AMPK-mediated potentiation of GABAergic signalling drives hypoglycaemia-provoked spike-wave seizures

Kathryn A. Salvati,1,2 Matthew L. Ritger,1 Pasha A. Davoudian,1,3 Finnegan O'Dell,1 Daniel R. Wyskiel,1 George M. P. R. Souza,1 Adam C. Lu,1 Edward Perez-Reyes,1 Joshua C. Drake,4,5 Zhen Yan1,5,6,7 and Mark P. Beenhakker1

Metabolism regulates neuronal activity and modulates the occurrence of epileptic seizures. Here, using two rodent models of absence epilepsy, we show that hypoglycaemia increases the occurrence of spike-wave seizures. We then show that selectively disrupting glycolysis in the thalamus, a structure implicated in absence epilepsy, is sufficient to increase spike-wave seizures. We propose that activation of thalamic AMP-activated protein kinase, a sensor of cellular energetic stress and potentiator of metabotropic GABA₉-receptor function, is a significant driver of hypoglycaemia-induced spike-wave seizures. We show that AMP-activated protein kinase augments postsynaptic GABA₉-receptor-mediated currents in thalamocortical neurons and strengthens epileptiform network activity evoked in thalamic brain slices. Selective thalamic AMP-activated protein kinase activation also increases spike-wave seizures. Finally, systemic administration of metformin, an AMP-activated protein kinase agonist and common diabetes treatment, profoundly increased spike-wave seizures. These results advance the decades-old observation that glucose metabolism regulates thalamocortical circuit excitability by demonstrating that AMP-activated protein kinase and GABA₉-receptor cooperativity is sufficient to provoke spike-wave seizures.

1 Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA
2 Epilepsy Research Laboratory and Weil Institute for Neurosciences, Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA 94143, USA
3 MD-PhD Program, Yale University School of Medicine, New Haven, CT 06520, USA
4 Department of Human Nutrition, Foods and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
5 The Robert M. Berne Center for Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville, VA 22908, USA
6 Department of Medicine, University of Virginia School of Medicine, Charlottesville, VA, USA
7 Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

Correspondence to: Mark P. Beenhakker
Department of Pharmacology
University of Virginia School of Medicine
Charlottesville, VA, 22908, USA
E-mail: markbeen@virginia.edu

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Abbreviations: AMPK = AMP kinase; ECoG = electrocorticogram; FRET = Förster resonance energy transfer; SWS = spike-wave seizure
Introduction

Epilepsy is a disorder defined by spontaneously recurring seizures. Identifying factors that drive neural circuits to very rapidly, yet transiently, seize remains a major challenge. The genetic generalized epilepsies comprise one-third of all epilepsy syndromes and stem from complex, polygenic insults whose mechanisms remain poorly understood. Absence seizures are commonly observed in the genetic generalized epilepsies and appear as bursts of rhythmic, high amplitude spike-wave discharges that arise from synchronous activity among cortical and subcortical circuits.1–3 Cortical structures are thought to initiate spike-wave seizures (SWSs), whereas thalamic structures serve as critical nodes for seizure generalization and maintenance.4,7

The brain consumes 20% of dietary glucose to sustain normal function.8 Unsurprisingly, glucose availability has a considerable effect on seizures.9–12 Over 80 years ago, Gibbs et al.13 showed that hypoglycaemia precipitates seizures in children with absence epilepsy. Since then, we have learned that nearly 10% of patients diagnosed with early-onset childhood absence epilepsy are deficient in glucose transporter-1 (GLUT1),9,14 the singular glucose transporter that shuttles glucose across the blood–brain barrier.15,16 Hypoglycaemia-sensitive seizures are also observed in GLUT1 deficient mice17 and a mouse model of SWS.11 The cellular mechanisms that transduce glucose availability into SWS are unknown.

The conversion of cellular energy state into neural activity is complex.18,19 The canonical transduction pathway involves the ATP-sensitive potassium (KATP) channel. Largely regulated by ATP, the KATP channel adjusts the membrane voltage of the cell in response to cellular energy state; ATP depletion activates the channel, causing membrane voltage hyperpolarization.20,21 KATP channels modulate neuronal activity in the hippocampus,22,23 hypothalamus,24,25 and brainstem,26 and are proposed to regulate limbic seizures.25,26 Metabolic regulation of neural activity can also arise from the cooperative action between the metabotropic, B-type GABA receptor (GABAB) and the energy-sensitive AMP kinase (AMPK). During cellular stress, activated AMPK (p-AMPK) phosphorylates the GABAB-receptor and potentiates GABA B-signaling in thalamocortical projections.27 This direct coupling between AMPK and GABAB receptors to provoke SWS.

Materials and methods

Animals

All procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of Virginia Animal Care and Use Committee (Charlottesville, VA, USA). Unless otherwise stated, animals were housed at 23–25°C under an artificial 12-h light/dark cycle with food and water provided ad libitum. Wistar Albino Glaxo/ from Rijswijk (WAG/Rij rats) were kindly provided by Dr Edward Bertram (University of Virginia). Non-epileptic Wistar IGS rats (stock no. 003) were purchased from Charles River. Wild-type DBA/2J mice (stock no. 000671) were purchased from The Jackson Laboratory. Both sexes were used in all experiments and no differences were observed. In total, 44 DBA/2J mice and 141 WAG/Rij rats were used.

