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Hyperosmolar sodium chloride is toxic to cultured neurons and causes reduction of glucose metabolism and ATP levels, an increase in glutamate uptake, and a reduction in cytosolic calcium

Cecilie Morland[a,b], Mi Nguyen Pettersen[a], Bjørnar Hassel[a,c,*]

[a] Norwegian Defence Research Establishment, Kjeller, Norway
[b] Oslo and Akershus University College of Applied Sciences, Oslo, Norway
[c] Department of Complex Neurology and Neurohabilitation, Oslo University Hospital and The University of Oslo, Oslo, Norway

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ABSTRACT

Elevation of serum sodium, hypernatremia, which may occur during dehydration or treatment with sodium chloride, may cause brain dysfunction and damage, but toxic mechanisms are poorly understood. We found that exposure to excess NaCl, 10–100 mmol/L, for 20 h caused cell death in cultured cerebellar granule cells (neurons). Toxicity was due to Na^+, since substituting excess Na^+ with choline reduced cell death to control levels, whereas gluconate instead of excess Cl^- did not. Prior to cell death from hyperosmolar NaCl, glucose consumption and lactate formation were reduced, and intracellular aspartate levels were elevated, consistent with reduced glycolysis or glucose uptake. Concomitantly, the level of ATP became reduced. Pyruvate, 10 mmol/L, reduced NaCl-induced cell death. The extracellular levels of glutamate, taurine, and GABA were concentration-dependently reduced by excess NaCl; high-affinity glutamate uptake increased. High extracellular [Na^+] caused reduction in intracellular free [Ca^{2+}], but a similar effect was seen with mannitol, which was not neurotoxic. We suggest that inhibition of glucose metabolism with ensuing loss of ATP is a neurotoxic mechanism of hyperosmolar sodium, whereas increased uptake of extracellular neuroactive amino acids and reduced intracellular [Ca^{2+}] may, if they occur *in vivo*, contribute to the cerebral dysfunction and delirium described in hypernatremia.

1. Introduction

An increase in the serum concentration of sodium ions above a physiological concentration, hypernatremia, may cause cerebral dysfunction, including cognitive dysfunction and delirium (for review, see Lieberman, 2007; Pendlebury et al., 2015), cerebral edema (Unal et al., 2008), and destruction of myelin, myelinolysis (Han et al., 2015). Hypernatremia may occur in dehydration, during intravenous infusion of hypertonic saline, or after excessive salt intake (Sterns, 2015), and serum values may in extreme cases exceed 200 mmol/L (Meadow, 1993; Ofran et al., 2004), which is approximately 60 mmol/L above normal serum sodium values. Dehydration may occur in elderly patients and the terminally ill (Bruera et al., 2005; Vidán et al., 2009), and dehydration is a common complication of prolonged fever and of diarrhoea, especially in children (World Health Organization, 2000). Therapeutically, intravenous infusion of hypertonic saline to raise serum sodium by 10–20 mmol/L is used to treat intracranial hypertension (for review, see Himmelseher, 2007; Ropper, 2012). Experimental studies have shown that an increase in the serum concentration of sodium leads to a similar increase in the extracellular fluid of the brain, with a time lag of about 30 min (Cserr et al., 1987, 1991).

In spite of the well described cerebral symptoms of hypernatremia (Lieberman, 2007; Vidán et al., 2009; Sterns, 2015) little is known about the effect of supranormal concentrations of sodium chloride on neuronal function and survival. Himmelseher et al. (2001) found that a 15-min pulse of hypertonic saline and hydroxyethyl starch (350 mOsm/L) was toxic to cultured hippocampal neurons, as could be seen from cell death 24 h later. Bhardwaj et al. (2000) found that hypernatremia aggravated neocortical damage after stroke in rats. Elliott et al. (2007) reported that hypertonic saline given immediately after traumatic brain injury in rats worsened brain damage. In human patients with traumatic brain injury hypernatremia is associated with increased mortality (Alharfi et al., 2013; Li et al., 2013).

Abbreviations: aCSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; TCA cycle, tricarboxylic acid cycle.

* Corresponding author at: Norwegian Defence Research Establishment, N-2027 Kjeller, Norway.
E-mail address: bjornar.hassel@ffi.no (B. Hassel).

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The aim of this study was to establish whether prolonged exposure of neurons to hyperosmolar NaCl is toxic in an *in vitro* model and to gain insight into the cellular responses of neurons to high concentrations of NaCl, including effects on energy metabolism, handling of extracellular amino acids, and intracellular free [Ca^{2+}].

2. Experimental procedures

2.1. Cell culture and incubation parameters

Cerebellar granule cells were cultured from the cerebella of 8-day-old rat pups according to Schousboe et al. (1989). Cells were seeded in poly-D-lysine-coated 24-well plastic trays or in plastic dishes (9 cm in diameter), and kept at 37 °C in an atmosphere of air:CO_2, 95:5. On day 2 cytosine D-arabinoside was added to a final concentration of 10 μmol/L to inhibit astrocytic growth. The cultures were used for experiments on day 8 *in vitro*.

For measurements of intracellular calcium and sodium, cerebellar granule cells were cultured on glass coverslips as described (Ring and Tansø, 2007; see below).

In experiments, the culture media were removed and replaced by artificial cerebrospinal fluid (aCSF) containing (in mmol/L) NaCl 94, KCl 25, $NaHCO_3$ 25, NaH_2PO_4 1.4, $CaCl_2$ 1.2, $MgCl_2$ 1.2, giving a total [Na^+] of 120.4. The high [K^+] promotes neuronal survival, and [Na^+] has to be reduced to achieve *iso*-osmolarity (Gallo et al., 1987; Schousboe et al., 1989; Van der Valk et al., 1991). However, in some experiments NaCl was 115 mmol/L (which together with $NaHCO_3$ and NaH_2PO_4 gave a total [Na^+] of 141.4 mmol/L), and KCl was 4 mmol/L; the other salts and glucose were kept constant.

Glucose was 1 mmol/L, because this is close to concentrations that have been measured extracellularly in the brain of wake rats (Kealy et al., 2013). Glucose was increased to 10 mmol/L in some experiments.

NaCl was added to give an additional concentration of 10, 30, 50, 60, or 100 mmol/L. In some experiments excess NaCl (50 mmol/L) was replaced by Na-gluconate or choline-Cl, 50 mmol/L, or mannitol or glycerol, 100 mmol/L. Incubations were done at 37 °C. In some experiments cells were incubated for 20 h with excess NaCl in the absence or presence of MK-801, 5 μmol/L (RBI, Natick, MA, USA) or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) 10 μmol/L (Tocris Bioscience, Bristol, UK). The solutions were equilibrated with O_2:CO_2 (95:5), and pH was adjusted to 7.3. Experimental incubations took place at 37 °C in an atmosphere of air and CO_2 (95:5).

