.

DISCUSSION

We have in the present study demonstrated that NKCC1 does not contribute to removal of K^+ from the extracellular space following neuronal activity in the rat hippocampus, whereas the combined action of the Na^+/K^+ -ATPase isoforms acts as key contributor. While Kir4.1 did not contribute to post-stimulus K^+ clearance, the peak $[K^+]_o$ was partially dependent on the action of Kir4.1 under conditions favoring spatial buffering. The concept of spatial buffering of K^+ via Kir4.1 has been forwarded as an important means of post-stimulus recovery of the extracellular K^+ concentration (Kofuji and Newman 2004; Walz 2000). It has been convincingly demonstrated that K^+ taken up in a local area of the astrocytic syncytium or retinal Müller cell is subsequently released at a distant site via Kir4.1 (Karwoski et al. 1989; Strohschein et al. 2011). A quantitative contribution of Kir4.1-mediated spatial buffering to K^+ clearance is uncertain: A range of experimental approaches with pharmacological block or genetic deletion of Kir4.1 have demonstrated minor, if any, delay in the rate of *post-stimulus* $[K^+]_o$ recovery (Chever et al. 2010; D'Ambrosio et al. 2002; Karwoski et al. 1989; Meeks and Mennerick 2007; Ransom et al. 2000) although another study reported decreased post-stimulus $[K^+]_o$ recovery in the Kir4.1 knock-out animal (Haj-Yasein et al. 2011).

Upon high-frequency stimulation of brain slice preparations, it is likely that the extracellular K^+ concentration is increased in a large volume of the tissue. The resultant global $[K^+]_o$ increase may prevent spatial buffering and a stimulation paradigm as the one used in the present study and in (Chever et al. 2010; Haj-Yasein et al. 2011; Meeks and Mennerick 2007; Ransom et al. 2000) may thus be ill-suited to experimentally determine the contribution of Kir4.1-mediated spatial buffering to clearance of excess $[K^+]_o$. To ensure an optimal experimental setting for detection of spatial buffering of K^+ , we therefore applied K^+ focally by means of iontophoresis. Inhibition of Kir4.1 did not significantly delay *post-stimulus* $[K^+]_o$ recovery but caused an increase in the peak $[K^+]_o$ *during* the K^+ application, the latter of which in agreement with an earlier report (Jauch et al. 2002). This observation aligns well with previous studies in retina, an experimental preparation conducive to Kir4.1-mediated spatial buffering due to its geometry and localized K^+ release: Kir4.1-mediated K^+ release into the vitreal body was apparent *during* the light stimulus whereas the vitreal K^+ concentration declined during post-stimulus $[K^+]_o$ -recovery (Karwoski et al. 1989). In addition, a Ba^{2+} -mediated delay in K^+ clearance in the inner plexiform layer of the retina was observed only *during* the light-evoked stimulus whereas the rate of post-light stimulus clearance of K^+ seemed unaffected by inhibition of Kir4.1 (Karwoski et al. 1989; Oakley II et al. 1992). Thus, it appears that Kir4.1-mediated spatial buffering enacts its function throughout the phase of continuous delivery of K^+ that takes place *during* neuronal activity and not in the post-stimulus phase of the $[K^+]_o$ recovery.

In the present experimental setting, inhibition of the Na^+/K^+ -ATPase caused a substantial delay in post-stimulus recovery of $[K^+]_o$ but did not prevent the associated shrinkage of the extracellular space. The Na^+/K^+ -ATPase thus appears as a prime molecular mechanism responsible for clearance of stimulus-induced increase in $[K^+]_o$ in agreement with previous reports on optic nerve and hippocampal CA3 stratum pyramidale (D'Ambrosio et al. 2002; Ransom et al. 2000). The ability of the Na^+/K^+ -ATPase to regulate $[K^+]_o$ in hippocampus was recently demonstrated; astrocytic Ca^{2+} signaling stimulated Na^+/K^+ -ATPase activity and thereby caused a reduction of $[K^+]_o$ (Wang et al. 2012). We demonstrate a partial role for $\alpha 2/\alpha 3$ in post-stimulus recovery of $[K^+]_o$. Complete inhibition of all three isoforms of the Na^+/K^+ -ATPase compromised slice viability but partial inhibition of $\alpha 1$ (in addition to the full inhibition of $\alpha 2/\alpha 3$) led to a further reduction in the rate of K^+ clearance. A problematic nature of the experimental design is the continued presence of ouabain in the test solution prior to induction of the stimulus protocol. This prolonged inhibition of Na^+/K^+ -ATPase activity (3 min) increased the baseline level of K^+ in the extracellular space of the brain slice, as observed here and in (D'Ambrosio et al. 2002; Ransom et al. 2000), which may affect the rate of K^+ clearance in a manner unknown.