Simultaneous video-ECoG/EMG recordings

Rat electrocorticography (ECoG) devices were purchased from Plastics One. Mouse devices were assembled from parts purchased at Digkey. Stainless-steel recording electrodes were implanted bilaterally in the cortex while a reference electrode was placed in the cerebellum; ECoG recordings were obtained from neck muscles. One week after surgery, animals were habituated to recording cages for 48 h. Filtered ECoG (0.3–100 Hz) and EMG (>100 Hz) signals were amplified with a model 3500 amplifier (A-M Systems) and sampled (200 Hz) with a PowerLab digitizer (ADI Instruments). All experiments occurred between Hours 2 and 10 of the 12-h dark cycle (i.e. Zeitgeber time 14–22). SWSs were scored manually by blinded individuals.

Fasted blood measurements

In both rats and mice, tail vein blood was collected and glucose levels were measured with a human glucometer (Nova Max Plus, Nova Biomedical Corporation). Serum was separated from the same blood sample to quantify β-hydroxybutyrate levels using a colorimetric assay kit (Cayman Chemical, Item Number 700190).

Insulin injection

Animals were injected intraperitoneally with either saline or insulin (3 IU/kg). Blood samples were collected in WAG/Rij rats 90 min before the start of the experiment, 90 min after injection and at experiment conclusion. Blood draws were not performed in mice during ECoG/EMG recordings due to elevated stress and casualty.

Combined ECoG/EMG and drug infusion

A custom length guide cannula (Plastics One) was implanted in the left ventral basal nucleus of the thalamus (anterior–posterior –2.7 mm; medial–lateral –2.7 mm). Before experimentation, the dummy cannula was removed and replaced with a delivery cannula. Vehicle, 2-deoxyglucose (2-DG); Millipore Sigma, Cat. no. D8375) or A-769662 (Tocris; Cat. no. 3336) solution was infused (50 nl/min) into the ventrobasal thalamus with a Microdialysis Syringe Pump (Harvard Apparatus).

Lactate measurements

An incision in the leg of anaesthetized rats exposed the femoral artery, enabling the insertion of a catheter (Clay Adams) that was pushed towards the abdominal aorta. Arterial lactate blood samples were measured using an iSTAT instrument (Abbott Instruments).
Immunohistochemistry

Anaesthetized animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Then 40-µm sections were cut from fixed brains with a Leica VT 1000S microtome (Leica Biosystems). All blocking and antibody solutions were prepared in an incubation buffer of 0.1% sodium azide and 2% normal goat serum. Primary antibody solutions containing rabbit anti-Kir6.2 (1:2000, Alomone Laboratories Cat. no. APC-020, RRID: AB_2040124) and mouse anti-parvalbumin (1:2000, Sigma-Aldrich Cat. no. P3088, RRID: AB_477329) were prepared and incubated for 2 days at 4°C. After PBS washes, sections were incubated overnight in secondary antibody solutions containing donkey anti-rabbit Cy3 (1:1000, Jackson ImmunoResearch Labs Cat. no. 711-165-152, RRID: AB_2307443) and donkey anti-mouse AlexaFluor 488 (1:1000, Jackson ImmunoResearch Labs Cat. no. 715-545-151, RRID: AB_2341099). Sections were imaged with a Z1 Axioimager (Zeiss Microscopy) and captured with consistent exposure settings.

Quantitative PCR with reverse transcription

Here, 400-µm brain sections were cut with a Leica VT 1200S microtome. Thalamus and somatosensory cortex were dissected and placed in sterile tubes containing RNAlater (Life Technologies). RNA was isolated using the RNAqueous 4PCR kit (Applied Biosystems) and/or RNAeasy mini kit (Qiagen) and DNase-treated. cDNA was prepared with the iScript cDNA Synthesis Kit (Bio-Rad) and/or RNAeasy mini kit (Qiagen) and DNase-treated. RNA was isolated using the RNAqueous 4PCR kit (Applied Biosciences Cat. no. B5399) adjacent to the soma of cells. To isolate these currents, kynurenic acid (1 mM, Millipore Sigma, Cat. no. K3375) and bicuculline methiodide (10 µM, Tocris, Cat. no. 2503, Bio-Techne Corporation, RRID: SCR_003689) were added to the artificial CSF. Internal solution included QX-314 (5 µM, Tocris, Cat. no. 1014) to suppress action potentials. Only cells producing initially robust GABAmediated currents were examined so that response rundown could be measured. AMPK-activating drugs were dissolved in the internal pipette solution and included: metformin (Millipore Sigma, Cat. no. D150959), AMP (Millipore Sigma, Cat. no. A1752) and A-769662 (Tocris). To assess KATP currents in ventrobasal nucleus neurons, glibenclamide (Abcam, Cat. no. ab120267) and/or diazoxide (Abcam, Cat. no. ab120266) was dissolved in the artificial CSF. Sucrose was added to low glucose-containing artificial CSF to compensate for osmolality changes.

Extracellular multi-unit recordings

Network activity was recorded in 400 µm thalamic slices within an interface chamber supplied with warm, oxygenated artificial CSF (31–33°C, 95% O2/5% CO2). Electrical stimuli were delivered to reticular thalamus with bipolar tungsten electrodes (FHC), while single tungsten electrodes placed in ventrobasal nucleus recorded extracellular activity. Filtered activity (0.1–3 kHz) was digitized and recorded with a Digidata 1440A and pClamp software. AMPK agonists and CGP-54626 (Tocris, Cat. no. 1088) were dissolved in the artificial CSF perfusate. Activity was quantified with custom MATLAB scripts. Voltage amplitude thresholds were applied to detect action potentials and were set at 3–4× the root mean square of the trace before stimulation. Oscillation duration was defined as the period between the stimulus onset and the last activity burst to occur within the specified inter-burst interval of 1 s.