2.2. Measurements of cell death

Cell death was assessed by the uptake of propidium iodide. Cell death was also assessed with trypan blue uptake (Supplemental Fig. 1), but results were similar to those obtained with propidium iodide; therefore only the latter are reported in the main text. More than 300 cells were counted per culture dish in the propidium uptake studies; more than 200 were counted in the trypan uptake studies. In some experiments cell death was measured as release of lactate dehydrogenase (LDH) to the extracellular fluid as described (Dreiem et al., 2005 and Supplemental Fig. 2). Previously, we have found excellent agreement between dye exclusion tests and LDH measurements in determination of cell death in cultured cerebellar granule cells (Olsen et al., 1999).

2.3. ATP measurements

For ATP measurements cerebellar granule cells were cultured in plastic dishes, 9 cm in diameter. Cells were exposed to aCSF, aCSF + NaCl, 50 mmol/L, or aCSF + mannitol, 100 mmol/L, for 3, 5, or 10 h. At the end of incubation, the media were removed, and the cultures were immersed in liquid N_2. One mL ice-cold perchloric acid, 7% (vol/vol), was sprayed onto the deep frozen cultures, which were then re-immersed in liquid N_2. Cells were scraped off together with the frozen perchloric acid, which was allowed to melt into slurry, transferred to a plastic tube, and centrifuged at 5000*g* to remove protein. The supernatants were brought to pH 8.1 with KOH, 9 mol/L. ATP in the extracts was analyzed fluorometrically according to Lowry and Passonneau (1972). Protein was analyzed according to Lowry et al. (1951).

2.4. Radiolabeling from [^{14}C]glucose. Measurement of amino acids, glucose, and lactate

For metabolic radiotracer studies the culture media were removed, and the cells were washed once with aCSF. Then cultures were incubated for 1 or 10 h with aCSF containing additional NaCl, 50 mmol/L, or mannitol, 100 mmol/L. Glucose was 1 mmol/L. Fifteen minutes before harvesting the cells, 1 μCi of [U-^{14}C]glucose, 3 μCi/μmol (American Radiolabeled Chemicals, St. Louis, MO, USA), was added to the incubation media. At the end of experiments the aCSF was removed, and cultures were washed once with 10 mL ice-cold aCSF before cells were harvested in 200 μL ice-cold perchloric acid, 3% (vol/vol), containing α-aminoadipate, 50 μmol/L, as an internal concentration standard. Protein was removed by centrifugation, and supernatants were neutralized with KOH, 9 mol/L. The precipitating $KClO_4$ was removed by centrifugation, and supernatants were lyophilized to dryness and redissolved in 60 μL double-distilled H_2O. The total levels of amino acids were quantified by HPLC and fluorescence detection after pre-column derivatization with *o*-phthaldialdehyde, as described (Hassel et al., 1995). Radiolabeling of glutamate and aspartate was determined after separation by HPLC as above. The HPLC eluate was collected in 1-minute fractions, and radiolabeling was measured by scintillation counting (Hassel et al., 1995).

Amino acid concentrations in aCSF were determined by HPLC and fluorescence detection as above after mixing the media 1:1 with α-aminoadipate, 50 μmol/L. Concentrations of glucose and lactate were measured by reflectance spectrophotometry with a DT 60 II Ektachem (Kodak, Rochester, NY, USA).

2.5. High-affinity uptake of ^{3}H-labeled glutamate

Cerebellar granule cells were washed once with aCSF and exposed to aCSF containing [2,3-^{3}H]glutamate (24Ci/mmol; final concentration 50 nmol/L; New England Nuclear, Boston, MA, USA) and increasing concentrations of sodium chloride (0–60 mmol/L excess) at room temperature. At 3 min the cultures were washed twice with ice-cold aCSF containing choline chloride instead of NaCl. Blank values were obtained by exposing cells to [2,3-^{3}H] glutamate in sucrose. Blank values were <10% of sodium-dependent values. Cells were harvested in 0.5 mL 70% ethanol, protein was removed by centrifugation, and radioactivity was measured by scintillation counting.

2.6. Measurements of intracellular free [Ca^{2+}] and [Na^+]

Intracellular free [Ca^{2+}] was measured with fura-2/AM (Molecular Probes, Leiden, The Netherlands). Primary cultures of cerebellar granule cells were cultured on 60 × 24 mm rectangular cover slips (Menzel, Germany). Cells were incubated at 37 °C in a

humidified 5% CO_2 atmosphere for 40 min with 5 μM fura-2/AM in the culture medium. Four coverslips were mounted in a custom-built chamber (Ring and Tansø 2007) giving 12 isolated wells on each coverslip and a total of 48 wells for each experiment. A plate reader (Fluostar Optima, BMG Labtechnologies, Germany), equipped with injectors, exposed cells to hypertonic or control solutions during the experiment. The extracellular concentration of sodium was increased by 50 mmol/L by the 1:5 addition of aCSF (with K^+ at 25 or 4 mmol/L, as appropriate) with excess NaCl, 250 mmol/L. For mannitol experiments aCSF with mannitol, 500 mmol/L, was added 1:5 (black columns). Excitation alternated between 340 nm and 380 nm and emission was measured at 510 nm. The bandwidth was 10 nm for all filters. Excitation and emission was from the bottom side of the glass coverslips. The system was calibrated with cells permeabilized with ionomycin and solutions containing EGTA, 1 mmol/L ($0Ca^{2+}$), or Ca^{2+}, 5 mmol/L, to obtain minimum and maximum calcium responses, respectively. Background autofluorescence was determined from coverslips with cells not preincubated with fura-2/AM. Intracellular $[Ca^{2+}]$ was estimated from the 340/380 fluorescence ratio, as described by Grynkiewicz et al. (1985). Because intracellular free $[Ca^{2+}]$ in cells exposed to control media ($[Na^+]$ 120.4 mmol/L, $[K^+]$ 25 mmol/L) varied between 140 and 150 nmol/L (see Results) data are given as percent of control. Intracellular sodium ions were monitored similarly with the use of SBFI/AM (Molecular Probes), excitation at 340 nm and 380 nm, and emission at 505 nm.

2.7. Data presentation and statistics

Data are given as means ± SD values. Statistical difference was assessed with Kruskal-Wallis test or one-way ANOVA with Dunnett's or Newman-Keuls' corrections for multiple comparisons, as appropriate, using GraphPad Prism 6. A p-value < 0.05 was considered significant.

3. Results

3.1. Toxicity of hyperosmolar NaCl

Incubation of cerebellar granule cells in artificial cerebrospinal fluid (aCSF), containing KCl, 25 mmol/L, and a total $[Na^+]$ of 120.4 mmol/L produced little cell death over 20 h, as could be seen from propidium iodide staining (Fig. 1a). Increasing NaCl caused cell death in a concentration-dependent manner; this effect was evident even when the concentration of NaCl was raised by as little as 10 mmol/L above control. Incubation of cells with 50 mmol/L excess NaCl for 10 h did not cause any cell death above control levels.