The cell-specific expression pattern of the Na^+/K^+ -ATPase α isoforms and the accessory β isoforms in the central nervous system remains unresolved due to the contradictory evidence in the literature. One emerging picture, however, assigns $\alpha 2$ and $\beta 2$ to glial cells, $\alpha 3$ to neurons and $\alpha 1$ and $\beta 1$ to both cell types although some $\alpha 2$ staining has been reported in neurons (Cameron et al. 1994; Cholet et al. 2002; Juhaszova and Blaustein 1997; Li et al. 2013; McGrail et al. 1991). We took advantage of the *Xenopus laevis* expression system to determine the isoform-specific characteristics of the different combinations of the Na^+/K^+ -

ATPase isoforms without significant contribution from contaminating native isoforms. The activity of $\alpha 1$ and $\alpha 3$ was saturated already at basal extracellular K^+ concentrations, whether combined with $\beta 1$ or $\beta 2$. When paired with $\beta 1$, $\alpha 2$ displayed a tendency towards lower K^+ affinity than $\alpha 1$, as previously observed (Blanco 2005; Horisberger and Kharoubi-Hess 2002; Jewell and Lingrel 1991), although in the present study the difference did not reach statistical significance. However, upon replacement of the household $\beta 1$ isoform with $\beta 2$, the K^+ affinity of $\alpha 2$ shifted towards lower affinity, which is in agreement with observations on the human isoforms of the Na^+/K^+ -ATPase (Crambert et al. 2000). Similarly, the voltage-sensitivity of $\alpha 2$ appeared more pronounced than that of $\alpha 1$ when paired with $\beta 1$ (Horisberger and Kharoubi-Hess 2002) although upon pairing of $\alpha 2$ with $\beta 2$, the voltage-sensitivity became significantly stronger than that obtained with $\alpha 1/\beta 1$. Notably, the apparent affinity for K^+ of the $\alpha 1$ and $\alpha 2$ isoforms (in combination with either β isoform but most prominently for $\alpha 2\beta 2$) increased as the membrane potential became more depolarized, i.e. at highly depolarized membrane potentials, these Na^+/K^+ -ATPase isoforms will reach their maximal transport rates at lower K^+ concentrations. Although the underlying transporter kinetics of this voltage-induced shift in $K_{0.5(K^+)}$ have yet to be resolved, one may speculate that $\alpha 2\beta 2$ in this manner is ensured of maximum transport activity during conditions of raised $[K^+]_o$. The glia-related $\alpha 2\beta 2$ isoform constellation thereby exhibited increased Na^+/K^+ -ATPase activity i) along physiologically relevant K^+ increments and ii) along the membrane depolarization inevitably associated with increased $[K^+]_o$. Taken together with the proposed localization of $\alpha 2$ in glial leaflets surrounding dendritic spines (Cholet et al. 2002), $\alpha 2\beta 2$, although not as a single major contributor, appears specifically geared to respond efficiently to stimulus-evoked increase of K^+ in the extracellular space.

Inhibition of NKCC1 has earlier been proposed to reduce stimulus-evoked shrinkage of the extracellular space in hippocampus and optic nerve (MacVicar and Hochman 1991; MacVicar et al. 2002; Ransom et al. 1985). NKCC1 would, as such, act as a potential additional mechanism for post-stimulus $[K^+]_o$ recovery (MacVicar et al. 2002; Walz 2000). Partly due to uncertainty regarding rat NKCC1's K^+ affinity and therefore its ability to increase its activity along physiologically occurring $[K^+]_o$ increments, its putative involvement in K^+ clearance has remained unresolved. We determined the apparent affinity of rat NKCC1 for K^+ with radioactive uptake experiments at a time point at which the uptake was a linear function of time and thus yielded a reliable $K_{0.5}$. The rat NKCC1, whether expressed natively in cultured astrocytes or heterologously in *Xenopus* oocytes, displayed increasing transporter activity along K^+ increments expected to occur in the perisynaptic extracellular space, albeit with different $K_{0.5}$ s. The different ionic compositions of intra- and extracellular solutions, putative accessory proteins, as well as the different membrane potentials of the two cell types may well underlie the differences in apparent affinity constants obtained in these two cellular systems. Either way, NKCC1 appears able to increase its transport activity along physiologically occurring K^+ increases in the extracellular space. Taken together with the robust NKCC1-mediated increase in cultured astrocytic cell volume upon an abrupt isotonic increase in $[K^+]_o$, NKCC1 displayed features that could account for glial clearance of excess $[K^+]_o$ in association with a shrinkage of the extracellular space. Irrespective thereof, NKCC1 did not contribute to the regulation of stimulus-evoked $[K^+]_o$ transients or extracellular space volume changes in the hippocampal