AMPKAR Förster resonance energy transfer imaging

We cloned AA9V-UPcamAMPKAR by moving the AMPKAR coding region from pFBbar2-403INES [a gift from Michiyuki Matsuda (Addgene_105241)] into the plasmid pENN.AAV.CamKII0.4.eGFP. WPRE.RB [a gift from James M. Wilson (RRID: Addgene_105541)]. The insert was amplified by PCR using primers that added Agel and BsrGI sites. The vector was digested with the same enzymes, thereby excising GFP and providing compatible restriction enzyme sites for the insert. Packaging in serotype AA9V particles was performed by Vigene Biosciences (titre 3.58 × 1015 vg/ml). P14-P21 rats
received bilateral stereotaxic injection of AAV.Camk2a. AMPKAR.WPRE.Rbg targeted to somatosensory thalamus (300 nl per injection site). Acute thalamic brain slices (250 µM) were prepared 2–4 weeks later and widefield imaged. Regions of interest were drawn in ImageJ and CFP/YFP signal intensities were measured using the FRETOffline plugin developed for ImageJ.39

Experimental design and statistical analyses

Statistical analyses were performed in MATLAB. Data normality was tested using a combination of the Lilliefors test, the Anderson–Darling test and the Jarque–Bera test. Statistical details are described in the ‘Results’ section and corresponding Supplementary material. Either parametric or non-parametric statistical analyses were performed, as indicated in the figure legends. A significance level was set at 0.05. Data are expressed as mean ± SEM. Error bars reflect 95% confidence intervals.

Data availability

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.7pvmcvsf. Custom analysis code is available on GitHub at https://github.com/blabuva.

Results

Overnight food withdrawal increases spike-wave seizures

Acute food withdrawal increases the number of spontaneous SWSs recorded in the DBA/2J strain of mouse.11 We first confirmed this result (Supplementary Fig. 1C–G) and then determined if acute hypoglycaemia also provokes SWSs in the WAG/Rij rat, a long-standing, validated model of absence epilepsy.40,41 We performed ECoG recordings in adult WAG/Rij rats for three consecutive days (Fig. 1A). Animals received food and water ad libitum for 2 days. On Day 3, animals fasted for 18 h, a duration required to achieve at least a 30% drop in blood glucose. We quantified SWSs during a 4-h period beginning at the same time of day for fed and fasted experiments. SWSs in WAG/Rij rats had an abrupt onset and occurred during behavioural arrest (Fig. 1B). Consistent with the efficacy of ethosuximide in treating human absence epilepsy, ethosuximide reduced SWSs in the rats (Fig. 1C). One hour before the fed and fasted recording sessions, we collected peripheral blood samples for blood glucose and serum ketone body (i.e. β-hydroxybutyrate) measurements. Ketone bodies represent an alternative fuel source for the brain during hypoglycaemia49 and are proposed to modulate seizures.43

Raster plots of individual SWSs (Fig. 1D and E) show that fasting increased SWSs relative to the fed state. Below each raster, we quantified the total number of SWSs per hour across the population; stacked histograms reveal the contribution of each animal to total SWS count for each 1-h-long bin. SWS count was nearly 2-fold higher during the fasted versus fed recording sessions for WAG/Rij rats (P = 0.0078, n = 13; Fig. 1F). Rats tested in the reverse order (i.e. fasted before fed) produced similar results (Supplementary Fig. 2). Seizure burden, measured as percentage duration seizing per recording session, was nearly 2-fold higher in WAG/Rij rats (P = 0.00061, n = 13; Fig. 1H) during fasted versus fed sessions. The increased seizure burden was largely driven by an elevated seizure count, not an increase in SWS duration (P = 0.17, n = 13; Fig. 1G).

Acute fasting elicited predictable changes in both blood glucose and β-hydroxybutyrate. Fasting decreased blood glucose (P = 0.00097, n = 11; Fig. 1I) and increased β-hydroxybutyrate (P = 0.002, n = 11; Fig. 1J), relative to the fed sessions. Fasting had similar effects on the DBA/2J mice (Supplementary Fig. 1H–J). We evaluated the correlation between SWS counts and glucose/β-hydroxybutyrate levels across both fed and fasted recording sessions. While correlation coefficients were not statistically significant, SWS count and β-hydroxybutyrate trended towards a positive correlation, whereas SWS count and blood glucose trended towards an inverse correlation (Fig. 1K). The observed trends suggested that either hypoglycaemia or ketosis—or both—might underlie the increase in SWS count following an acute fast.

Elevated spike-wave seizure count associates with low glucose, not elevated ketone bodies