When neurons were kept for 20 h in aCSF with KCl, 4 mmol/L, and a total $[Na^+]$ of 141.4 mmol/L, cell death was high (Fig. 1b). An excess NaCl of 50 mmol/L reduced cell death under these circumstances. To be able to study possible toxic effects of hyperosmolar sodium chloride we therefore proceeded with aCSF with KCl at 25 mmol/L and a total $[Na^+]$ of 120.4 mmol/L in control solutions.

3.2. Identification of hyperosmolar Na^+ as neurotoxic

Replacement of excess NaCl, 50 mmol/L, with choline chloride resulted in cell death at control levels, whereas replacement with sodium gluconate caused as much cell death as did the excess NaCl, 50 mmol/L (Fig. 1c). Results were the same when cells were exposed to excess NaCl, 100 mmol/L, or choline chloride or sodium gluconate, 100 mmol/L (not shown). Challenging the cells with osmotic agents (mannitol, 100 mmol/L, or glycerol, 100 mmol/L) did not cause significant cell death (Fig. 1c). These findings

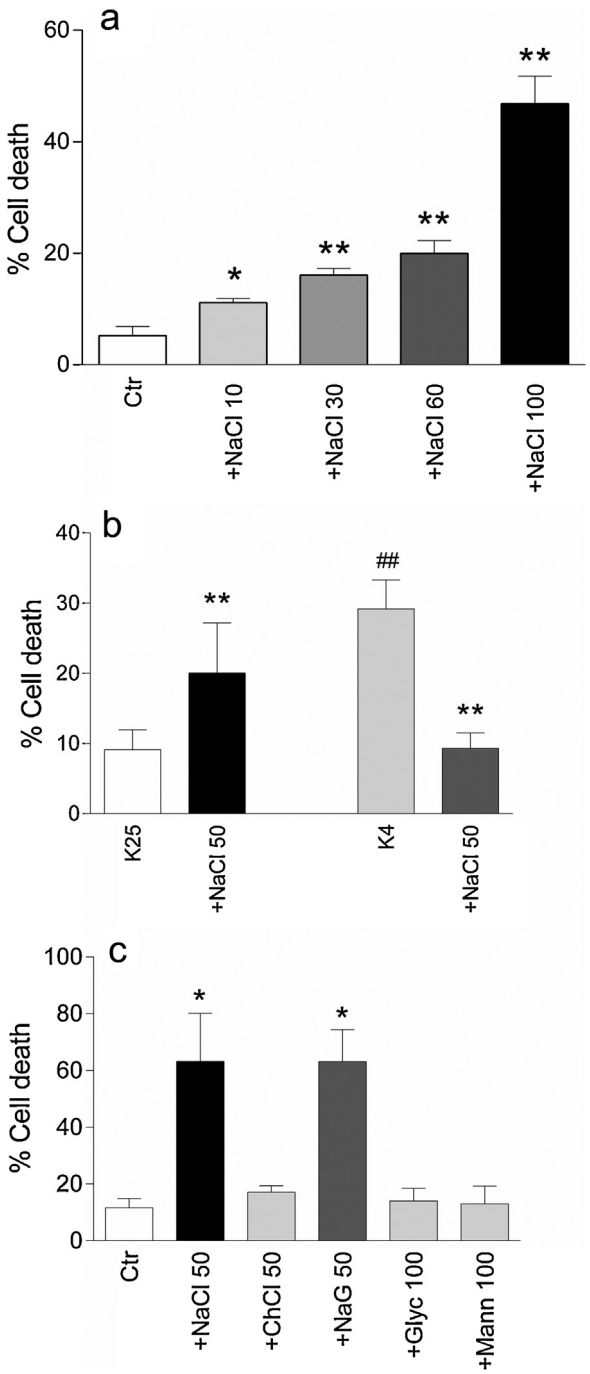

Fig. 1. Neurotoxic effect of sodium chloride. a: Cerebellar granule cells were incubated for 20 h in aCSF with $[Na^+]$ 120.4 mmol/L and $[K^+]$ 25 mmol/L (Ctr). NaCl (Na) was added in excess at 10, 30, 60, or 100 mmol/L. b: Cerebellar granule cells were incubated in aCSF with $[K^+]$ 25 mmol/L (two left columns) and $[Na^+]$ 120.4 mmol/L (white column) or 170.4 mmol/L (black column) or in aCSF with $[K^+]$ 4 mmol/L (two right columns) and $[Na^+]$ 141.4 mmol/L (light grey column) or 191.4 mmol/L (dark grey column). c: Cerebellar granule cells were incubated in control aCSF as in a. NaCl, choline chloride (ChCl), or sodium gluconate (NaG) were added at 50 mmol/L, whereas glycerol (Glyc) or mannitol (Mann) were added at 100 mmol/L. Necrotic cell death was determined from nuclear staining with propidium iodide. Data are percent dead cells, mean + SD values (N = 4–7). Data in Fig. 1a and c were analysed by one-way ANOVA and Dunnett's correction for multiple comparisons; *: different from control, $p < 0.05$, **: $p < 0.01$. Data in Fig. 1b were analysed by Newman-Keuls all pairwise comparison; **: different from corresponding aCSF control without added NaCl, $p < 0.01$; ##: different from control aCSF with $[K^+]$ 25 mmol/L, $p < 0.01$.

indicated that excess Na^+ was responsible for the neurotoxicity of hyperosmolar NaCl.

3.3. Effect of hyperosmolar NaCl on extracellular levels of amino acids

Hyperosmolarity induced by addition of sucrose has been used to elicit glutamate release in brain slices (Stevens and Williams, 2000), and an increase in extracellular glutamate may cause neuronal cell death through excitotoxicity (Choi, 1987). Cultured neurons give off amino acids to the extracellular fluid even under control conditions (Hassel et al., 1995). We therefore measured the extracellular concentration of glutamate and other amino acids after exposure to hyperosmolar NaCl. The extracellular concentration of glutamate decreased progressively with increasing concentrations of NaCl (Fig. 2a), as determined after 1 h of incubation.

