Supplementary Material

Supplementary Materials and Methods

Data analysis and statistical inference

In-vivo experiments with urethane/α-chloralose anesthesia were randomized and blinded (randomization script in R). In-vivo experiments with isoflurane as well as brain slice experiments were not blinded. To minimize the effects of subjective bias during analysis, experimental datasets were processed in batches without emphasis on a single experiment or group and whenever possible, custom scripts (Python, MATLAB, R) were used to iterate over all files in a data folder. In-vivo SD threshold experiments were excluded from analysis when the threshold current exceeded 1 mA (total: n=6; $\alpha1^{+/KOE15}$, n=2, $\alpha1^{+/+}$, n=1; $\alpha2^{+/KOE4}$, n=1; $\alpha3^{+/KOI4}$, n=1; $\alpha3^{+/+}$, n=1). The majority of these cases resulted from insufficient contact of the bipolar stimulation electrode through the dura exposed at the occipital burr hole. The contact between electrode tips and dura was improved in the isoflurane-based in-vivo experiments using conductive electrode cream (see Supplementary Material). Two experiments were excluded due to malfunction of the battery-driven stimulus isolator ($\alpha3^{+/KOI4}$, n=1; $\alpha3^{+/+}$, n=1). One experiment was excluded because of epileptiform activity in the extracellular voltage recording ($\alpha3^{+/KOI4}$, n=1). ISME ($[K^+]_o$, DC) data was not included when there was an obvious discrepancy between the $[K^+]_o$ and the DC signal of the same electrode, indicating malfunction e.g. caused by clogging of the tip. LDF data was only accepted for analysis when the typical multiphasic rCBF change during SD was present. In-vivo experiments using LASCA were only accepted when premature SDs were absent during preparation, as evidenced by similar perfusion levels of both hemispheres and the typical multiphasic rCBF response of the first SD.[1]

The experimental unit for all descriptive statistics and statistical testing was a single animal, not a brain slice or an event. When several brain slices from the same animal were analyzed, the mean of the measurements was used. Two groups were compared using the Mann-Whitney U test. A significance level of $P<0.05$ was considered statistically significant.

K^+-sensitive microelectrodes

Changes in $[K^+]_o$ and field potentials were measured using double-barreled K^+-sensitive/reference microelectrodes at a depth of ~100 µm as described previously.[2-4] In short, ion-sensitive microelectrodes are prepared using double-barreled theta glass (Fa. H. Kuglstatter, Garching, Germany)[5, 6] using a vertical pipette puller (custom build) or a Flaming/Brown micropipette puller (P-97, Sutter Instrument). One barrel was filled with 154 mM NaCl and served as the reference. The other barrel was silanized (5% trimethyl-1 chlorosilane in 95% CCl4) and filled with a potassium ion exchanger resin (Fluka 60031 Potassium ionophore I - cocktail A, Fluka, Honeywell, Bucharest, Romania). Electrodes were tested as described[7] prior to the experiment and yielded a potential of 59±1 mV for a 10-fold concentration change (3 and 30 mM K^+) at ~25 °C. $[K^+]_o$ was calculated using a modified Nernst equation[8]: $\log_{10}([ION]_a) = E / (s * v) + \log_{10}([ION]_r)$, with E: recorded potential; s: electrode slope obtained at calibration; v: valence of the measured ion; $[ION]_r$: extracellular ion concentration at rest; $[ION]_a$: ion concentration at activation.

$[K^+]_o$ threshold for SD induction

In coronal brain slices, SD was triggered by raising the potassium concentration in the ACSF ($[K^+]_{ACSF}$) in steps of 2.5 mM at 30-min intervals, starting from 10 mM.[4, 9-11] The high $[K^+]_o$ solution was made by an equimolar replacement of KCl for NaCl. To measure field potential and threshold $[K^+]_o$, an ion-sensitive microelectrode (ISME) was placed in neocortical layer 2/3

in a dorsal position and a second ISME was placed ventral of the rhinal fissure. In a different slice from the same animal 5 µM ouabain was added to the high $[K^+]_{ACSF}$. The SD threshold was defined as the $[K^+]_o$ at the time point of the first SD occurrence measured by two ISMEs. The duration of SD's negative direct current (DC) shift was measured at 25% of the maximum DC shift amplitude. Latency until SD initiation was defined as the time elapsed between the high $[K^+]_o$ wash-in until the onset of the first SD.

