Research Article

Inadequate Brain Glycogen or Sleep Increases Spreading Depression Susceptibility

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Objective: Glycogen in astrocyte endfeet contributes to maintenance of low extracellular glutamate and $K^+$ concentrations around synapses. Sleep deprivation (SD), a common migraine trigger induces transcriptional changes in astrocytes reducing glycogen breakdown. We hypothesize that when glycogen utilization cannot match synaptic energy demand, extracellular $K^+$ can rise to levels that activate neuronal pannexin-1 channels and downstream inflammatory pathway, which might be one of the mechanisms initiating migraine headaches.

Methods: We suppressed glycogen breakdown by inhibiting glycogen phosphorylation with 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and by SD.

Results: DAB caused neuronal pannexin-1 large-pore opening and activation of the downstream inflammatory pathway as shown by procaspase-1 cleavage and HMGB1 release from neurons. Six-hour SD induced pannexin-1 mRNA. DAB and SD also lowered the cortical spreading depression (CSD) induction threshold, which was reversed by glucose or lactate supplement, suggesting that glycogen-derived energy substrates are needed to prevent CSD generation. Supporting this, knocking-down neuronal lactate transporter, MCT2 with an anti-sense oligonucleotide or inhibiting glucose transport from vessels to astrocytes with intracerebroventricularly given phloretin reduced the CSD threshold. In vivo recordings with a $K^+$-sensitive/selective fluoroprobe, APG-4 disclosed that DAB treatment or SD caused significant rise in extracellular $K^+$ during whisker-stimulation, illustrating the critical role of glycogen in extracellular $K^+$ clearance.

Interpretation: Synaptic metabolic stress caused by insufficient glycogen-derived energy substrate supply can activate neuronal pannexin-1 channels as well as lowering the CSD threshold. Therefore, conditions that limit energy supply to synapse (e.g. SD) may predispose to migraine attacks as suggested by genetic studies associating glucose or lactate transporter deficiency with migraine.
INTRODUCTION

Glycogen metabolism is tightly regulated in register with synaptic activity by a restricted number of neurotransmitters, including noradrenaline, vasoactive intestinal peptide and adenosine. Pointing to its imperative role in synaptic function, glycogen has been shown to be crucial in the hippocampus for long-term memory formation. Glycogen is also needed for maintenance of low extracellular glutamate and K^+ concentrations around synapses especially during intense glutamatergic transmission. The coupling of glycogen utilization with synaptic transmission appears so fundamental that even under conditions where the energy production via glucose breakdown is sufficient, glycogen is still required for glutamate uptake.

Increased extracellular K^+, glutamate and ATP concentrations and intracellular Ca^{2+} rises can activate neuronal pannexin-1 (Panx-1) channels. The above conditions are present during cortical spreading depression (CSD), the putative cause of migraine aura. Indeed, CSD has been shown to activate neuronal Panx-1 channels in the mouse brain in vivo and, this has been proposed as a potential mechanism initiating a parenchymal-meningeal inflammatory cascade that lead to activation of meningeal trigeminal afferents and, hence, headache following migraine aura. As similar conditions may emerge around intensely active synapses when glycogen-derived glucose and lactate cannot match the increased energy demand for restoration of the transmembrane gradients of glutamate, K^+ and Ca^{2+}, we hypothesize that neuronal Panx-1 channels can be activated and, this might be one of the potential mechanisms that sleep deprivation, a well-known migraine trigger, can initiate the inflammatory cascade leading to headache without requiring aura (i.e. without CSD).

Sleep deprivation (i.e. extended wakefulness) induces astrocytic expression of the enzyme Protein Targeting to Glycogen (PTG), a non-catalytic subunit of the protein phosphatase-1 that results in an increased activity of glycogen synthase, the enzyme responsible for incorporation of glucose into glycogen. Therefore, sleep deprivation may limit the capacity to maintain low extracellular glutamate and K^+ concentrations during sustained excitatory transmission by driving glycogen metabolism towards synthesis rather than its utilization as a glucose and lactate source. Hence, a decreased capacity for such compensation (e.g. due to genetic predisposition or hormonal factors) may make some migraineurs more vulnerable to changes in sleep rhythm either by predisposing to synaptic conditions that can activate large pore channels such as neuronal P2X7/Panx-1 complex and the downstream inflammatory cascade (migraine without aura) or by lowering the threshold for CSD generation (migraine with aura). In both cases, Panx-1 channels may serve as sensors that detect the neuronal stress induced by metabolically uncoupled synaptic transmission or CSD and, initiate the inflammatory cascade that can potentially activate the trigeminal system.

To test these hypotheses, we have investigated whether or not the impairment of glycogen-derived energy supply to synapses can directly activate Panx-1 channels or lower the CSD induction threshold in the mouse cortex in vivo. To reduce glycogen mobilization, we used a pharmacological inhibitor of glycogen phosphorylase 1,4-dideoxy-1,4-imino-D-arabinitol (DAB). Since the energy metabolite transferred from astrocytes to neurons upon glycogen breakdown is lactate, we also used an anti-sense oligonucleotide (ODN) for the neuronal monocarboxylate transporter MCT2. Finally, we induced sleep deprivation of 6 hours to reduce glycogen availability for supporting excitatory synaptic transmission. We have found that these approaches reducing glycogen mobilization can initiate an inflammatory cascade downstream to Panx-1 complex by directly triggering caspase-1
cleavage and HMGB1 release from neurons even in the absence of CSD. We have also found that insufficient glycogen-derived synaptic energy supply can reduce the CSD threshold.