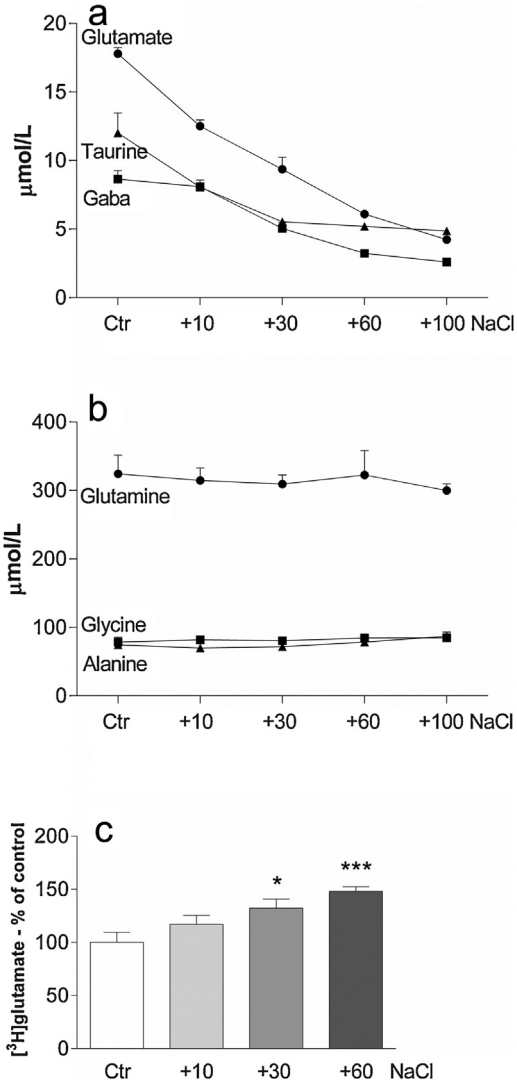

Fig. 2. Effect of hyperosmolar NaCl on extracellular levels of amino acids and high-affinity glutamate uptake. (a) Level of glutamate, taurine, and GABA, and (b) level of glutamine, glycine, and alanine in the incubation medium at 1 h of incubation at 37 °C of cerebellar granule cells with aCSF supplemented with excess NaCl, 0–100 mmol/L. Data are µmol/L, mean + SEM values. All amino acid concentrations in (a) were significantly reduced by excess NaCl ($p < 0.001$; one-way ANOVA, Dunnett's method), except GABA at excess NaCl, 10 mmol/L. c: Uptake of ^{3}H-labeled glutamate by cerebellar granule cells at room temperature during 3 min in the presence of excess NaCl, 0–60 mmol/L. Data are percent of control, mean + SD values (N = 4-6 per value). Asterisks: significantly different from control value; *: $p < 0.05$; ***: $p < 0.001$; one-way ANOVA, Dunnett's method.

A decrease was also seen for GABA and taurine. Aspartate was not detectable extracellularly in control cultures or cultures exposed to hyperosmolar NaCl. The extracellular concentrations of alanine, glycine, or glutamine were not significantly altered by hyperosmolar sodium chloride (Fig. 2b).

Increasing concentrations of NaCl augmented the uptake of ^{3}H-labeled glutamate into cultured granule cells (Fig. 2c), an effect that may explain the lowering of extracellular glutamate with increasing concentrations of sodium chloride (compare Fig. 2a and c).

To see if glutamate toxicity might contribute to the toxic effect of hyperosmolar Na^+ in spite of a reduced extracellular concentration of glutamate, we incubated cells in aCSF with excess NaCl, 100 mmol/L, in the presence or absence of *N*-methyl-D-aspartate (NMDA) receptor blocker MK-801, 5 µmol/L, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), 10 µmol/L. None of the glutamate receptor blockers reduced cell death induced by hyperosmolar NaCl (data not shown).

3.4. The effect of hyperosmolar NaCl on glucose metabolism

Because inhibition of energy metabolism could be a cause of neurotoxicity, we exposed neuronal cultures to aCSF with excess NaCl, 50 mmol/L, or mannitol, 100 mmol/L, for 1 or 10 h and added ^{14}C-labeled glucose during the last 15 min of incubation. There was no cell death at either time point, as could be observed in sister cultures. At 1 h, no significant differences were seen in the concentration of glucose or lactate in the incubation media (not shown). However, at 10 h, diminished consumption of glucose was evident from a higher level of glucose and a lower level of lactate in the incubation media of cells exposed to excess NaCl (Fig. 3a). After 1 h of incubation with excess NaCl, 50 mmol/L, the intracellular concentration of aspartate had increased (Fig. 3b), and this tendency was even more pronounced after 10 h (Fig. 3c). At this latter time point the intracellular concentration of glutamate was also increased. The radiolabeling of intracellular amino acids from ^{14}C-labeled glucose was not significantly changed after 1 h of incubation (Fig. 3d), but at 10 h the formation of radiolabeled glutamate from [U-^{14}C]glucose was reduced by 29% (Fig. 3e). In contrast, addition of mannitol, 100 mmol/L, did not cause significant changes in glucose metabolism or intracellular levels of glutamate or aspartate (Fig. 3a–e).

3.5. Effect of pyruvate on cell death caused by hyperosmolar NaCl

To see if inhibition of glycolysis might underlie the toxic effect of hyperosmolar Na^+ we added Na-pyruvate, 10 mmol/L, during exposure to excess NaCl, 50 mmol/L. This treatment reduced cell death at 20 h significantly (Fig. 4). Increasing the concentration of glucose in the aCSF from 1 to 10 mmol/L also reduced cell death caused by hyperosmolar NaCl (Fig. 4).

3.6. Effect of hyperosmolar NaCl on the level of ATP

To see whether the reduction in glucose metabolism caused by hyperosmolar NaCl was associated with changes in ATP levels, we incubated cells with excess NaCl, 50 mmol/L, or mannitol, 100 mmol/L, and determined neuronal ATP content at various time points. After 3 h of exposure to excess NaCl, 50 mmol/L, there was no significant reduction in ATP, but after 5 and 10 h of exposure the level of ATP had fallen by 18% and 29%, respectively (Fig. 5). At neither time point was there any cell death, as could be determined in sister cultures. Exposure of cells to aCSF with mannitol,

100 mmol/L, did not cause any reduction in ATP levels compared to control (Fig. 5).

3.7. Hyperosmolar solutions and intracellular free [Ca^{2+}]

In cells incubated under control conditions ([Na^+] 120.4 mmol/L, [K^+] 25 mmol/L), mean intracellular free [Ca^{2+}] was 145–165 nmol/L in the various experiments. In cells incubated with [Na^+] 141.4 mmol/L and [K^+] 4 mmol/L, mean intracellular free [Ca^{2+}] was approximately 80 nmol/L. Below, intracellular free [Ca^{2+}] is given as percent of values obtained with [Na^+] 120.4 mmol/L and [K^+] 25 mmol/L.

Both acute (10 min) and long-term (3 and 10 h) exposure to excess NaCl, 50 mmol/L, led to a significant reduction in intracellular free [Ca^{2+}] (Fig. 6a). This effect was highly reproducible in six separate experiments, and it was seen, although at smaller magnitude, with 25 mmol/L excess NaCl (Fig. 6b). However, the reduction in intracellular free [Ca^{2+}] was not specific to sodium chloride, but was also seen in cells exposed to mannitol, 100 mmol/L (Fig. 6a).