brain slice. Thus, while NKCC1 is highly expressed in cultured astrocytes (this study and (Su et al. 2002a; Su et al. 2002b; Walz 1992)), absence of astrocytic NKCC1 expression in native rat brain tissue has previously been reported (Clayton et al. 1998; Plotkin et al. 1997) but see Randall et al. (1997). This pattern is in line with NKCC1 being recognized for its functional up regulation in cultured cells of different lineages (Raat et al. 1996). However, it is entirely possible that during brain pathologies, i.e. epilepsy, stroke, and edema, NKCC1 may be up-regulated and thereby contribute to K^+ and water homeostasis.

The stimulus-evoked shrinkage of the extracellular space, taking place during the neuronal activity, terminates with the stimulus-induced $[K^+]_o$ increase, i.e. the post-stimulus K^+ recovery is associated with a *normalization* of the size of the extracellular space. Therefore, it must be emphasized that the mechanism responsible for post-stimulus restoration of basal $[K^+]_o$ does not mediate net astrocytic influx of osmotic particles. The extracellular space volume response remained intact following pharmacological blockade of Kir4.1, in agreement with a previous report carried out with Kir4.1 $^{-/-}$ mice (Haj-Yasein et al. 2011). Genetic deletion of AQP4, in addition, failed to abolish stimulus-induced shrinkage of the extracellular space (Haj-Yasein et al. 2012). A functional coupling of Kir4.1 and AQP4, in terms of stimulus-evoked glial cell swelling, therefore cannot be reconciled with published experimental data nor with the concept of Kir4.1-mediated spatial buffering precluding intracellular accumulation of ions (and thus osmotic particles). The molecular mechanism underlying the stimulus-evoked shrinkage of the extracellular space, which has recurrently been observed (Dietzel et al. 1980; Ransom et al. 1985) thus remains elusive.

In conclusion, we have demonstrated that NKCC1 does not contribute to K^+ clearance following neuronal activity in the hippocampus whereas the Na^+/K^+ -ATPase acts as a key mechanism. Kir4.1 enacts its contribution to K^+ -buffering *during* the delivery of K^+ to the extracellular space. We must, however, emphasize that different clearance mechanisms may dominate in other brain regions. Increased Na^+/K^+ -ATPase activity generates intracellular K^+ accumulation and its molecular characteristics therefore aligns well with the reported transient increase in intracellular K^+ concentration of astrocytes during the burst of activity which, however, is immediately followed by post-stimulus restoration of neuronal $[K^+]_i$ (Ballanyi et al. 1987; Grafe and Ballanyi 1987). The glial-specific isoform constellation of the Na^+/K^+ -ATPase, $\alpha 2\beta 2$, appears to be specifically geared towards clearance of excess K^+ from the extracellular space due to its lower K^+ affinity and higher voltage-sensitivity.

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Figure 1.

K^+ -stimulated activity of NKCC1 and the Na^+/K^+ -ATPase. (A-C) $^{86}Rb^+$ uptake in primary cultures of rat astrocytes: The NKCC1-dependent uptake was obtained as the bumetanide-sensitive (10 μ M) fraction (A) after ensuring time-linearity (insert) and the Na^+/K^+ -ATPase-dependent uptake as the ouabain-sensitive (1 mM) fraction (B). The individual experiments were fitted according to Michaelis-Menten kinetics and plotted as the average % of V_{max} as a function of $[K^+]_o$ ($n = 4$ experiments carried out in triplicate with the $K_{0.5}$ obtained from each individual experiment prior to averaging). (C) The fractional contribution of NKCC1 and Na^+/K^+ -ATPase to the total overall uptake at each K^+ concentration. (D) The bumetanide-sensitive (10 μ M) uptake in rNKCC1-expressing *Xenopus laevis* oocytes were fitted according to Michaelis-Menten kinetics and plotted as the average % of V_{max} as a function of $[K^+]_o$ whereas the $K_{0.5}$ was obtained from each individual experiment prior to averaging ($n = 3$ experiments with 10 oocytes per condition).