RESULTS

Inhibition of glycogen use or its depletion by synaptic activity opens neuronal Panx-1 channels

We used a well-characterized glycogen phosphorylase inhibitor, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) to suppress glycogen use during synaptic activity in mice under anesthesia. We examined Panx-1 activation one hour after i.c.v. administration of DAB (0.25 M in 0.75 µl) slowly injected within 10 minutes with a micromanipulator through a cannula placed one day prior to the experiment to minimize the effect of any potential injection-induced injury. It had been established that one-hour was required for an effective suppression of glycogen use in astrocytes by DAB. The membrane impermeable fluorescent agent propidium iodide (PI) was slowly administered i.c.v. one hour after DAB through the same cannula to monitor Panx-1 channel activation as large-pore openings allow fluorescent dyes smaller than 1 KDa to enter the cell as shown in vivo as well as in vitro. Five minutes were allowed for distribution and detectable influx of PI to cells before sacrifice by intracardiac perfusion fixation, which terminates cellular activities in situ and, hence, prevents changes in the brain that might be induced by anoxia and hypoperfusion during sacrifice. The inhibition of glycogen use by DAB (but not i.c.v. saline injection) led to widespread PI influx to neurons (NeuN+ cells) in cortical and subcortical areas (n=3) (fig. 1A-D). The intensity of labeling paralleled DAB distribution after i.c.v. injection, being more intense on the injection side hemisphere as well as in neurons closer to the injected ventricle. In the sham group, we found PI labeling only around the cannula tract but not other brain areas in animals that were subjected to the same procedures except DAB injection (n=3) (fig. 1B). Further supporting the connection between DAB and activation of Panx-1 channels, carbenoxolone (CBX, slowly administered i.c.v. through the cannula 10 minutes before PI) completely inhibited PI influx in another set of DAB-injected mice (n=3) (fig. 1D). The i.c.v. dose of CBX used had previously been shown to block Panx-1 channels without affecting gap junction communication in the mouse brain in vivo. DAB injection also caused activation of the downstream inflammatory pathway from Panx-1 as shown by appearance of the cleaved active form of caspase-1 and loss of nuclear HMGB1 staining in neurons with immunohistochemistry (n=3 for each group) as well as by release of HMGB1 to CSF as detected with Western blotting (CSF from 3 mice were pooled for each of DAB and saline injection groups) (fig. 1E-O).

Inhibition of glycogen use by DAB or Sleep Deprivation Lowers CSD threshold

We observed spontaneous CSDs in 3 out of 8 mice within 24-55 minutes after DAB administration, possibly caused by reduced uptake of K+ and glutamate to astrocytes. Accordingly, we investigated the effect of DAB on the CSD induction threshold in mice in which spontaneous CSDs were not observed during continuous DC potential recordings starting after DAB injection. Moreover, the CBF response recorded by laser speckle ensured whether or not any CSDs had preceded the one evoked by KCl, as CBF responses to the first and second CSDs are clearly distinguishable in the mouse. We used increasing concentrations of KCl to detect the CSD induction threshold because we obtained a highly reproducible threshold value at 0.15 M KCl in naïve control mice unlike the highly variable threshold observed with electrical stimulation reported by several laboratories. Strictly applying the...
measures described in the methods, we were able to evoke the first CSD with 0.15 M KCl in every mouse in the control group (n=15). After establishing a highly reproducible normal standard, we tested the effect of DAB on the CSD induction threshold and modulation of this action by glucose and lactate in groups of 6 mice in which no spontaneous CSD was observed. We found that i.c.v. injection of DAB (0.25 M in 0.75 μl saline) with a high-precision micromanipulator slowly over 8-10 minutes one hour prior to the test significantly lowered the CSD threshold to 0.075 M KCl compared to i.c.v. vehicle-injected sham controls (threshold 0.15 M, p=0.001, fig. 2B).