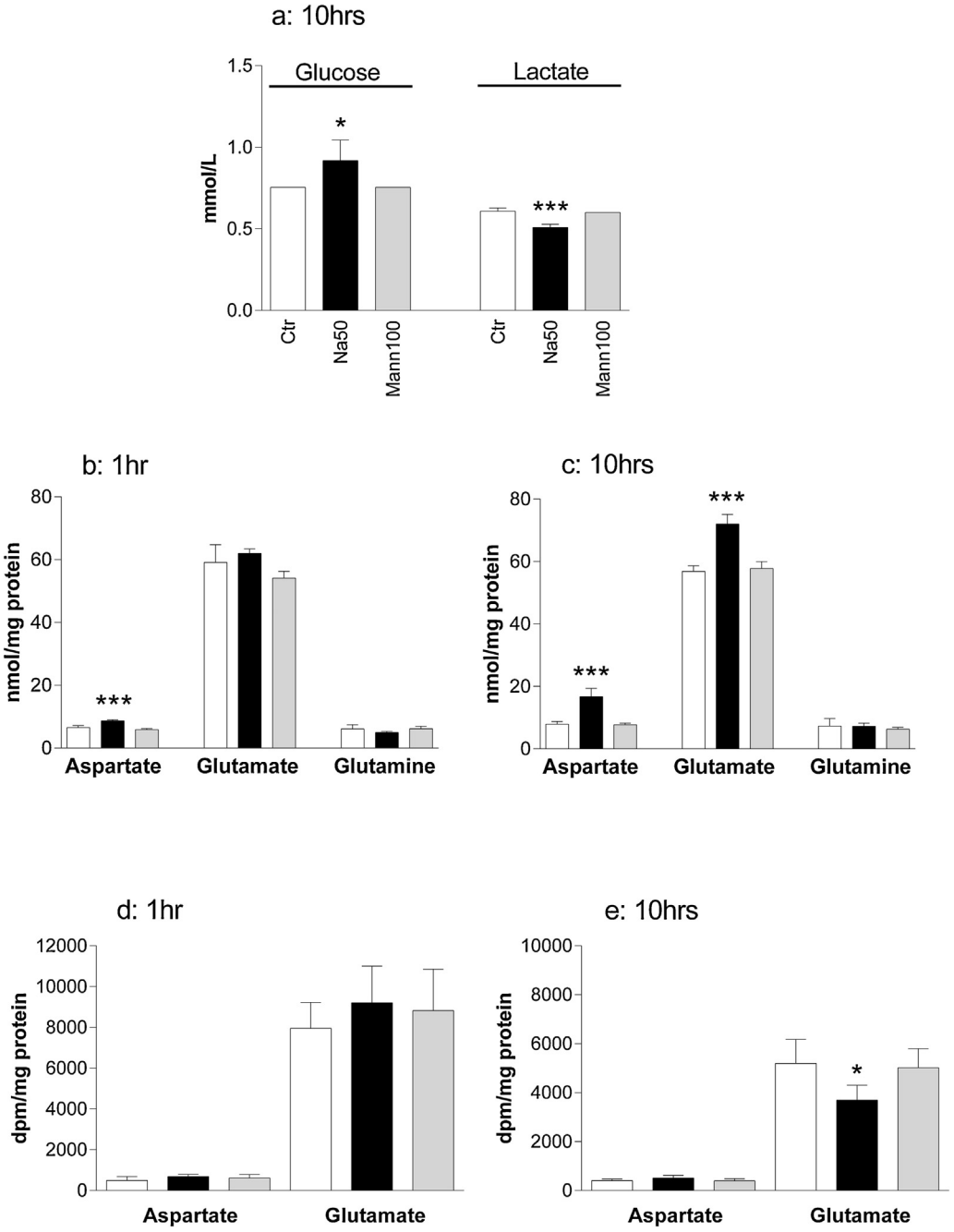

Fig. 3. Effect of hyperosmolar NaCl on glucose metabolism in cerebellar granule cells. Cerebellar granule cells were incubated for 1 or 10 h in aCSF with excess NaCl, 50 mmol/L (black bars), or mannitol, 100 mmol/L (grey bars). Glucose was 1 mmol/L. Fifteen minutes before harvesting the cells [U-^{14}C]glucose, 1 μCi, was added. a: Concentration of glucose and lactate in the aCSF after 10 hrs of incubation. b: Levels of intracellular aspartate (Asp), glutamate (Glu) and glutamine (Gln) after 1 h of incubation c: Levels of intracellular amino acids after 10 h of incubation. d: Radiolabeling of amino acids from [U-^{14}C]glucose after 1 h of incubation. e: Radiolabeling of amino acids from [U-^{14}C] glucose after 10 h of incubation. Intracellular amino acids are nmol/mg protein, radiolabeling is dpm/mg protein, glucose and lactate in the aCSF are mmol/L. All data are means + SD values; N = 6). Asterisks: different from control value; *: $p < 0.05$; ***: $p < 0.001$; one-way ANOVA, Dunnett's method.

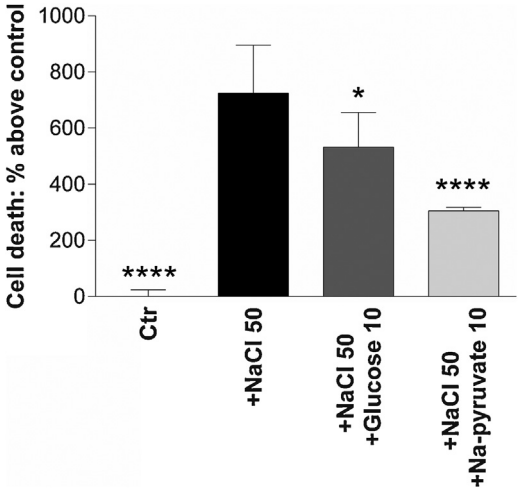

Fig. 4. Neuroprotective effect of glucose and pyruvate on cerebellar granule cells exposed to excess NaCl, 50 mmol/L, for 20 h. Cell death was determined from lactate dehydrogenase (LDH) release. Values are percent of control without addition of glucose or pyruvate; means + SD. Asterisks: difference from cultures exposed to excess NaCl, 50 mmol/L; *: $p < 0.05$; ****: $p < 0.0001$; ANOVA, Dunnett's method.

With $[K^+]$ 4 mmol/L, and $[Na^+]$ 141.4 mmol/L, intracellular free $[Ca^{2+}]$ was approximately 50% of the value obtained with high $[K^+]$ and lower $[Na^+]$. Raising extracellular NaCl by 50 mmol/L caused intracellular free $[Ca^{2+}]$ to decrease further (Fig. 6c).

3.8. Effect of hyperosmolar sodium chloride on cytosolic $[Na^+]$

Excess NaCl, 50 mmol/L, produced an increase in intracellular $[Na^+]$ within 10 min of exposure to NaCl, as could be seen from an increase in SBFI signal (Fig. 7). This effect was seen whether the incubation medium had high potassium (25 mmol/L) or low (4 mmol/L), and it was also seen with addition of mannitol, 100 mmol/L. At later time points (2 and 4 h) during exposure to excess NaCl or mannitol no increase in SBFI signal was seen (data not shown).