Figure 2.

K⁺-induced NKCC1-mediated astrocytic cell swelling. Stable baseline recordings of cell volume of cultured astrocytes were obtained in a microfluidic cell volume sensor with a control solution containing 0 mM K⁺. (A) The cells were abruptly exposed to an isosmotic test solution containing 15 mM K⁺ as indicated by the black bar in the left panel. Following return to 0 mM K⁺ for 15 min, exposure to 15 mM K⁺ induced a similar rate of astrocytic cell swelling (middle panel) compared to the one obtained in the initial trace. The K⁺-induced astrocytic cell swelling was abolished by addition of bumetanide (10 μM), right panel. (B) As in (A) but with 3 mM K⁺ in the control solution followed by an isosmotic challenge with a test solution containing 7 mM K⁺ (left panel). The K⁺-induced astrocytic cell swelling was abolished by addition of bumetanide (10 μM), right panel. (C) A schematic drawing of the microfluidic cell volume sensor with the two outer electrodes, between which the current is passed, and the two inner electrodes, between which the voltage is measured.

Figure 3.

NKCC1 does not contribute to K^+ -clearance and extracellular space shrinkage in hippocampal brain slices. Ion-sensitive microelectrodes were employed to simultaneously measure extracellular $[K^+]_o$ and the relative size of the extracellular space (upon addition of 1.5 mM TMA⁺ to the test solution) in rat hippocampal slices. **(A)** Electrical stimulation (10 sec at 20 Hz) of stratum radiatum in CA1 (insert) generated field potentials. Representative traces of stimulus-evoked changes in $[K^+]_o$ prior (black trace) and post (grey trace) exposure to bumetanide (10 μ M). The dashed lines mark the linear section ($r^2 = 0.99$ in all experiments) used for quantification of the K^+ decay rate. **(B)** Representative recordings of the relative extracellular space obtained simultaneously in the same local area as (A); the black trace illustrates the control trace and the grey trace the one obtained in the presence of bumetanide. **(C)** Normalized and summarized data on the post-stimulus $[K^+]_o$ decay rate (left panel) and the rate of extracellular space shrinkage (right panel): Bumetanide had no effect on the $[K^+]_o$ decay rate (96.7 ± 8.8 % of control, $n = 4$) or the relative extracellular

space ($94.3 \pm 4.8\%$ of control, $n = 4$). Data presented as mean $\pm$ SEM and statistical significance determined with Student's paired t-test.

Figure 4.

Kir4.1 does not contribute to post-stimulus K⁺-clearance and extracellular space shrinkage in hippocampal brain slices. Ion-sensitive microelectrodes were employed to determine the effect of Ba²⁺-mediated inhibition of Kir4.1 on K⁺ clearance and extracellular space shrinkage, essentially as in Fig. 3. (A) Representative traces of stimulus-evoked changes in [K⁺]_o prior (black trace) and post (grey trace) exposure to BaCl₂ (100 μM), left panel. The dashed lines mark the linear section (with $r^2 = 0.99$ in all experiments) used for quantification of the K⁺ decay rate. The data obtained in the presence of BaCl₂ were