Next, we investigated whether the synaptic energy insufficiency caused by preventing glycogenolysis could be reversed by superfusing the cortex with concentrated glucose solution (0.5 M) starting 10 minutes before recording. As glucose could penetrate through the dura, we did not remove the dura to maintain the cortical physiology. D-glucose (0.5 M) completely reversed the DAB-induced threshold drop, suggesting that the effect of DAB was indeed due to lack of sufficient energy substrate to match the energy demand for clearing extracellular K⁺ and glutamate and preventing synchronized depolarization leading to CSD (fig. 2B). The effect of glucose was not due to an increased osmolarity because superfusion with L-glucose of the same concentration failed to reverse the DAB effect. Superfusion with an equimolar concentration of L-lactate (0.5 M) also reversed the effect of DAB whereas its optical isomer was ineffective (fig. 2B). On equicaloric basis, glucose (0.25 M) was less effective in reversing DAB’s effect possibly because, unlike lactate, it is preferentially used for glycogen synthesis before being used as a fuel owing to significant differences in the reaction rates of enzymes involved in glycogen synthesizing and glycolytic pathways³⁷ (fig. 2B). Further supporting the important role of lactate as an energy substrate needed to prevent synchronized depolarization and CSD, knocking-down MCT2 with an antisense ODN injected intracortically where recordings were performed, significantly lowered the CSD induction threshold. The control scrambled antisense oligonucleotide had no significant effect (n=6 for each of ODN and scrambled ODN groups)(fig. 2C). The ODN sequence used and injection 1 hour before the synaptic activity had been previously established to reduce expression of MCT2 by Western blotting and to impair long-term memory formation⁴.

Since gentle sleep deprivation (GSD) hampers glycogen use by inducing transcriptional changes in several enzymes and proteins involved in glycogen metabolism in astrocytes¹⁸⁻²⁰, we next tested whether or not GSD of 6 hours, a physiological disturbance, could also modify the CSD threshold. The term “gentle” here refers to gentle stimulation of the animal to keep it awake without inducing stress; hence, GSD of 6 hours duration is highly reminiscent of a common form of lack of sleep (insomnia) in humans. GSD significantly reduced the CSD induction threshold compared to the control group (p=0.001). This effect was reversed by 3h sleep and also by D-glucose or L-lactate superfusion, suggesting that the sleep deprivation-induced suppression of glycogen use caused the threshold change indeed by leading to synaptic energy substrate deficiency (n=6 for each group)(fig. 2D).

Since GSD induces rapid transcriptional changes in several genes involved in glycogen metabolism, which may create a stress on excitatory transmission by hampering K⁺ and glutamate clearance, we also looked at the expression level of mRNAs encoding Panx-1 (a stress sensor) and Panx-2 in the cortex at the end of 6h of GSD. Compared with circadian control at ZT6 (Zeitgeber Time 6 i.e. 6h after the beginning of the light period), Panx-1 mRNA expression was significantly induced by 12±2.8% (p<0.0001), whereas the mRNA encoding Panx-2 exhibited no significant changes (-2 ±1.3%)(Fig 2E).

**DAB or sleep deprivation reduced K⁺ clearance during synaptic activity**
We have adapted a recently developed K⁺-fluoroprobe (APG-4) to monitor extracellular K⁺ changes during whisker stimulation in vivo through a cranial window. First, we monitored the K⁺ rise during whisker stimulation over the barrel cortex area, which displayed little change in fluorescence (0.15±0.04 % increase). However, in the presence of DAB, APG-4 fluorescence intensity changed from hardly noticeable rise to a robust increase (7.9±0.6%) during whisker stimulation. This confirms that K⁺ uptake was significantly slowed down during synaptic activity when glycogen mobilization was inhibited by DAB. Similarly, a striking K⁺ rise (4.8±1.3% increase in fluorescence intensity) was observed during 5-min whisker stimulation in sleep-deprived mice tested at the end of 6-hours GSD (Figure 3A-C). In line with these findings, both DAB and GSD prolonged the duration of CSDs without changing the CSD propagation speed and frequency. We confirmed that prolongation of the CSD duration was caused by an extended K⁺ rise during CSD by comparing APG-4 fluorescence intensity change during CSD evoked by KCl (1 M) in DAB-treated and untreated control mice (333±28 vs. 162±11 seconds, P=0.05)(Figure 3D-E, Table).

Inhibition of vascular glucose transport lowers CSD threshold

Next, we specifically tested the effect of inhibition of glucose transport from blood into astrocytes on the CSD threshold as glucose is necessary for glycogen synthesis. Since systemic administration of insulin has several peripheral and central actions other than reducing glucose supply to brain, we chose to directly inhibit glucose transport to the brain from the abluminal surface of the endothelium and, selectively reduce glucose supply to astrocytes and neurons by intracerebroventricularly administering GLUT-1 inhibitor phloretin without significantly compromising endothelial metabolism. Unlike its vehicle (DMSO, 1μl), phloretin (2M in 1μl) significantly lowered the CSD threshold 30 minutes after i.c.v. injection, suggesting that a constant supply of glucose to astrocytes and neurons is needed to prevent CSD generation (n=6 for each group)(fig. 2F). Interestingly, contrary to their deficiency illustrated by several approaches above, excess glucose and lactate supplementation had no effect on the CSD threshold: Naïve animals superfused with D-glucose or L-lactate did not show significantly different thresholds when compared to the control group (p=0.317 and 0.317, respectively, fig. 2B), suggesting that under resting conditions synaptic energy metabolism is balanced not to allow CSD generation (n=6 for each group).