Cells incubated in aCSF with $[K^+]$ at 4 mmol/L and $[Na^+]$ 141.4 mmol/L had a higher intracellular $[Na^+]$ than cells incubated in aCSF with $[K^+]$ at 25 mmol/L and $[Na^+]$ at 120.4 mmol/L, as could be seen from the SBFI signal in the former group being $109 \pm 7\%$ of the signal in the latter group ($p = 0.02$, Student's *t*-test).

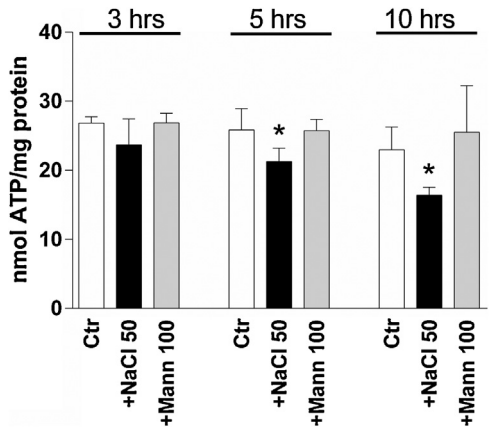

Fig. 5. Levels of ATP in cells exposed to excess NaCl, 50 mmol/L, or to mannitol, 100 mmol/L, for 3, 5, or 10 h (before measurable hyperosmolarity-induced cell death). Data are nmol/mg protein, mean + SD values; N = 4–6 per value. Asterisks: difference from control; *: $p < 0.05$; Kruskal-Wallis test, Dunn's correction. Note that while excess NaCl, 50 mmol/L, leads to a reduction in ATP levels, there is no such effect of mannitol.

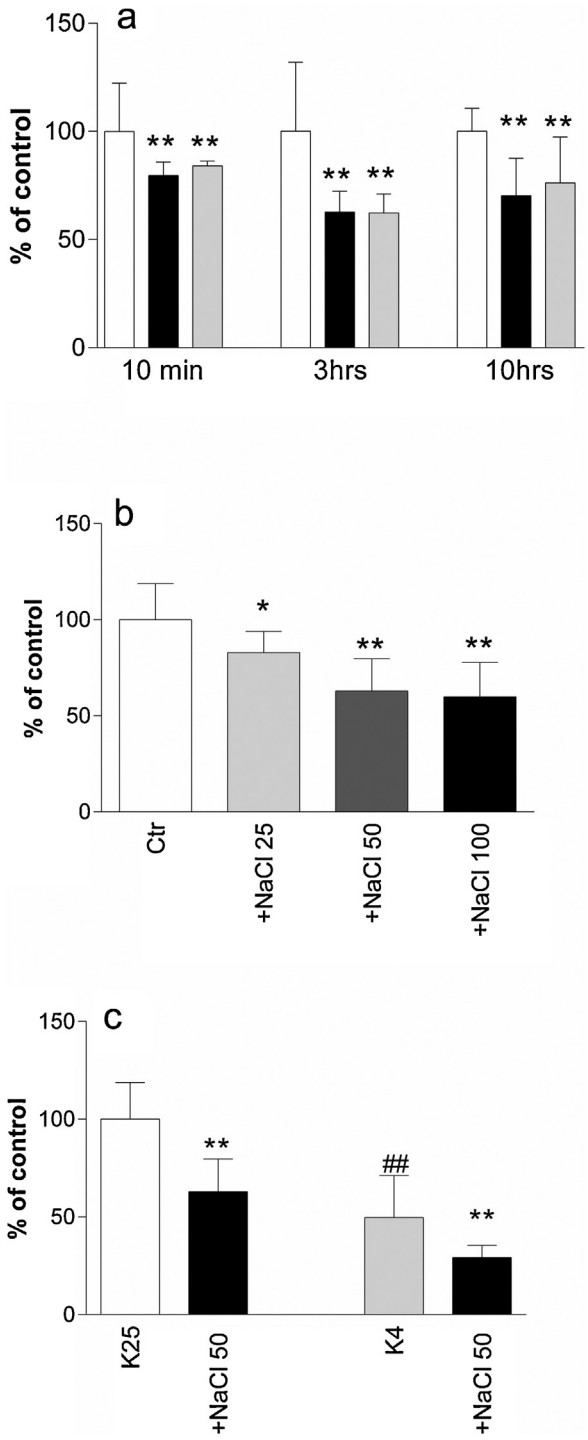

Fig. 6. Effect of hyperosmolar NaCl on intracellular free $[Ca^{2+}]$ detected by Fura-2. a: Intracellular free $[Ca^{2+}]$ after 10 min, 3, or 10 h of exposure to aCSF with excess NaCl, 50 mmol/L (black columns) or mannitol, 100 mmol/L (grey columns). White columns are controls. b: Intracellular free $[Ca^{2+}]$ after 5 h' exposure to aCSF with excess NaCl, 25, 50, or 100 mmol/L. c: Intracellular free $[Ca^{2+}]$ in cultures incubated with 25 mmol K^+/L (two left columns) or 4 mmol K^+/L (two right columns) and the effect of excess NaCl, 50 mmol/L (values at 10 min). Mean intracellular free $[Ca^{2+}]$ in control cultures incubated with 25 mmol K^+/L (a–c) was 145–160 nmol/L. Values are percent of control, means + SD (N = 7–12 per value). Asterisks: different from corresponding control group; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ##: different from control group incubated with 25 mmol K^+/L: $p < 0.01$; one-way ANOVA, Dunnett's or Newman-Keuls methods, as appropriate.

4. Discussion

4.1. Evaluation of the model

The present study is based on conventional incubation of cerebellar granule cells with a high concentration of KCl (25 mmol/L) and an adjusted sodium concentration to achieve physiologic osmolarity (Gallo et al., 1987; Schousboe et al., 1989; Van der Valk et al., 1991; Hassel et al., 1995), leading to a total [Na^+] of 120.4 mmol/L in the control situation. It may be argued from a physiological point of view that increasing [Na^+] in this situation would be to normalize extracellular [Na^+]. However, cultured neurons will remain electrophysiologically inactive at [K^+] at 4 mmol/L and will lack the trophic effect of depolarization, including NMDA receptor activation (Gallo et al., 1987; Van der Valk et al., 1991; Zhou et al., 2015). This was probably why, in the present study, incubation of cerebellar granule cells in a more 'physiologic' aCSF, with [K^+] at 4 mmol/L and [Na^+] at 141.4 mmol/L led to pronounced cell death, and increasing extracellular [Na^+] under these circumstances reduced cell death. To be able to describe possible neurotoxic effects of hyperosmolar NaCl we therefore chose to use the model which allowed cell survival in the control situation and which produced cell death with increasing concentrations of NaCl.