normalized to control and summarized for the rate of decay of $[K^+]_o$ following stimulus (88.7 ± 10.6 % of control, $n = 5$) and iontophoresis of K^+ (111.0 ± 7.4 % of control, $n = 4$), right panel. **(B)** Representative recordings of the relative extracellular space obtained simultaneously in the same local area as (A); the black trace illustrates the control trace and the grey trace the one obtained in the presence of $BaCl_2$, left panel. The rate of stimulus-induced shrinkage of extracellular space obtained in the presence of $BaCl_2$ was normalized to control and summarized (98.0 ± 9.0 % of control, $n = 3$) in the right panel (two of these were carried out simultaneously with K^+ measurements and one was carried out with individual monitoring of the extracellular space). **(C)** Focal application of K^+ was obtained with iontophoresis with a 5 sec current pulse (indicated by the black bar) in the presence of an inhibitor cocktail containing (AP-5 40 μ M, NBQX 10 μ M, PiTX 100 μ M, CGP-55845 1 μ M, TTX 1 μ M). Consecutive iontophoretic pulses gave rise to identical increases in $[K^+]_o$ (insert). Representative traces of changes in $[K^+]_o$ prior (black trace) and post (grey trace) exposure to $BaCl_2$ (100 μ M), left panel. The dashed lines mark the linear section (with r^2 0.99 in all experiments) used for quantification of the K^+ decay rate which, in this set of experiments, covers a span of 1 sec due to the fast decay and thereby loss of linearity, see (A), right panel for summarized data. Peak $[K^+]_o$ values obtained in the presence of $BaCl_2$ was normalized to that of control and summarized (122.1 ± 5.1 %, $n = 4$) in the right panel. Data presented as mean $\pm$ SEM and statistical significance determined with one-way ANOVA with Dunnett's multiple comparison *post hoc* test and Student's paired t-test (**; $p < 0.01$).

Figure 5.

Inhibition of the Na⁺/K⁺-ATPase delays K⁺-clearance in hippocampal brain slices. Ion-sensitive microelectrodes were employed to determine the effect of ouabain on K⁺ clearance following electrical stimulation, essentially as in Fig. 3. **(A)** Representative traces of stimulus-evoked changes in [K⁺]_o in control solution (black trace), after exposure to 5 μM ouabain (dark grey trace) and subsequently to 50 μM ouabain (light grey trace). The dashed lines mark the linear 2-sec section (with $r^2 = 0.99$ in all experiments) used for quantification of the K⁺ decay rate. **(B)** Representative recordings of the relative extracellular space in an

independent experiment; the black trace illustrates the control trace and the grey trace the recording after application of 5 μM ouabain. Insert depicts a similar experiment obtained with 50 μM ouabain (light gray trace). (C) The $[\text{K}^+]_o$ decay rate obtained in the presence of ouabain was normalized to control and summarized (69.3 ± 9.3 % of control for 5 μM ouabain, $n = 7$ and 31.5 ± 7.8 % of control for 50 μM ouabain, $n = 5$), left panel. The rate of stimulus-induced shrinkage of the extracellular space in the presence of ouabain was normalized to control and summarized (99.3 ± 4.4 % of control, $n = 3$ for 5 μM ouabain and 131 ± 15 % of control, $n = 4$ for 50 μM ouabain), right panel. Data presented as mean $\pm$ SEM and statistical significance determined with one-way ANOVA with Dunnett's multiple comparison *post hoc* test (*; $p < 0.05$, ***, $p < 0.001$).

Figure 6.

The subunit constellation of the Na^+/K^+ -ATPase determines its K^+ - and voltage-sensitivity. Various subunit isoform compositions of the Na^+/K^+ -ATPase ($\alpha 1\beta 1$, $\alpha 1\beta 2$, $\alpha 2\beta 1$, $\alpha 2\beta 2$, $\alpha 3\beta 1$, $\alpha 3\beta 2$) were heterologously expressed in *Xenopus laevis* oocytes and their activity determined with two-electrode voltage clamp. **(A)** From a holding potential of -50 mV, the membrane potential was stepped to 0 mV and subsequently in 20 mV increments to -100 mV for 200 msec (step protocol depicted in insert). The panel illustrates representative current traces from an $\alpha 2\beta 2$ -expressing oocyte prior to (upper trace) and following exposure to 1 mM ouabain (lower trace). **(B)** The Na^+/K^+ -ATPase activity was determined as a function of $[\text{K}^+]_o$ (at $V_m = -80$ mV) and fitted according to Michaelis-Menten kinetics. The graph depicts the obtained activity at each $[\text{K}^+]_o$ as % of $V_{max} \pm \text{SEM}$ whereas the affinity constants were obtained from each individual oocytes prior to averaging, $n = 4-7$. **(C)** The $K_{0.5}$ s for each subunit isoform composition was plotted as a function of the membrane potential (V_m), $n = 4-7$. **(D)** The ouabain-sensitive I/V relationships for each of the subunit compositions were obtained in the presence of 5 mM K^+ ($n = 6-8$ of each). Data was normalized to the current obtained at a membrane potential of -60 mV. Data presented as

mean $\pm$ SEM and statistical significance determined with one-way ANOVA with Tukey's Multiple Comparison *post hoc* test or Student's t-test.