We next used a blunt manipulation—insulin injection—to disambiguate the effects of glucose and β-hydroxybutyrate on SWSs. In fed animals, insulin reduces blood sugar44,45 without, presumably, affecting serum ketone bodies; although insulin can inhibit ketogenesis, this effect is primarily observed in fasted or diabetic animals.46,47 We therefore tested the capacity of acute insulin administration in fed animals to reduce blood glucose and increase SWSs, without elevating serum β-hydroxybutyrate. We recorded SWSs in WAG/Rij rats during two 4-h recording sessions, each separated by a day of rest. One hour into the recording session, the animals received either insulin injection (3 IU, intraperitoneal) or volume-matched saline injection (Fig. 2A); we opted for a high insulin dose to induce a rapid and precipitous drop in glucose levels. We obtained peripheral blood samples 90 min after insulin injection to measure blood glucose and β-hydroxybutyrate (Fig. 2A). The 90-min time point aimed to capture the peak hypoglycaemic response induced by insulin.48 SWS count, represented in stacked histograms, shows the contribution of each rat following either saline (Fig. 2B) or insulin (Fig. 2C) injection. To evaluate the effect of insulin on SWS count, we compared the mean number of SWSs observed during the 3 h following saline versus insulin injection. Relative to saline, insulin increased SWS count (P = 0.036, n = 12; Fig. 2D) and SWS burden in the rats (P = 0.012, n = 12; Fig. 2F); SWS duration was not affected (P = 0.92, n = 12; Fig. 2E). Rats tested in the reverse order (i.e. insulin before saline) produced similar results (Supplementary Fig. 3). Insulin injection produced a large reduction in blood glucose (P = 0.0039, n = 9; Fig. 2G), whereas β-hydroxybutyrate remained unchanged relative to saline injection (P = 0.36, n = 9; Fig. 2H).

We made similar observations for SWSs, blood glucose and β-hydroxybutyrate in DBA/2J mice (Supplementary Fig. 4C–G). As in Fig. 1K, we evaluated the correlation between SWS count and glucose or β-hydroxybutyrate levels. This analysis revealed that SWS count and blood glucose were significantly and inversely correlated (R = −0.48, P = 0.046), similar to the trend we observed during acute fasting (cf. Fig. 2I and Fig. 1K). As the inverse relationship between SWS count and blood glucose was consistent across fasting and insulin experiments, we concluded that hypoglycaemia is sufficient to increase SWSs.

Intrathalamic 2-DG increases spike-wave seizures

Reduced peripheral blood glucose also reduces cerebral glucose.49 Moreover, neural activity in the human thalamus, a structure critical for SWS generation, is particularly susceptible to moderate levels of acute hypoglycaemia.50 Therefore, we tested the
Figure 1 Overnight fasting increases spike-and-wave discharges. (A) Seizure activity was evaluated for multiple days. Animals had access to food ad libitum before the overnight fast on Day 3. Before control and fasting experiments, blood was drawn for glucose and β-hydroxybutyrate (βHB) measurements. (B) Top: Continued
hypothesis that selective disruption of thalamic glycolysis is sufficient to provoke SWSs. We used a local drug delivery system to infuse 2-DG into the thalamus of WAG/Rij rats. 2-DG disrupts glycolysis by competing with native glucose as a substrate for glycolytic metabolism.51 We targeted our local, unilateral 2-DG delivery to the somatosensory thalamus, a well-characterized SWS node,52 while recording ECoG signals (Fig. 2J). After positioning a solution-filled cannula into the thalamus and recording basal activity for 3 h, we delivered either 2-DG (27 μM)53 or saline for an additional 3 h. Figure 2K shows the number of SWSs contributed by each animal to total SWS count per hour during intrathalamic infusion of saline or 2-DG. Comparing total SWS count during active delivery (i.e. pump turned on) showed that 2-DG infusion doubled SWS count, relative to saline (P = 0.010, n = 9; Fig. 2L). 2-DG did not affect SWS duration (P = 0.56, n = 9; Fig. 2M). Thus, targeted disruption of thalamic glycolysis is sufficient to provoke SWSs.

K\textsubscript{ATP} channels are well-characterized, glucose-sensitive ion channels21,24 implicated in epilepsy.23,26 Therefore, we determined whether thalamic neurons express K\textsubscript{ATP} channels, and whether these channels confer glucose-sensitivity to thalamic neurons. Whereas immunohistochemical (Supplementary Fig. 5A and B), quantitative PCR (Supplementary Fig. 5C) and electrophysiological (Supplementary Fig. 5D) assays support the conclusion that thalamocortical neurons express K\textsubscript{ATP} channels, low glucose challenges did not affect the intrinsic excitability of these neurons in either WAG/Rij rats (Supplementary Fig. 5H and J) or DBA/2J mice (Supplementary Fig. 5K and L), indicating that ion channel activity in thalamocortical neurons is unaffected during hypoglycaemic conditions. We therefore turned our attention to possible changes in synaptic properties induced by hypoglycaemia.

**Activated AMPK potentiates GABA\textsubscript{B}-receptor-mediated currents in the thalamus**

AMPK serves as a master metabolic regulator24,55 in many tissues, including brain,56–60 by responding to the ratio of AMP to ADP to ATP within cells. As ATP levels drop, AMP or ADP activates AMPK, which is rapidly mobilized to restore the balance of cellular energy.55 The kinase, therefore, functions specifically as a sensor of low levels of intracellular ATP and, more generally, as a sensor of energetic stress in the cell. Reliable cellular stressors that activate (i.e. phosphorylate) AMPK include hypoxia,60,61 and hypoglycaemia.56,62 We first used western blots to determine whether AMPK activation is inducible in acute WAG/Rij rat, thalamic brain slices. We applied two indirect activators of AMPK, 2-DG and metformin, as well as the potent, direct activator A-769662.63,64 We generated a virally deliverable AMPK activity reporter driven by the excitatory neuron CaMKII promoter (Fig. 3A); thalamocortical neurons express CaMKII. AAV.Camk2a.AMpkAR.WPRE.Rbg was stereotaxically delivered to the somatosensory nucleus of the thalamus of WAG/Rij rats. Following expression, we extracted acute brain slices containing the thalamus and measured AMPKAR FRET efficiency. Within 20 min, application of either metformin (10 mM; P = 0.0002, n = 13; Fig. 3B and C) or A-769662 (100 nM; P = 0.0129, n = 10; Fig. 3B and D) increased FRET efficiency relative to baseline, indicating elevated AMPK activity.