Stimulus-induced $[K^+]_o$ increases

In horizontal slices with preserved hippocampal circuitry from $\alpha2^{+/KOE4}$ and $\alpha2^{+/+}$ mice (n=32), the Schaffer collateral pathway was stimulated with intensities set to levels eliciting 25, 50, 75, and 100% of the maximal population spike amplitudes to achieve similar efficacy in all experiments. Stimulus trains (20 Hz, 10 s duration, 100 µs single pulse length) were delivered through bipolar stimulation electrodes (platinum wire, 25 µm diameter, 100 µm tip separation). Orthodromic excitatory postsynaptic potentials in stratum radiatum, population spikes in stratum pyramidale, and the accompanying $[K^+]_o$ were assessed using K^+-sensitive/reference microelectrodes. Decay time was defined as the time elapsed from the termination of the stimulus train until $[K^+]_o$ has decayed to 1/e of its amplitude. Rise time was defined as the time elapsed from the start of the stimulus train to 1-1/e of the $[K^+]_o$ amplitude.

KCl microinjection SD threshold

To minimize KCl solution spill-over on the slice surface, SD was triggered focally by injecting a small amount of 1 M KCl solution into the neocortex of transverse brain slices from $\alpha2^{+/KOE4}$ and $\alpha2^{+/+}$ mice (n=15). Micropipettes were pulled (P-97, Sutter Instrument, Novato, CA, USA) from borosilicate glass capillaries (GB150F-10P, Science Products, Hofheim, Germany) and the tip was broken back to a diameter of ~7.0-7.5 µm. KCl-filled pipettes were connected to a

pressure ejection system (Ionophor 3, Science Products) which was adjusted to produce a pressure output of 1 bar. The tip of the pipette was inserted into the tissue to yield a depth of ~100 µm. Pressure pulses of exponentially increasing duration (20, 40, 75, 140, 250, 430, 680, 1000 ms) were applied at 120 s intervals until SD was triggered. Two recording electrodes were placed in neocortical layer 2/3 ~1.5 mm medial and dorsal from the injection site.

Intrinsic optical signal (IOS)

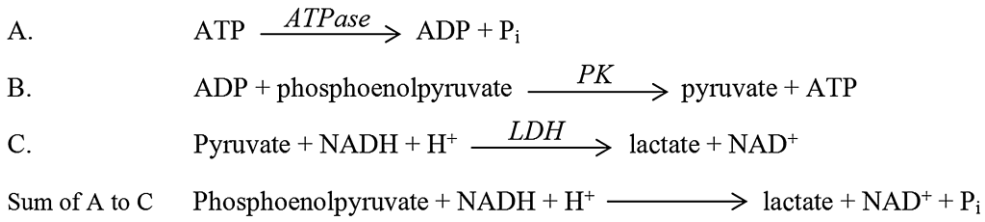
Brain slices for IOS imaging experiments were placed on 0.4 µm culture plate inserts (Millicell, Merck Chemicals, Darmstadt, Germany) glued to platinum frames and the slices were aligned with the light guide of the recording chamber. Single slices were trans-illuminated from below with a cold-light source (KL 1500 LED, Schott, Mainz, Germany) and light transmission changes were recorded using a CCD camera (DFC360 FX, Leica Microsystems, Wetzlar, Germany) mounted on a stereomicroscope (MZ-6, Leica Microsystems) to allow for assessment of the IOS[4, 10, 12-15] The IOS was defined as $\Delta T = ((T_1 - T_0) / T_0) * 100$ to yield the normalized light transmittance change from baseline in percent, where T_1 and T_0 are transmitted light intensity of a region of interest in the field of view at a certain time point and 30 s before SD onset, respectively. Single images were acquired at a frame rate of 5 Hz and stored for off-line analysis. SD speed was based on wave front progression and assessed separately to yield a dorsal and a ventral SD component relative to the microinjection point by dividing the propagated distance by elapsed time. SD area was calculated from the two-dimensional projection of the cumulative extent of SD spread, where the relative IOS change exceeded 3% from baseline.