DISCUSSION

Our data show that inhibition of glycogen use in the intact brain in vivo causes Panx-1 large-pore channel opening in neurons. Glycogen granules in perisynaptic astrocyte endfeet around excitatory synapses are used for uptake of glutamate and K⁺ released during synaptic activity. Since glycogenolysis is stimulated by even small increases in [K⁺], glycogen appears to be a preferential energy substrate for maintaining low synaptic [K⁺] levels. Accordingly, inhibition of glycogen use by DAB or GSD causes rise of [K⁺], toward higher values than normal during synaptic activity as clearly shown with our in vivo studies using a K⁺-sensitive/selective fluoroprobe. Studies in brain slices have established that accumulation of [K⁺] over 10 mM can activate Panx-1 large-pore channel. Owing to very narrow extracellular space around synapses, inefficient K⁺ removal caused by DAB may readily result in K⁺ accumulation to concentrations above 10 mM and activate Panx-1 channels. Similarly to K⁺, extracellular glutamate buildup may contribute to activation of Panx-1 channels by activating NMDA receptors.

In addition to demonstration of Panx-1 activation during synaptic energy insufficiency in vivo, these findings also provide strong support to the idea that glycogen-derived energy is indispensable for maintaining low concentrations of synaptic extracellular K⁺ and glutamate throughout the brain. Therefore, we conclude that when glycogen availability decreases as for example due to prolonged and/or intense neural stimulation or to sleep deprivation,
neuronal Panx-1 channels may be activated under physiological conditions as also suggested by induction of Panx-1 expression by sleep deprivation. Cleavage of caspase-1 in these neurons and release of HMGB1 suggest that Panx-1 activation may serve to report the stress created by synaptic metabolism-activity mismatch.

Data from familial hemiplegic migraine (FHM) 1 and 2 knock-in mice point out that unregulated glutamate increases in the synaptic cleft of recurrent synapses between pyramidal cells may culminate in simultaneous depolarization of an aggregate of neurons and trigger CSD\(^4,46\). Excess extracellular glutamate may also trigger regenerative glutamate release by stimulating presynaptic NMDA receptors, which may contribute to rapid escalation of synchronized network excitation to a spreading depolarization wave\(^47\). Knock-in mice carrying human PQ type calcium channel mutations (FHM1) have a lower CSD threshold compared to their wild type littermates\(^48\) possibly because of excess glutamate release during synaptic activity. In FHM2, haploinsufficiency of \(ATP1A2\) (gene that encodes a form of the \(Na^+\)/\(K^+\) ATPase-alpha 2 selectively localized in astrocytes and shown to be activated by glutamate uptake into astrocytes\(^49\) also leads to a reduced capacity to remove glutamate and, to a lesser extent \(K^+\), hence, FHM2 knock-in mice exhibit a low CSD threshold\(^33\). Therefore, one may posit that conditions that decrease glycogen utilization may similarly predispose the cortical neurons to generate CSD during intense prolong synaptic activity due to accumulation of glutamate and \(K^+\). Our findings strongly support this view by showing that interfering with glycogen use either with pharmacological blockade (DAB) or sleep deprivation lowers the CSD induction threshold. Prolongation of the duration of CSDs and associated extracellular \(K^+\) rise\(^14,50-53\) supports this idea. The predisposition to CSD generation created by DAB or sleep deprivation is likely to be caused by insufficient energy supply during synaptic activity because the CSD threshold drop was completely reversed by supplying extra lactate or glucose to the interstitium. Similarly, the lower CSD threshold observed after inhibition of glucose transfer to astrocytes with phloretin or knocking-down MCT2, the transporter that supply lactate to pre- and post-synaptic compartments, are in line with the idea of an insufficient energy supply to match synaptic activity leads to uncontrollable synchronous excitability\(^54,55\). Decrease in the CSD threshold induced by knocking-down MCT2 suggests that sufficient energy supply to pre- and postsynaptic compartments is important to prevent CSD generation in addition to maintaining low extracellular glutamate and \(K^+\) concentrations. Restoration of the reduced CSD threshold induced by insufficient glycogen availability with lactate as well as glucose conforms with the view that glycogen is used to match the rapidly emerging energy demand during intensive synaptic activity by supplying lactate as well as glucose to pre- and postsynaptic compartments\(^56\). It appears so that the fine-tuning of the excitatory synapses terminating on excitatory as well as inhibitory neurons within a network to prevent synchronized network depolarization (which may spread to the neighboring networks once generated) is energy dependent such that insufficient acceleration of energy substrate supply from perisynaptic astrocyte processes may lead to build up of glutamate and \(K^+\) concentrations to supra-normal levels in the synaptic cleft, favoring synchronized excitation in cortical networks as observed in FHM knock-in animals.