Next, we tested whether AMPK activation by metformin alters the intrinsic excitability of WAG/Rij rat thalamocortical neurons. We recorded individual thalamocortical neurons in the current clamp configuration while injecting a family of hyperpolarizing and depolarizing current steps (Supplementary Fig. 6C–E). Hyperpolarizing current injection was used to evaluate post-inhibitory rebound burst firing by thalamocortical neurons, a firing mode associated with SWSs.9 Depolarizing current injection was used to evaluate general neuronal excitability. Rebound burst strength following a 2-s, −140 pA, hyperpolarizing current injection was unaltered during 10 mM metformin application (P = 0.70, n = 13; Supplementary Fig. 6D). In contrast, 10 mM metformin produced a modest increase in excitability during depolarizing current injections [repeated-measures two-way ANOVA: F(1,245) = 15.3, P = 0.0001, n = 13; Supplementary Fig. 6E]. The physiological relevance of the observed increase in spike count during a sustained, depolarizing current injection remains unclear as rebound burst firing mode, not tonic firing mode, is proposed to drive SWSs.5,65

AMPK-mediated enhancement of inhibitory GABA\textsubscript{B}-receptor signalling serves to curtail the damaging effects of ischaemia, a stressor that activates the kinase.27,61 AMPK phosphorylates S783 of the R2, GABA\textsubscript{B}-receptor subunit that, in turn, potentiates receptor coupling to G-protein inwardly rectifying K\textsuperscript{+} channels (GIRKs).27,66 The slow, long-lasting hyperpolarizing current that results from GIRK channel activation counters ischaemia-associated excitotoxicity.27 As enhanced postsynaptic GABA\textsubscript{B}-receptor function increases SWS occurrence,29,67 we proposed that hypoglycaemia-provoked SWSs rely on similar AMPK-GABA\textsubscript{B}-receptor cooperativity during energetic stress.

We evaluated the effects of AMPK on postsynaptic GABA\textsubscript{B}-receptor function by manipulating the internal pipette solution of
voltage-clamped neurons recorded in the whole-cell patch-clamp configuration. We evoked GABAB-receptor-mediated GIRK currents in WAG/Rij thalamocortical neurons by pressure ejecting the GABAB-receptor agonist baclofen (100 μM, 5 ms) proximal to the recorded neuron every 3 min (Fig. 3E). Similar to observations made in cultured hippocampal neurons,27 successive baclofen applications produced progressively smaller responses in thalamocortical neurons recorded under control conditions (i.e. internal solution containing 2 mM ATP; Fig. 3F–H, black), a response rundown attributed to cell surface instability of the receptor.27,28 AMPK activation in cultured hippocampal neurons increases cell surface GABA<sub>B</sub>-receptor stability and counteracts baclofen response rundown.27,28 Similarly, when included in the internal pipette solution of recorded thalamocortical neurons, AMPK activators metformin29 and A-76966230 eliminated rundown to repeated baclofen application (Fig. 3G and H, red); inclusion of AMP produced a non-significant trend of this rundown elimination (Fig. 3F). We quantified these observations by normalizing the amplitude of all baclofen responses to the amplitude of the first baclofen response. Thus, under control conditions, baclofen-activated GIRK currents exhibited rundown, relative to starting responses, while inclusion of AMP in the recording pipette produced responses that trended larger (repeated-measures two-way ANOVA: F<sub>(5,65)</sub> = 2.21, P = 0.06; Fig. 3F). Rundown was significantly mitigated when 1 mM metformin (repeated-measures two-way ANOVA: F<sub>(5,75)</sub> = 2.79, P = 0.022; Fig. 3G) or 100 nM A-769662 (repeated-measures two-way ANOVA: F<sub>(5,65)</sub> = 2.83, P = 0.022; Fig. 3H) was added to the internal pipette solution. Thus, AMPK activation potentiates postsynaptic GABAB-receptor-mediated GIRK currents in thalamocortical neurons.

AMPK agonists intensify GABA<sub>B</sub>-receptor-dependent epileptiform oscillations

We next tested whether thalamic circuit oscillations recorded in vitro are also sensitive to AMPK activation. Electrical stimulation of
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corticothalamic afferents in acute brain slices containing somatosensory thalamus is a well-established model of both sleep spindles and SWSz21–73 and provides a measure of thalamic circuit excitability. Several studies demonstrate that spindle-like activity evoked in the thalamic slice preparation relies on A-type GABA (GABA_A) receptor-mediated signalling, whereas SWS-associated epileptiform activity relies on robust activation of GABA_B receptors expressed by thalamocortical neurons.30,34,35 Using this model, we tested the sensitivity of both GABA_B-receptor-dependent and -independent activity to AMPK activation.