In-vivo: isoflurane anesthesia

Heterozygous knock-out mice (n=111) were anesthetized using isoflurane for the duration of the surgical procedure and during the subsequent experiment. The isoflurane concentration was adjusted to 4% during anesthesia induction, 2% during the surgical procedure and to yield a burst-suppression pattern in the ECoG recording during the SD induction protocol and the recording (1.2%-1.6% isoflurane in 30 ml/min N_2O and 15 ml/min O_2). Mice were placed in a stereotaxic frame, an incision was made in the scalp, and the eyes were covered with ointment containing 5% dexpanthenol to reduce drying of the eyes (Bayer Vital, Berlin, Germany). Two holes were drilled over the right hemisphere, one for the stimulation electrode (diameter 1.3 mm, posterior 2.6 mm, lateral 1.5 mm from bregma) and another smaller one for recoding of the epidural ECoG (diameter 0.5 mm, anterior 1 mm, lateral 2 mm from bregma). The dura was left intact and the stimulation site was covered with a thin film of mineral oil to reduce tissue drying. To lower the resistance between stimulation electrode and dura, the tips of the stimulation electrode were dipped into conductive electrode cream (SYNAPSE, Kustomer Kinetics, Arcadia, CA, USA). Cerebral perfusion of both hemispheres was monitored during preparation and recording using laser speckle contrast analysis (LASCA) imaging (PeriCam PSI HR, Perimed Instruments, Järfälla, Sweden). The near infrared (785 nm) laser allowed for assessment of perfusion (imaging area: 1.3 x 1.1 cm, sampling rate: 0.5 Hz, spatial resolution: 20 µm, averaged frames per image: 8) through the intact mouse skull and permitted the detection of premature SDs during the preparation phase. Animals with accidentally initiated SDs were excluded from the analysis. SD was induced electrically using the same stimulation electrode and protocol as described above but the duration of the biphasic stimulation was increased to 200 ms (±100 ms). In addition, a mains-operated stimulus isolator (SIU-102, Warner Instruments, Hamden, CT, USA) was employed to ensure a consistent compliance voltage of 100 V. Animals were killed after the experiment by decapitation under 6% isoflurane.

Na^+/K^+-ATPase activity assay

To investigate the consequences of genetic α2 and α3 deficiency on actual Na^+/K^+-ATPase activity, we performed a real time coupled enzyme assay on brain homogenates from knockout mice and WT.[9, 16] The enzyme assay couples formation of adenosine diphosphate (ADP) by ATPases to nicotinamide adenine dinucleotide (NADH) oxidation in the presence of excess pyruvatekinase (PK), lactatedehydrogenase (LDH), and phosphoenolpyruvate (PEP):



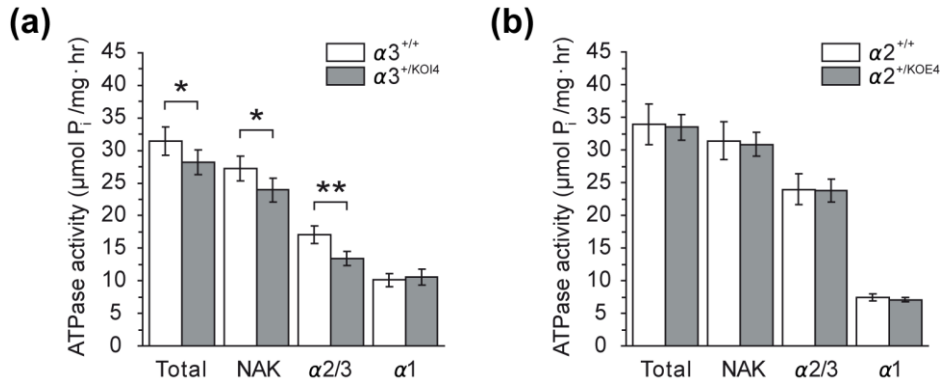
The assay measures the rate of NADH absorbance decrease, which is proportional to the rate of steady-state ATP hydrolysis. NADH oxidation was continuously monitored spectrophotometrically at 340 nm in the presence and absence of ouabain. ATPase activity was calculated from the slope of the linear portion of the tracing, the NADH millimolar extinction coefficient, the volume of the reaction mixture, and the total amount of protein in the reaction mixture as shown below:

$$\text{ATPase activity } (\mu\text{mol } P_i/\text{mg} \cdot \text{hr}) = \frac{\text{slope (OD units/hr)}}{6.22 \left(\frac{(\text{OD units} \cdot \text{ml})}{\mu\text{mol}} \right)} \times \frac{\text{volume (ml)}}{\text{protein (mg)}}$$