All together these observations with DAB, sleep deprivation, MCT2 knockdown and phloretin conform with the hypothesis that glycogen in astrocyte processes is strategically positioned to secure the energy demand required for rapid removal of glutamate and \(K^+\) from synaptic cleft\(^57\) (Fig 2G). Glucose transported from the circulation can then be directly metabolized by astrocytes and used to restore the glycogen stores\(^58\). This metabolic safety system over the excitatory synaptic activity may ensure that the cortical networks sustain their activity in a desynchronized state. Given the importance of CSD in the development of migraine aura and headache, therefore, an adequate glycogen turnover appears to be critical such that an insufficient mobilization of glycogen created by e.g.
sleep deprivation or conditions that reduce glucose transport to the brain such as glucose transport protein type-1 (GLUT-1) deficiency syndrome may predispose to CSD generation and headache (migraine with aura). Interestingly, when K⁺ and glutamate accumulation caused by insufficient glycogen availability induces Panx-1 activation without CSD, then the inflammatory cascade downstream from Panx-1 activation may trigger headache without aura. Supporting this view, several cases of GLUT-1 deficiency syndrome have been reported to suffer from migraine with and without aura attacks, which were successfully prevented with ketogenic diet. Similarly, patients with monocarboxylate transporter 1 (MCT-1, which transports lactate from astrocyte to synapses) deficiency have been reported to have migraines. Therefore, our results may have important clinical implications: The genetic findings from familial monogenic disorders can be extended to non-familial migraine cases by including several other genes involved synaptic energy metabolism. Several biological factors on a genetically susceptible background may account for why insufficient sleep triggers migraine to varying degrees among patients. Ketogenic diet could be tested on sleep-deprived patients to see if it prevents migraine attacks by providing ketone bodies as alternative energy substrates for synaptic transmission. Similarly, the effect of glucose supplement or ketogenic diet on reducing the photosensitivity in migraine patients could also be tested.

In conclusion, our findings demonstrate how Panx-1 channels can be activated and upregulated under stressful metabolic conditions during synaptic activity and suggest that migraine with and without aura could be triggered by synaptic metabolic stress.
SUBJECTS/MATERIALS AND METHODS

Animals, Surgery and Intracerebroventricular Injections

Swiss albino mice (n= 209) weighing 25 to 36 g were housed under diurnal lighting conditions (12-hour darkness and 12-hour light) and fed ad libitum before the experiment. Animal housing, care, and application of experimental procedures were all carried out in accordance with the institutional guidelines and approved by Hacettepe University Animal Experiments Local Ethics Committee (2009/38-2). Animals were anesthetized with urethane/xylazine (ip. 1.25 g/kg and 10 mg/kg) during the experiments. Brief isoflurane (1-2%) anesthesia was used for i.c.v. cannula placement a day before the experiment. Oxygen (2 L/min) was supplied throughout the procedure. Body temperature was kept at 37.0±0.1°C with a homeothermic blanket and a rectal probe. Tissue oxygen saturation and pulse rate were monitored with a pulse-oxymeter. Mice were placed in a stereotactic frame after the induction of deep anesthesia. A frontal burr hole was drilled over the right hemisphere for CSD induction with an epidural KCl-soaked cotton ball.

For intracerebroventricular injections of propidium iodide, DAB and carbenoxolone, a Hamilton syringe with a 26G needle was slowly inserted through the cannula placed a day before the experiment except for the CSD induction threshold experiments. For the threshold experiments, DAB or phloretin was slowly injected into the left ventricle with help of a micromanipulator within 8-10 minutes at coordinates: 0.1 mm posterior, 0.9 mm lateral, 3.1 mm deep with reference to bregma. DAB (0.25 M, 0.75 µl; dissolved in saline; Sigma) or saline (0.75 µl) was injected 1 hour before the threshold test. During the 1-hour waiting period, DC potential changes were continuously monitored. Phloretin (2 M, 1 µl; dissolved in DMSO; Sigma) or DMSO (1 µl) was injected 30 minutes before threshold experiments (fig. 2A). All solutions were heated to 37°C before injection.

Propidium iodide injection

We assessed the opening of pannexin-1 channels with propidium iodide (PI) after DAB administration or whisker stimulation. PI is a membrane impermeable dye, however, it can pass through large-pore channels when they are open and give fluorescence by interacting with nucleic acids after entering the cell. A one mm-thick (OD) polyethylene cannula was slowly inserted 2.1 mm deep into the left lateral ventricle through a burr hole drilled over the left parietal bone under isoflurane anesthesia. The day after cannula implantation, animals were placed in a stereotaxic frame under urethane/xylazine anesthesia. PI (0.5µL, 1mg/ml in distilled water) was injected with 26G needle of a Hamilton syringe slowly inserted through the cannula by a micromanipulator either 2 minutes before whisker stimulation or 55 min after DAB or saline (as control for DAB experiments) injection. The tip of the needle was adjusted to target the ventricle 3.1 mm deep with reference to dura. As a sham control, PI was administered without whisker stimulation in another group of mice. Mice were sacrificed by transcardial perfusion with heparinized saline and 4% PFA 5 minutes after whisker/sham stimulation or 1 h after DAB/saline injection (n = 3 in each group). Brains were quickly removed and kept in 4% PFA overnight followed by 30% sucrose for 2 days. Twenty µM-thick sections were cut on a freezing cryostat and mounted in glycerol/PBS (1:1) medium containing 12.5 mg/ml sodium azide and 1 µl/ml Hoechst-33258. PI fluorescence was evaluated on 3 consecutive images of coronal brain sections under 400X magnification with appropriate filter sets using a fluorescence microscope (Nikon E600).
Carbenoxolone injection

In order to inhibit Panx-1 channels, 400 ng carbenoxolone (CBX, in 2µL distilled water, n = 3) was slowly injected into the left lateral ventricle with the needle of a Hamilton syringe driven by a micromanipulator through a polyethylene cannula placed a day before as described above. An equal volume of distilled water (n = 3) was injected to control animals. After 10 minutes, PI was slowly injected through the same cannula as described above to monitor Panx-1 activation in both groups. To investigate the effect of CBX on DAB-induced PI influx, CBX or distilled water (control) was injected through the cannula 45 min after DAB injection, which was followed by PI injection 10 minutes later. Mice were sacrificed by transcardial perfusion with heparinized saline and 4% PFA 5 minutes after PI injection and the above tissue processing protocol was applied for cryoprotection, sectioning and evaluation of the PI-positive cells.