Pilot studies suggested that 10 µM A-769662 potentiates evoked oscillations generated in slices wherein GABA_A and GABA_B receptors are both functional (Supplementary Fig. 7A). Next, to dissect the contribution of GABA_A and GABA_B receptors to this effect, we evoked thalamic, SWS-like oscillations in WAG/Rij brain slices by stimulating neurons of the reticular thalamic nucleus in the presence of the GABA_A receptor antagonist, bicuculline (10 µM; Fig. 4A and B). Oscillations were evoked once per minute and action potential activity was recorded with extracellular field electrodes placed within somatosensory thalamus. In the absence of any additional pharmacological manipulations, oscillations evoked during bicuculline application are generally stable36 but can exhibit some rundown74 (Supplementary Fig. 7B). Following a baseline period wherein oscillations were evoked in 10 µM bicuculline, we co-applied either metformin (5 mM) or A-769662 (10 µM) to the perfusate for 40 min, followed by a 20-min washout period. Both metformin (Fig. 4C) and A-769662 (Fig. 4G) prolonged evoked oscillations. Plotting binned spikes in a greyscale heat map showed that oscillation prolongation occurred within 10 min of metformin (Fig. 4D) or A-769662 (Fig. 4H) application. Such was also the case when pooling all slice experiments and tracking oscillation duration across time (metformin: Fig. 4E; A-769662: Fig. 4I). The pooled data showed that oscillation duration increased by 20% during metformin application (P = 0.0027, n = 11; Fig. 4F) and 12% during A-769662 application (P = 0.0082, n = 15; Fig. 4I). These results indicate that AMPK-GABA_B cooperativity modulates thalamic oscillations.

We also evaluated the sensitivity of evoked network activity during AMPK activation when GABA_A, not GABA_B, receptors were blocked. GABA_A-receptor blockade produces oscillations that are disorganized and short in duration.34,35 Nonetheless, activity evoked during GABA_A-receptor blockade provides an opportunity to evaluate the sensitivity of thalamic circuits to AMPK activity in the absence of GABA_A-receptor activity. We recorded thalamic network activity in the presence of 20 nM CGP-54626, a potent GABA_A-receptor blocker,75 using the same stimulation paradigm described previously. As expected, evoked activity was less oscillatory and shorter in duration, relative to oscillations evoked during GABA_A receptor blockade (cf. Fig. 4K and Fig. 4C and G). Importantly, application of the potent, direct AMPK activator...
Figure 4 AMPK activators intensify thalamocortical oscillations in vitro and SWS in vivo. (A) Thalamocortical oscillations were electrically evoked in acute brain slices containing reticular thalamus (RT) and ventrobasal nucleus (VB) thalamus. (B) Top: A single stimulus evoked seconds-long bursting activity in ventrobasal nucleus that was evaluated by measuring interspike intervals (see ‘Materials and methods’ section). Bottom: Histogram of detected spikes. Bin size = 100 ms. (C) Representative oscillations and (D) spike raster during control and 5 mM metformin. Oscillations were evoked Continued
A-769662 did not affect activity duration ($P = 0.43$, $n = 11$; Fig. 4K–N). Thus, prolongation of oscillations evoked in the acute thalamic slice requires functional GABA$_B$ receptors.

The observation that AMPK activation specifically modulated SWS-like activity in the WAG/Rij rat thalamic slice motivated us to determine whether A-769662 is sufficient to provoke SWSs recorded in vivo. We compared the actions of saline and 10 μM A-769662 on SWS occurrence in cannulated (somatosensory thalamus, unilateral) WAG/Rij rats (Fig. 4O). Figure 4P shows the contribution of each rat to total SWS count per hour before, during and after intrathalamic infusion of saline or A-769662. Several rats produced an unusually high number of SWSs before pump activation, an effect that possibly reflects passive diffusion of the potent AMPK activator. Nonetheless, relative to saline infusion, A-769662 infusion produced more SWSs ($P = 0.017$, $n = 10$; Fig. 4Q), but did not change SWS duration ($P = 0.72$, $n = 10$; Fig. 4R). These data demonstrate that like fasting (Fig. 1), insulin injection (Fig. 2B) and thalamic 2-DG infusion (Fig. 2K–M), thalamic AMPK activation is sufficient to increase SWSs in WAG/Rij rats.

Metformin increases spike-wave seizures and causes status epilepticus in WAG/Rij rats

Similar to A-769662, metformin potentiated postsynaptic GABA$_B$-receptor function in thalamocortical neurons and strengthened SWS-like oscillations. We therefore hypothesized that metformin would also exacerbate SWSs. To test this hypothesis, we administered metformin systemically (intraperitoneal) to WAG/Rij rats and monitored SWSs. We delivered metformin systemically as this route is more comparable to its oral administration in patients. We tested metformin at 150 and 200 mg/kg, two doses used in previous rat studies.76–78 While the lower dose of metformin produced a trend towards increased SWSs (see next), WAG/Rij rats injected with 200 mg/kg metformin advanced through a stereotyped progression of worsening seizure phenotype. Within 20 min of 200 mg/kg metformin injection, SWS count increased by several-fold and the rats quickly entered a state of nearly continuous SWSs for 30 min (i.e. absence status epilepticus, Fig. 5A). Within 70 min of metformin injection, all animals experienced generalized tonic-clonic seizures (convulsive status epilepticus, Fig. 5A) for ~20 min, after which five of six animals died. The one surviving rat remained in convulsive status epilepticus for 11 h before seizures subsided. Figure 5B shows binned SWS counts for baseline (i.e. no injection) and saline injection, whereas Fig. 5C shows SWS counts before/after 150 and 200 mg/kg metformin injections, respectively. Binned SWS counts for non-epileptic Wistar rats, the strain of rat from which WAG/Rij rats are derived,79 are shown in Fig. 5D and E. We compared the total SWS count for each animal during the 2 h following saline versus metformin injection (Fig. 5F and G). WAG/Rij rats injected with 200 mg/kg metformin produced more SWSs, relative to saline ($P = 0.036$, $n = 6$; Fig. 5F). The 200 mg/kg metformin did not provoke seizures in non-epileptic Wistar rats (Fig. 5G) and 150 mg/kg metformin produced a trend towards increased SWSs in WAG/Rij rats ($P = 0.22$, $n = 6$; Supplementary Fig. 8B) relative to saline. In Wistar rats, 150 mg/kg metformin did not affect SWS count (Supplementary Fig. 8E).