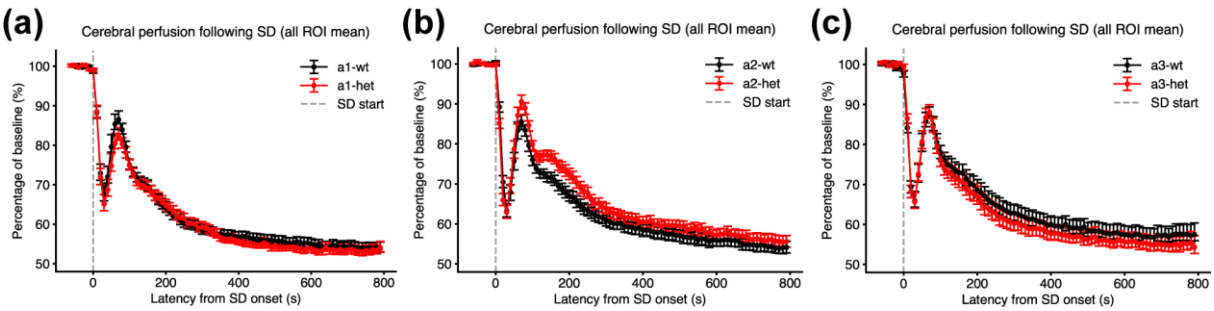
Na^+/K^+-ATPase activity represents the ouabain-suppressible portion of total ATPase activity. To distinguish between α1 and α2/3 isoforms of the Na^+/K^+-ATPase different concentrations of ouabain (10 mM and 10 µM, respectively) were present in the reaction mixture. Nonfasted adult male Na^+/K^+-ATPase $\alpha2^{+/-}$, $\alpha3^{+/-}$ knockout mice and their wild-type littermates (n=28) were deeply anesthetized with isoflurane (4%) and sacrificed by decapitation. For the preparation of the standard homogenate forebrains of C57BL/6J mice were used. Brains were

quickly removed and transferred into carbogenated (5% CO_2 and 95% O_2), ice-cold ACSF. The ACSF contained (in mmol/L) 129 NaCl, 3 KCl, 1.8 $MgSO_4$, 1.6 $CaCl_2$, 1.25 NaH_2PO_4, 21 $NaHCO_3$, and 10 glucose (pH 7.4). The cerebellum was excised and the forebrains were transferred immediately in liquid nitrogen. Brain tissue was homogenized in 10 ml solution per 1 g wet weight containing 0.25 M sucrose, 1.25 mM EGTA, and 10 mM Tris, pH 7.0, at 25 °C, by eight strokes in a precooled PTFE-glass Potter-Elvehjem homogenizer. The brain homogenate was centrifuged at 750 *g* for 5 minutes (4 °C). Analysis of Na^+/K^+-ATPase activity was performed on the supernatant without further dilution. Stock solutions of reaction buffer (with 10 µM, 10 mM or without ouabain), auxiliary enzymes and substrates were prepared in advance. The final reaction mixture contained 125 mM Tris buffer, 1 mM EGTA, 120 mM NaCl, 12.5 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, 5 mM ATP, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH and 15 units each of LDH (type XI) and PK (type III). Brain homogenate samples, auxiliary enzymes and substrates were added to the reaction buffer stock solutions omitting only ATP. The mixture was preincubated 5 min at 37 °C in the reading compartment of a temperature controlled multiwell plate reader (Berthold Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). The reaction was initiated by adding 10 µl of ATP-Na_2 solution to each well employing a reagent injector to assure a constant temperature of 37 °C from the beginning of the reaction. A standard was run simultaneously with the samples. Protein concentration was determined with Bradford Reagent using bovine serum albumin as a standard.