Immunohistochemistry

Since caspase-1 is reportedly cleaved 5 min after pannexin-1 activation\textsuperscript{16}, mice were sacrificed 65 min after DAB/saline injection (n=3/group). After cryoprotection, 40 µm-thick coronal sections were cut on a sliding microtome. As HMGB1 is reportedly released 30 minutes after pannexin-1 activation, another set of mice was sacrificed 90 minutes after DAB injection. These mice were transcardially perfused with heparinized saline and 4% PFA. Brains were quickly removed, post-fixed in the same solution overnight and cryoprotected in 30% sucrose solution for two days. Twenty µm-thick coronal sections were cut on a freezing cryostat. Sections were immunostained with either goat polyclonal cleaved caspase-1 (1:200, Santa Cruz), rabbit polyclonal HMGB1 (1:200, Abcam) or mouse monoclonal NeuN antibody (1:200, Chemicon) followed by secondary labeling with donkey antim-goat IgG-FITC (1:200, Santa Cruz), goat anti-rabbit Cy2 antibody (1:200, Jackson Immunoresearch) or goat-antimouse Cy3 antibody (1:200, Jackson Immunoresearch). Primary antibody omissions were performed to test the specificity of immunoreactivity. Sections were mounted in glycerol/PBS (1:1) medium containing 12.5 mg/ml sodium azide and 1 µl/ml Hoechst-33258 and, were examined under a fluorescence and laser-scanning confocal microscope with appropriate filter sets.

CSF Collection and HMGB1 Western Blotting

For the detection of HMGB1 release, CSF was collected through an i.c.v. cannula placed into left lateral ventricle a day before the experiment. One hour after DAB injection through the same cannula, 2.5 µl CSF was collected within 30 minutes (n = 3 mice). For sham control experiments (n = 3 mice), saline was injected instead of DAB. A CSF sample was discarded if it was not crystal clear to avoid blood contamination. CSF was frozen immediately and stored at -80°C until use. CSF samples were pooled and were mixed with 4 µl of 4x sample buffer, heated at 90 °C for 10 minutes, and separated by 4-12% SDS-PAGE. They were then transferred to PVDF membranes. The blotted membrane was heated in boiling PBS for 5 minutes to enhance the signal\textsuperscript{23}. The other steps were performed with primary (rabbit polyclonal anti-HMGB1, 1:1000, Abcam) and secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, 1:3000, Cell Signaling) as described elsewhere\textsuperscript{16}. Briefly, nonspecific protein binding was blocked by incubating the PVDF membranes in 3% BSA in TBS-T for 1 hour. Membranes were incubated with rabbit polyclonal anti-HMGB1 overnight at 4°C. Next day, after washing with TBS-T, the membrane was incubated with secondary antibody for 1 hour at room temperature. The protein bands
were visualized by chemiluminescence (Super Signal West-Femto; Pierce). Images were captured by Kodak 4000MM image station. Carestream Molecular Imaging Software was used for data analysis.

**Whisker stimulation**

Whiskers on the left side were stimulated with a custom made brush driven by a motor at a rate of 10 Hz for 5 min. These stimulation parameters were first shown to induce a robust cortical blood flow (CBF) increase in the contralateral barrel cortex by laser speckle imaging. For examining PI influx, mice were transcardially perfused with 4% PFA 5 minutes after stimulation. Brains were removed and kept in 4% PFA overnight followed by 30% sucrose for two days. Twenty µM-thick sections were then obtained with a cryostat.

**Electrophysiological Recordings**

For extracranial recording of direct current (DC) potentials, two Ag-AgCl pellet electrodes (Warner Instruments, E205, 1 mm diameter) were placed over the thinned skull (fig. 2A). EEG gel applied to the electrode tip to obtain good electrical contact. For intracortical recordings, a cranial window (2 mm in diameter) was opened over the parietal cortex and its borders were sealed with dental cement. Two 0.5 M NaCl filled borosilicate glass microelectrodes were used for intracortical recordings. Microelectrodes were prepared by pulling glass capillaries (Harvard Apparatus, GC150F-10) with a horizontal puller (Kopf Instruments) and then the tips were broken into 5-10 µm so that the impedance would be 0.5-1 MΩhm. A chlorinated silver wire (Warner Instruments, AG15W, 0.375 mm diameter) was inserted into the glass microelectrodes. Tip of the microelectrodes were lowered 400 µm deep into the cortex with a micromanipulator (Narishige, MMO220A). An Ag-AgCl plated disk electrode was placed under the neck muscles to be used as the ground lead. All the electrodes were connected to a data acquisition system (Powerlab 8/30, ADInstruments) via gold pins and jacks (Warner Instruments, WC1-10 and PJ1-10). Signals were digitized, displayed, and stored on a computer.