At high doses, metformin causes lactic acidosis.80 Therefore, we measured blood lactate after metformin/saline injection in a separate cohort of WAG/Rij and Wistar rats. The 1-h time point aligned with the pronounced change in SWSs and the emergence of tonic-clonic seizures in the WAG/Rij rat. Lactate did not increase in WAG/Rij rats injected with 150 mg/kg metformin ($P = 0.16$, $n = 7$; Supplementary Fig. 8C). After 200 mg/kg injection, we observed a 3-fold increase in lactate ($P = 0.016$, $n = 7$; Fig. 5F) and all rats perished within 1 h. The 200 mg/kg metformin also increased lactate in Wistar rats ($P = 0.03$; $n = 5$; Fig. 5G). Post-metformin lactate levels in WAG/Rij and Wistar rats were similar ($P = 0.64$).

As the WAG/Rij seizure response to lactate-producing doses of metformin is complex, we speculate that lactate’s actions on WAG/Rij neural circuits are likewise complex. Nonetheless, we tested whether GABA$_B$-receptor-dependent oscillations evoked in acute thalamic slices from WAG/Rij rats were sensitive to activation of HCAR1, a receptor for lactate that, like AMPK, has recently been shown to augment GABA$_B$-receptor function.81–83 SCI-50H-BA, a selective HCAR1 receptor agonist, prolonged evoked oscillations by 40% ($P = 0.025$, $n = 4$; Fig. 5H and I). Thus, both p-AMPK and lactate are sufficient to augment GABA$_B$-receptor function and exacerbate thalamic network oscillations associated with SWs. However, it remains formally possible that p-AMPK actions on GABA$_B$-receptor function are indirect, resulting from its ability to elevate lactate.

Discussion

Here, we first advance the observation that hypoglycaemia provokes SWS by showing that selective blockade of thalamic glycolysis in the WAG/Rij rat is sufficient to provoke SWS. Specifically, we show that hypoglycaemia probably activates AMPK in the thalamus, and that activated AMPK augments postsynaptic GABA$_B$-receptor signalling in thalamocortical neurons. AMPK-GABA$_B$-receptor enhancement strengthens epileptiform, GABA$_B$-receptor-dependent oscillations in acute thalamic slices and elevates SWS in vivo. Last, we report that metformin, a common diabetes treatment and AMPK activator,84–85 powerfully instigates SWS in the WAG/Rij rat. These findings provide the first molecular framework for understanding how glucose availability regulates generalized SWSs.

Hypoglycaemia and spike-wave seizures

Glucose fuels the brain and is the most robust energy source for generating ATP. Moreover, glucose-derived metabolites are...
required for the synthesis of several neurotransmitters that regulate neuronal excitability, including glutamate and GABA. SWS exacerbation by hypoglycaemia was first reported nearly 80 years ago, and impaired glucose handling continues to associate with the genetic generalized epilepsies today. Mutations in SLC2A1, the gene encoding GLUT1, are found in 1% of all genetic generalized epilepsy patients and specifically account for 10% of early-onset childhood absence epilepsies. Moreover, nearly half of GLUT1 deficient patients produce 2.5–4 Hz spike-wave discharges, electrographic patterns similar to SWS associated with the absence epilepsies. The SLC2A1 mutation impairs glucose transport across the blood–brain barrier. As GLUT1-deficient patients present with CSF containing <60 mg/dl glucose, these patients lack the necessary cerebral glucose to maintain normal brain function.

An effective therapy for GLUT1 deficiency syndrome is the ketogenic diet, a high-fat, low carbohydrate diet that switches the body's fuel source from glucose to ketone bodies. As the brain shifts towards a reliance on ketone bodies for fuel, a change that takes several days, seizures in GLUT1 patients on the diet eventually abate. Indeed, the anti-convulsant effects of the ketogenic diet extend well beyond GLUT1 deficiency. Several studies...
specifically attribute the anti-seizure effects of the diet to elevated ketone bodies.94–96 And yet, here we report that acute fasting—a manipulation that elevates ketone bodies—exacerbates SWS. We speculate that during acute hypoglycaemia, the pro-SWS actions of hypoglycaemia outweigh the anti-SWS mechanisms of ketosis, if any, to provoke SWS; notably, the diet is considered only moderately effective in treating the absence epilepsies.92,93 If true, then evaluating whether absence seizure occurrence in patients correlates with normal, diurnal fluctuations in blood glucose may ultimately provide novel insights into improving seizure control.

Curiously, our observations that hypoglycaemia, 2-DG and metformin can aggravate absence seizures appear to contradict the proposed, seizure-suppressing use of these drugs. Indeed, both 2-DG94,95 and metformin96 show great promise in treating temporal lobe seizures. However, both 2-DG and metformin may ultimately belong to a sizeable list of anti-seizure drugs that counterintuitively worsen absence epilepsy. Drugs in this list—carbamazepine, oxcarbazepine, phenytoin, vigabatrin and tiagabine—are used to treat temporal lobe seizures and yet aggravate absence seizures.96–101 Indeed, both vigabatrin98 and tiagabine100 can induce absence status epilepticus, a state of continuous and prolonged SWS and the EEG pattern we observed in WAG/Rij rats following a high metformin dose. Both vigabatrin and tiagabine increase the availability of extracellular GABA and are hypothesized to exacerbate absence seizures by promoting GABA<sub>B</sub>-receptor activation.95,98,100 Thus, the finding that 2-DG and metformin may in fact both reduce temporal lobe seizures and aggravate absence seizures may ultimately align with our current understanding of some anti-seizure drugs.