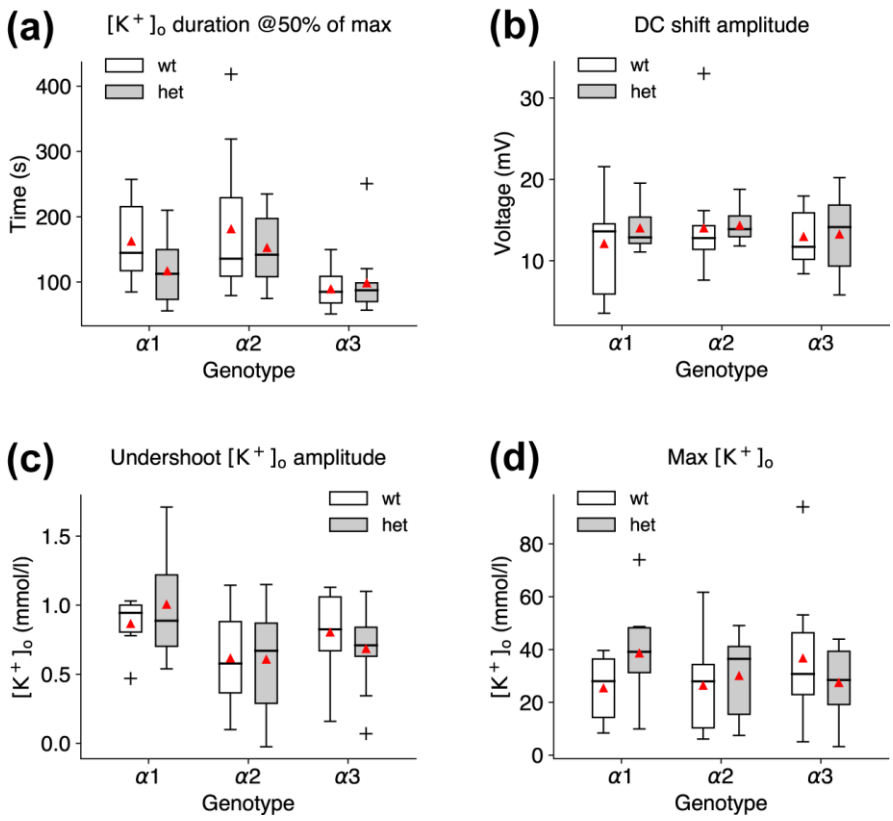
Supplementary Figures



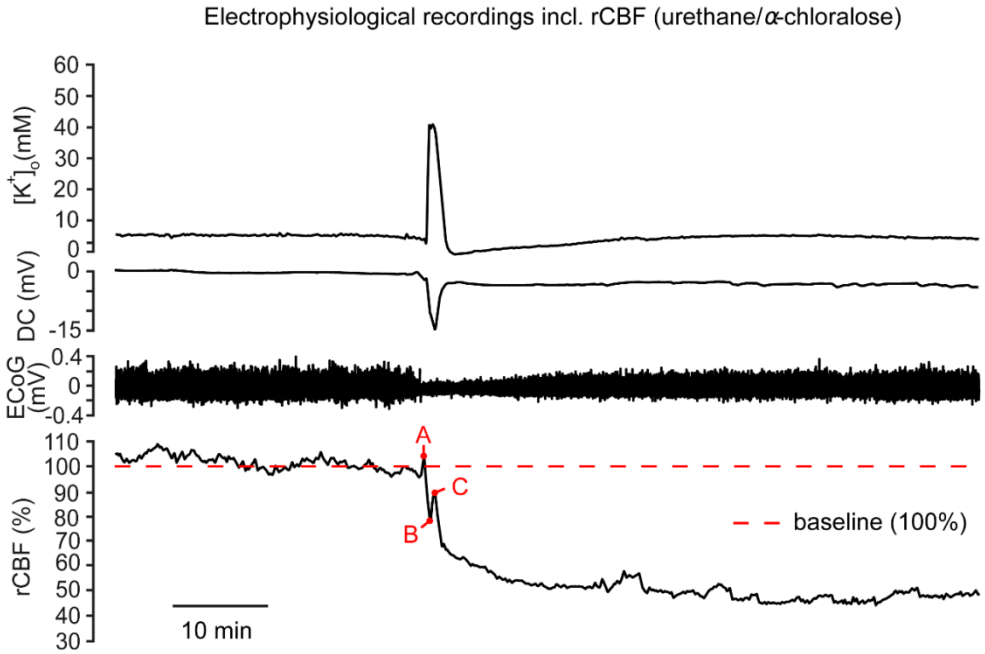
Supplementary Figure 1. ATPase activity in whole brain homogenates. (a) In a coupled enzyme assay, $\alpha3^{+/KOI4}$ mice showed a significant gene dosage effect **(Supplementary Table 1)**. Mice deficient of the α3 isoform displayed a ~22% reduction of ATPase activity in the α2/3 portion of the Na^+/K^+-ATPase with no effect on the α1 portion. **(b)** Despite reduced protein expression in hippocampal extracts by ~50%,[17] no significant Na^+/K^+-ATPase activity reduction could be detected in the brains from $\alpha2^{+/KOE4}$ mice when compared to WT. Similar difficulties in showing differences in actual Na^+/K^+-ATPase activity have been reported in α2 isoform deficient mice.[18] The reasons for this discrepancy are unclear. A possible explanation could be that the Na^+/K^+-ATPase α2 isoform constitutes only a fraction of astrocytic Na^+/K^+-ATPase protein (~20%)[19] and may contribute perhaps even less to the overall Na^+/K^+-ATPase activity (~10%) [20]. *P < 0.05, **P < 0.001; Mann-Whitney U test; mean ± standard deviation; n=4-10 experiments were performed in triplicate.