**Assessment of the CSD threshold**

Cotton balls soaked with increasing concentrations of KCl (0.05, 0.075, 0.1, 0.15, 0.25 M) were applied on intact dura over the frontal cortex 5 minutes apart. The first concentration that induced a CSD was taken as the threshold. During preliminary experiments, we observed that the KCl-induced CSD threshold was dependent on the location of the burr hole where the KCl-soaked cotton ball applied and, it could be lowered by dural damage and bleedings. Therefore, we discarded animals even with a minor dural damage and, used a standard location and size for the cotton ball. KCl was applied with a calibrated micropipette in a fixed volume (10 µL). To obtain a highly reproducible threshold, we also used male mice and allowed them free access to food until the experiment to minimize potential variations that might be caused by hormonal changes and fasting.

**Glucose and Lactate Superfusion**

After intracortical placement of glass electrodes, 10 µl of either D-glucose (0.5 M; Sigma, France), L-glucose (0.5 M; Sigma, UK), L-lactate (0.5 M; Sigma, Switzerland) or D-lactate (0.5 M; Sigma, Switzerland) was applied for 10 minutes before recordings. The solutions were left over the dura through the entire recording. In these experiments, the CSDs were recorded by intracortical electrodes because intracortical electrodes allowed recording
more focally from the cortex superfused and also eliminated the possibility of contact of the surface pellet electrodes with the superfusion fluid leaking through the cranial window despite measures taken.

**Anti-sense knockdown of MCT2**

MCT2 antisense oligodeoxynucleotide (MCT2-ODN; 5’-GACTCTGATGGCATTTCTGAG-3’) and relative scrambled ODN (SC2-ODN; 5’-GGTTTACGAGTCGTCCGTAAT-3’), which showed no homology to any mammalian sequence were previously used in the brain in vivo with success by Suzuki et al. In order to protect ODNs from nuclease degradation, ODNs were phosphorothioated on the three terminal bases at each end. ODNs were purchased from Gene Link (Hawthorne, NY) as reverse phase cartridge-purified. ODN’s were dissolved in PBS, pH 7.4. After anesthesia, 4 nmol/µl of ODN solution was injected intracortically 1 mm deep at two different points at a rate of 0.1µl/min by a 26-gauge Hamilton syringe. Coordinates of the injection sites were; 0.5 mm posterior, 2 mm lateral to bregma and, 1 mm anterior, 2 mm lateral to lambda. The needle was removed slowly over 5 minutes to allow for the diffusion of the ODNs into cortical layers. The CSD threshold was assessed with above protocol 1 hour after injection.

**Sleep Deprivation**

Mice were housed in reverse 12 hours light-dark cabinet and they were kept in this condition for at least one week for adjustment. On the day of the experiment, mice were kept awake for 6 hours from the beginning of light period using the “gentle sleep deprivation” method described by Tobler et. al. Briefly, animals were kept awake by providing new nesting material or new objects into their home cage when they adopted a sleeping posture of more than 5 sec of immobility. Mice were not touched or stimulated by moving the cage to avoid stress as far as possible (hence, this method of keeping mice awake is called gentle). Control mice were left undisturbed in their home eages and sacrificed at the same time point. These mice were used in experiments to test the CSD threshold.

For detecting sleep-deprivation induced Panx transcription, 14 adult male C57BL6/j mice (8-10 weeks old, 22-25 g, Charles River, France) were single housed in their home cage with 12 hours light/dark cycle (lights on at 07:00, ZT0) in standard conditions (temperature at 23±1 °C, food and water ad libitum). Following 7 days of habituation, mice were randomly divided into two groups (n=7 per group). One group underwent a 6 hour GSD beginning at ZT0 (SD6 group) and another group in which mice were let undisturbed for 6 hours (ZT6). Mice of each group were alternatively sacrificed by decapitation. These experiments were performed in LNDC, Brain Mind Institute, EPFL, Lausanne, Switzerland by Dr. Petit according to the European Community’s Council Directive (86/609/EEC) and with the authorization of the veterinary services of the Canton of Vaud (n°224).

**Pannexin gene expression analysis**

For each mouse, brain was rapidly dissected out on ice and cerebral cortex were homogenized (Ultraturax, IKA, Germany) in ice-cold TRIzol™ and stored at -80°C until RNA extraction. Total RNA was extracted from tissue samples according to the TRIzol™ manufacturer’s protocol. The concentration of each sample was measured with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), and the purity was assessed using the ratios of optic density at (260/280) nm and (260/230) nm.