GABA<sub>B</sub>-receptor mediated inhibition in the thalamus

Our results support the hypothesis that energetic stress activates thalamic AMPK that, in turn, upregulates GABA<sub>B</sub>-receptor function. Promoting GABA<sub>B</sub>-receptor function elevates SWS counts,92,67,105 whereas inhibiting GABA<sub>B</sub>-receptor function dampens SWS.92,67,106 The long-lasting and powerful inhibition produced by postsynaptic GABA<sub>B</sub> receptors recruits low threshold, T-type calcium channel activity to produce robust post-inhibitory rebound bursts that probably sustain SWS.5,45 Consistent with these observations, we show that activated AMPK potentiates postsynaptic GABA<sub>B</sub>-receptor signalling in thalamocortical neurons; strengthens GABA<sub>B</sub>-receptor-dependent, epileptiform oscillations recorded in thalamic brain slice preparations and increases SWS. Postsynaptic GABA<sub>B</sub> receptors have a moderate affinity for GABA (1 µM107) and are largely localized to extrasynaptic dendritic regions of thalamocortical neurons.58 Thus, the receptors are largely inactive during basal conditions or during moderate synaptic activity.108–110 However, if GABAergic inputs are sufficiently active, then released GABA can spillover to extrasynaptic regions to activate GABA<sub>B</sub> receptors.75 We propose that AMPK-mediated potentiation of thalamic GABA<sub>B</sub>-receptor activity during hypoglycaemia reduces the threshold for such receptor activation.

Herein, we focused on the potential contribution of the thalamus to hypoglycaemia-provoked SWS. Notably, however, SWS result from complex interactions between multiple structures, including the cortex and thalamus. According to the cortical focus theory, the somatosensory cortex provides the initiating drive to thalamic circuits that then generate hypersynchronous, rhythmic activity that is rapidly generalized throughout widespread regions of the cortex.111,112 While our findings demonstrate that selective disruption of glucose metabolism in the thalamus is sufficient to elevate SWS in the seizure-prone animal, future studies are warranted to determine whether selective cortical disruption is likewise sufficient. Nonetheless, our current observations suggest that glucose-sensitive mechanisms in the thalamus might establish a threshold necessary to produce generalized SWS, and that hypoglycaemia reduces this threshold by enhancing thalamic GABA<sub>B</sub>-receptor function. This hypothesis is consistent with the observation that the human thalamus is uniquely sensitive to even moderate levels of hypoglycaemia.113

**Metformin and spike-wave seizures**

Metformin exacerbated SWS and, at high doses, evoked a profound and fatal seizure response in seizure-prone animals. Metformin inhibits complex I of the mitochondrial electron transport chain, an effect that reduces ATP production and elevates AMP levels that facilitate AMPK activation.82 Metformin increases insulin sensitivity, enhances glucose uptake in muscle tissue, and blocks gluconeogenesis in the liver; collectively, all actions result in lower blood glucose82 and make metformin the most common and highly effective pharmacological treatment for type 2 diabetes.82,114 With its ability to pass the blood–brain barrier, metformin also has potential therapeutic effects in Huntington’s disease,115 Alzheimer’s disease116 and some forms of epilepsy.76–78,117 While adverse actions of metformin on absence epilepsy are not reported, several reasons may obscure any possible links. First, the high metformin dose used in our study was roughly twice that of the equivalent dose used in humans and therefore may not have a clinical equivalence; however, metformin overdose occurs in humans.114,118 Second, despite the drug’s ubiquity, metformin is generally not prescribed to children; the mean age of onset for childhood absence epilepsy is 4–7 years of age.119 Thus, future studies are required to fully resolve the clinical ramifications of our unexpected observations.

Herein, the effects of metformin on SWS probably result from a combination of actions. We propose that lower doses of metformin activate AMPK to augment GABA<sub>B</sub>-receptor mediated signalling and increase SWS. The actions of high doses of metformin are probably multifaceted. Following a high dose of metformin, SWS counts not only increased, but all animals quickly transitioned into a state of near-continuous spike-wave activity (i.e. absence status epilepticus). We speculate that metformin-induced absence status epilepticus in rats results from the converging and enhancing actions of AMPK and lactate82 on thalamic GABA<sub>B</sub>-receptor function. The subsequent progression into convulsive, tonic-clonic seizures (status epilepticus) and death probably results from the actions of elevated lactate on multiple brain structures. Indeed, the drug’s capacity to produce lactic acidosis provides the basis for the FDA’s black box warning. Nevertheless, the intense seizure response was not observed in non-epileptic Wistar rats. Thus, SWS predisposition appears necessary for a metformin-provoked seizure response. Clearly, the actions of metformin are complex and varied, and fully testing this hypothesis will ultimately benefit from transgenic approaches that modify AMPK’s activation capability.

**Conclusions**

In aggregate, our study addresses a growing number of observations that glucose availability regulates SWS. Despite such conclusions, the mechanisms that enable hypoglycaemia to regulate SWS remain entirely unknown. We now provide data in support of the hypothesis that glucose-mediated regulation of SWS results from p-AMPK potentiation of GABA<sub>B</sub>-receptor-mediated signalling.
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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

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