The model allows a description of various cellular reactions to hyperosmolar NaCl that take place before the cells die, that is, within 10 h of exposure to hyperosmolar solutions, including changes in glucose metabolism, ATP levels, uptake of amino acids, and intracellular free [Ca^{2+}]. However, necrosis, which occurred after more than 10 h of exposure to hyperosmolar NaCl, and which was measured at 20 h, entails disruption of the cell membrane and hence a loss of both cell content and ion gradients, including loss of glutamate to the extracellular fluid and cytosolic calcium overload (Choi, 1987; Schramm et al., 1990). Thus, the 20-h incubation period consisted of two distinct phases: a first phase in which cells reacted to hyperosmolar NaCl by reduced glycolysis, increased uptake of glutamate, and lowered intracellular free [Ca^{2+}], but without cell death, and a second, necrotic, phase, which occurred after more than 10 h of incubation.

4.2. Hyperosmolarity caused by Na^+ is neurotoxic

This study shows that prolonged exposure to hyperosmolar NaCl causes cell death in cultured cerebellar granule cells. An increase in extracellular NaCl of as little as 10 mmol/L caused a small, but significant increase in cell death. Na^+ was clearly the neurotoxic agent, as replacement with choline reduced cell death to control values, whereas replacement of the excess chloride ions with gluconate did not. Hyperosmolarity *per se* (with mannitol or glycerol) was without neurotoxic effect. Previously, Himmelseher et al. (2001) reported that a transient (15-min) exposure of cultured hippocampal neurons to increased osmolarity, partly due to NaCl, caused cell death at 24 h. Their study addressed the question of whether therapeutic hypernatremia in neurocritical care may be harmful to the brain. The results of the present study may add to the worry that such treatment has untoward effects, but given the limitations of the *in vitro* model (see Section 3.1), further studies are necessary to elucidate this question.

4.3. Hyperosmolar NaCl inhibits glycolysis and reduces ATP levels

Incubation of cerebellar granule cells with excess NaCl caused reduction in glucose metabolism, as could be seen from the reduced consumption of glucose and the diminished formation of lactate, indicating lower glycolytic activity. The reduced ^{14}C-labeling of glutamate from ^{14}C-glucose is consistent with this interpretation. The reduction in glycolytic activity probably contributed to the reduction in ATP levels in cells exposed to hyperosmolar NaCl and it was probably an important cause of necrosis in these cells, as suggested by the reduction in cell death by addition of pyruvate, which bypasses glycolysis as an energy substrate.

The increase in intracellular aspartate in response to hyperosmolar NaCl probably reflected two distinct mechanisms. First, increased uptake of glutamate and GABA from the extracellular fluid would lead to flooding of the tricarboxylic acid (TCA) cycle with intermediates, causing build-up of oxaloacetate as the terminal intermediate of the TCA cycle (Fig. 8). Oxaloacetate will only be metabolized further to citrate if acetyl-CoA is available, the formation of which is controlled by pyruvate dehydrogenase, a rate-limiting enzyme for TCA cycle activity in the brain (Lai et al., 1977; Morland et al., 2007). Oxaloacetate is in equilibrium with aspartate, so when the former builds up, so does the latter. Second, build-up of aspartate is a key metabolic event during inhibition of glycolysis (Engelsen et al., 1986; Gundersen et al., 2001): when availability of acetyl-CoA (derived from glycolytic end product pyruvate) is reduced, oxaloacetate (and hence aspartate) accumulates (cf. Fig. 8). A similar increase in aspartate, was seen in brains of acutely hypernatremic mice (Thurston et al., 1986). This effect of hypernatremia was ascribed to a reduction in serum glucose (Thurston et al., 1986), but treatment with glucose did not reverse symptoms or biochemical abnormalities, lending support to the view that the glycolytic pathway itself was inhibited. The higher level of glutamate in cells after 10 h of incubation in hyperosmolar sodium chloride could be explained by the higher uptake of extracellular glutamate in these cultures. A similar increase in brain glutamate was seen in hypernatremic mice *in vivo* (Thurston et al., 1986).

We cannot at present explain how hyperosmolar NaCl leads to inhibition of glycolysis. A high [Na^+] has been shown to inhibit hexokinase *in vitro* (Olsen et al., 2007), and an increase in extracellular [Na^+] may reduce intracellular pH (Okada et al., 2001; Rose and Karus, 2013), which in turn could affect the activity of phosphofructokinase (Trivedi and Danforth, 1966). However, the increase in intracellular [Na^+] was only transient, and mannitol also caused an increase in intracellular [Na^+] without affecting glycolysis or causing cell death. Probably, this transient increase reflected the efflux of water from the cells as a response to the increased osmolarity in the extracellular fluid, producing a higher concentration of intracellular sodium ions. Further, increasing intracellular [Na^+] has previously been shown to stimulate rather than inhibit glycolytic activity in synaptosomes (Erecińska et al., 1991). Alternatively, the reduction in intracellular free [Ca^{2+}] may have reduced the activity of glyceraldehyde-3-phosphate dehydrogenase, which, at least in muscle, is activated by calcium in a calmodulin-dependent manner (Singh et al., 2004); however, mannitol also caused a reduction in intracellular free [Ca^{2+}], without affecting glycolysis or causing cell death. Because the intracellular effects of hyperosmolar NaCl were also seen with mannitol, whereas only hyperosmolar NaCl caused inhibition of glycolysis and cell death, it is possible that hyperosmolar NaCl inhibited glycolysis through an extracellular effect, e.g. on the neuronal glucose transporter, GLUT3. In agreement with this interpretation, Himmelseher et al. (2001) observed a reduction in the neuronal uptake and accumulation of ^3H-labeled 2-deoxyglucose after exposure to a 15-minute pulse of hypertonic saline and hydroxyethyl starch (350 mOsm/L) indicative of reduced uptake of glucose into neurons. Accumulation of 2-deoxyglucose reflects the combined activity of the glucose transporter and the phosphorylating activity of hexokinase (Sokoloff et al., 1977). The possibility that hexokinase was inhibited by hyperosmolar NaCl, appears less attractive, given that, in the present study,

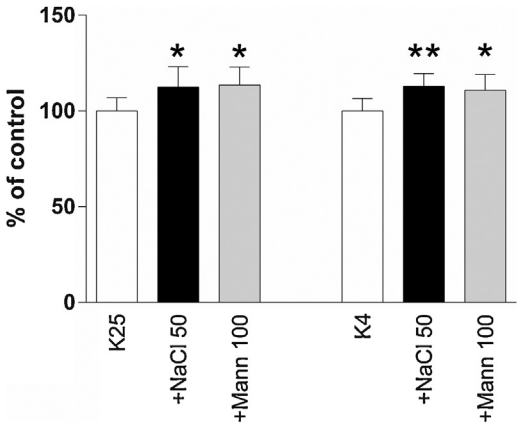

Fig. 7. Effect of hyperosmolar NaCl on intracellular free [Na^+]. Cultured cerebellar granule cells were kept in aCSF with 25 mmol K^+/L (three left columns) or 4 mmol K^+/L (three right columns). The extracellular concentration of sodium was increased by 50 mmol/L (black columns), or mannitol was added at a final concentration of 100 mmol/L (grey columns). Incubation time was 10 min. At later time points (2 or 4 h) no difference was seen between groups (not shown). Values are percent of the corresponding control group, means + SD (N = 8 per value). Asterisks: different from corresponding control group; *: $p < 0.05$, **: $p < 0.01$; one-way ANOVA, Dunnett's method.

hyperosmolar NaCl and mannitol produced the same transient increase in intracellular NaCl, whereas only hyperosmolar NaCl caused reduction in glucose consumption. We therefore hypothesize that hyperosmolar Na^+ somehow reduces the activity of GLUT3. Further studies are needed examine the relationship between glucose transport and extracellular [Na^+].