Supplementary Figure 2. rCBF following SD in-vivo (isoflurane anesthesia). LASCA imaging was used to map perfusion changes during and following SD onto the cortical surface through the intact mouse skull. In accordance with the lack of differences in SD threshold and speed, the rCBF changes in the wake of SD were not significantly different in Na^+/K^+-ATPase alpha isoform deficient mice compared to their wild-type littermates.



Supplementary Figure 3. Electrophysiologic recordings in-vivo (urethane/α-chloralose anesthesia). ISMEs were placed in a dorsal and ventral position and DC shift and $[K^+]_o$ were recorded during SD **(Figure 5a)**. No significant differences were observed between Na^+/K^+-ATPase alpha isoform deficient mice and their wild-type littermates in **(a)** SD duration, **(b)** DC shift amplitude, **(c)** undershoot amplitude, and **(d)** max. $[K^+]_o$ amplitude. Sample sizes: $\alpha1^{+/KOE15}$/ $\alpha1^{+/+}$, n=7-10; $\alpha2^{+/KOE4}$/$\alpha2^{+/+}$, n=9-13; $\alpha3^{+/KOI4}$/ $\alpha3^{+/+}$, n=9-13.



Supplementary Figure 4. Exemplary in-vivo recording (urethane/α-chloralose anesthesia). Typical recording with characteristic changes during SD in $[K^+]_o$, DC, ECoG and rCBF. The different phases of the multiphasic rCBF response are marked with capital letters. Changes in rCBF were calculated from the baseline perfusion prior to the SD.

Supplementary Table

Genotype	ATPase activity (μmol Pi/mg · hr)			
	Total	Na,K-ATPase	α2/3	α1
$\alpha3^{+/-}$ (n = 10)	28.2 ± 1.9	23.9 ± 1.9	13.4 ± 1.1	10.6 ± 1.2
wild type (n = 10)	31.4 ± 2.2	27.2 ± 1.9	17.1± 1.3	10.1 ± 1.1
$\alpha2^{+/-}$ (n = 4)	33.5± 2.0	30.9± 1.8	23.8 ± 1.7	7.1± 0.3
wild type (n = 4)	34.0 ± 3.1	31.4 2.9	24.0± 2.4	7.4± 0.6

Supplementary Table 1. ATPase activity in whole brain homogenates. Total: entire ATPase activity in the homogenate (not specific for Na^+/K^+-ATPase); α2/3: alpha2 and alpha3 portion of the Na^+/K^+-ATPase activity; NAK: total Na^+/K^+-ATPase activity (α2/3 + α1).

Supplementary references

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