The first strand of cDNA was synthesized from 200ng of total RNA using TaqMan RT-reagents (Applied
Biosystem, Foster City, USA) after incubation for 45 min at 48°C followed by 5 min at 95°C and finally stored at 4°C. Then, 2 µL of RT-reagents were added to 0.5 µL of forward and reverse primers and to 23 µL of Sybr-Green PCR MasterMix (Applied Biosystem, Foster City, USA) following manufacturer’s instructions. Forty cycles of amplification were then performed in an ABI Prism 7900 (Applied Biosystem, Foster City, USA) with 384-well plate, which allowed the analysis of three genes simultaneously including β-actin for normalization in each plate. Each RT (from the cortex of one animal) was tested in triplicate. Primer sequences were designed using Primer Express (3.0) software (Applied Biosystem, Foster City, USA). Oligonucleotide primers have been synthesized by Microsynth (Balgach, Switzerland) and designed according to the published cDNA sequences (NCBI database). The forward and reverse primers for Panx1 were 5'-CCCACGTCCCTACAGACCAAm3' and 5'-AGTTCTTCTCCCCATTGTTTGCm3' respectively. The forward and reverse primers for Panx2 were 5'-CTGCTGGTCACCCTGGTCTTm3' and 5'-CCTGATCACGGGTGAAGTTGTm3' respectively. The β-actin, a housekeeping gene classically used in sleep deprivation experiments, was used as internal control to normalize data. The forward and reverse primers for β-actin (Actb) were 5'-GCTTCTTTGCAGCTCCTTCGTm3' and 5'-ATATCGTCATCCATGGCGAACm3' respectively. Finally, data were obtained using the SDS sequence detector software (Applied Biosystem, Foster City, USA). Expression level of target genes was normalized by β-actin level according to geNorm software (version 3.3; http://medgen.ugent.be/~jvdesomp/genorm/). Results were finally expressed as percentage of mean change (± SEM) relative to the control group (ZT6).

Detection of extracellular K⁺ by APG-4 fluoroprobe

In order to detect the extracellular K⁺ changes after DAB injection or GSD, a set of experiments were performed with a fluorescent potassium indicator, Asante Potassium Green (APG)-4 (Teflabs, USA). APG-4 was prepared in 2% DMSO and artificial cerebrospinal fluid (aCSF) to yield a final concentration of 250 µM. Anesthetized (urethane) mice were placed in a stereotactic frame. A cranial window was opened over the right barrel cortex leaving duramater intact, sealed with dental acrylic and filled with aCSF to form a pool and closed with a coverslip. After obtaining baseline fluorescent images under a stereomicroscope (Nikon SMZ 1000, Japan), the aCSF was replaced with APG-4 solution for 1 hour. At the end of 1-hour incubation period, the dye was washed out by irrigating with acSF and then the cranial window was refilled with acSF. Subsequently, whiskers on the left side were stimulated at 10 Hz for 5 minutes. Time series images were taken for 30 seconds before stimulation, 5 minutes during stimulation and 1.5 minutes after the end of stimulation. This cycle of imaging was repeated 3 times with 5-minute intervals. Epifluorescent imaging was performed with 488/517 nm excitation and 540 nm emission filter sets by a monochrome DS-Qi1mc CCD camera (Nikon, Japan) set at 250 ms exposure time. Images were captured with NIS Elements Software (Nikon, Japan). This whisker stimulation protocol was performed on 3 groups (n=3 mice per group); naive mice, DAB-treated mice (incubation with solution containing 0.25 M DAB in 250 uM APG-4 for one hour) and mice subjected to 6-hour GSD. Baseline images obtained before APG-4 application were subtracted mathematically from all time series images and every 10 subtracted images were averaged by NIS Elements 3.2 AR in order to minimize the pulsation artifacts. Two regions of interest were chosen over barrel cortex and fluorescent intensity changes (ΔF/Fo) were calculated by dividing all images to basal fluorescent intensity. In a separate group of mice treated with topical DAB in APG-4 solution or only APG-4 solution through a cranial window over right parietal cortex, we measured the duration of APG-4 fluorescence increase during passage of a CSD wave evoked by KCl (1M) (n=3/group).
Statistical Analyses

Electrophysiological properties of CSD and duration of CBF changes are expressed as mean and standard error of the mean (mean±sem). Multiple group comparisons were performed by means of Kruskal–Wallis variance analysis followed by Mann–Whitney’s U-test. Threshold values are expressed as median (minimum-maximum). Analyses were performed using Fisher’s exact test. For Panx transcription experiments the normalized mRNA levels of each gene in the GSD and control groups were statistically compared using a one-way ANOVA. Similar variances in each group were assessed using the Bartlett’s test. A Bonferroni’s multiple comparison test was used as post-hoc test. GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for calculations. A p value ≤ 0.05 was regarded as statistically significant.

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Author contributions: TD, HK, KK, PJM, JMP and YGO contributed to the study concept and design; KK, HK, BDD, EEK, JMP and AC performed data acquisition and analysis; TD, KK, AC, JMP and HK contributed to drafting the manuscript and preparing the figures.

Potential Conflicts of Interest: Authors disclose no conflict of interest.
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**Table 1.** Electrophysiological characteristics of CSDs in DAB-treated or sleep-deprived mice.

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