4.4. Intracellular free [Ca^{2+}] responds to hyperosmolarity

Intracellular free [Ca^{2+}] was reduced in response to high extracellular NaCl and mannitol alike, suggesting that it represented a response to hyperosmolarity rather than hyperosmolar NaCl specifically. An increased intracellular free [Ca^{2+}] is part of the volume regulatory response to hypo-osmolarity (Morales-Mulia et al., 1998; Okada et al., 2001). To our knowledge, the present study is the first to demonstrate a decrease in intracellular [Ca^{2+}] in neurons in response to extracellular hyperosmolarity.

4.5. Can effects of hyperosmolarity help explain cerebral dysfunction in dehydration?

Hypernatremia may cause cognitive dysfunction and delirium (for review, see Lieberman, 2007; Vidán et al., 2009). In our study hyperosmolar NaCl led to an increase in glutamate uptake and to reduced extracellular concentrations of glutamate, GABA, and

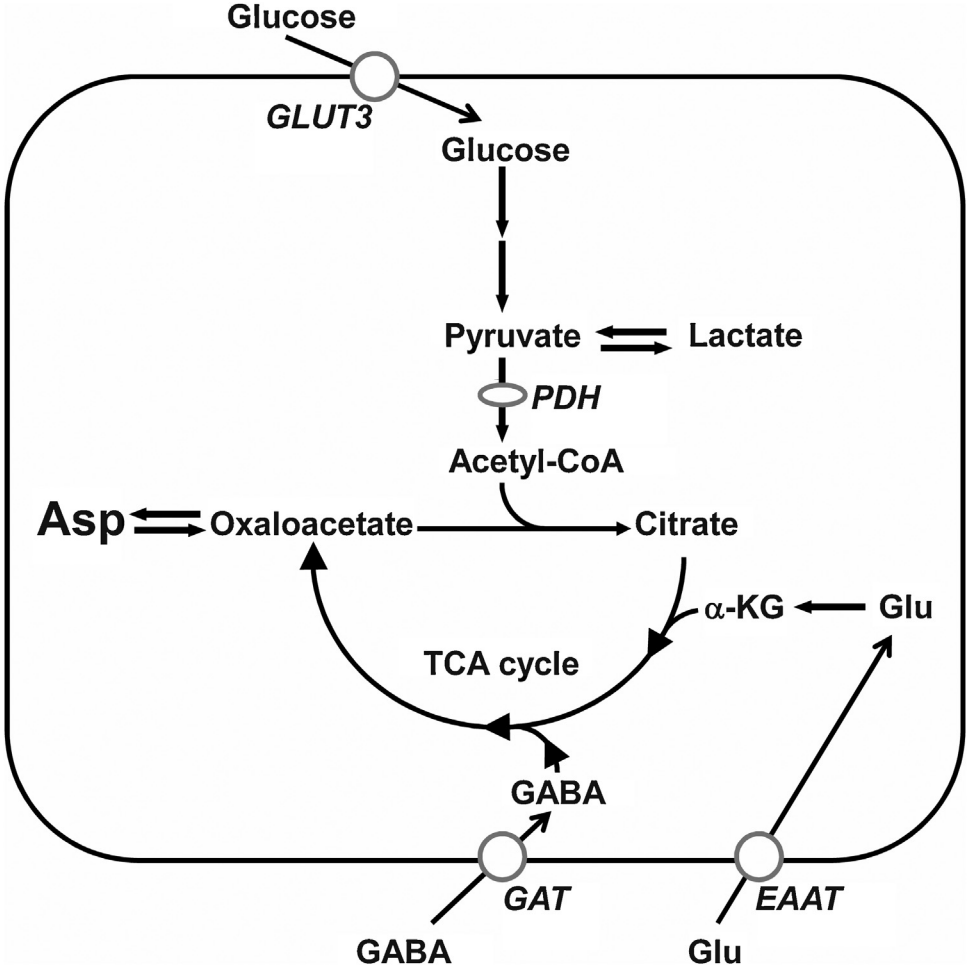

Fig. 8. Simplified scheme of glycolysis and TCA cycle activity, showing how aspartate builds up when oxaloacetate level exceeds the availability of acetyl-CoA. Diminished glycolytic activity leads to reduced formation of acetyl-CoA, while increased uptake of glutamate and GABA floods the TCA cycle with intermediates, leading to accumulation of oxaloacetate, because pyruvate dehydrogenase (PDH) is rate-limiting (Lai et al., 1977; Morland et al., 2007). Oxaloacetate is transaminated to aspartate. Glutamate transporters (EAAT) and GABA transporters (GAT) are sodium-dependent and respond to increased extracellular [Na^+] with an increase in transport activity. Abbreviations: Asp: aspartate; EAAT: excitatory amino acid transporter; GAT: GABA transporter; α-KG: α-ketoglutarate; PDH: pyruvate dehydrogenase.

taurine; these effects were probably due to the Na^+ dependence of the transporters for these amino acids (for review, see Kanner, 1994). An increased uptake of taurine during hyperosmolarity has been reported previously for cultured astrocytes (Beetsch and Olson, 1996). Conversely, efflux of these amino acids has been reported in cultured brain cells exposed to hypo-osmolarity (Pasantes-Morales et al., 1993). If the effect of hyperosmolar NaCl on extracellular amino acids in neuronal cultures translates to the intact brain, a reduction in the levels of extracellular glutamate and GABA could lead to reduction in glutamate and GABA receptor activation in hypernatremia, given that CSF sodium varies with serum sodium (Cserr et al., 1987, 1991). A reduction in the extracellular level of taurine, an inhibitory neuromodulator in the brain (Oja and Saransaari, 2013), could also influence neuronal activity during hypernatremia. A reduction in intracellular free calcium, as that seen during exposure to high NaCl in the present study, could, if it took place in the intact brain, affect intracellular calcium signalling. Such effects could contribute to the cerebral dysfunction seen in dehydration, manifesting as cognitive dysfunction and delirium (Lieberman, 2007; Vidán et al., 2009).

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.03.005.

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