



Glycolysis regulates neuronal excitability via lactate receptor, HCA₁R

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Repetitively firing neurons during seizures accelerate glycolysis to meet energy demand, which leads to the accumulation of extracellular glycolytic by-product lactate. Here, we demonstrate that lactate rapidly modulates neuronal excitability in times of metabolic stress via the hydroxycarboxylic acid receptor type 1 (HCA₁R) to modify seizure activity.

The extracellular lactate concentration, measured by a biosensor, rose quickly during brief and prolonged seizures. In two epilepsy models, mice lacking HCA₁R (lactate receptor) were more susceptible to developing seizures. Moreover, HCA₁R deficient (knockout) mice developed longer and more severe seizures than wild-type littermates. Lactate perfusion decreased tonic and phasic activity of CA1 pyramidal neurons in genetically encoded calcium indicator 7 imaging experiments. HCA₁R agonist 3-chloro-5-hydroxybenzoic acid (3CL-HBA) reduced the activity of CA1 neurons in HCA₁R WT but not in knockout mice. In patch-clamp recordings, both lactate and 3CL-HBA hyperpolarized CA1 pyramidal neurons. HCA₁R activation reduced the spontaneous excitatory postsynaptic current frequency and altered the paired-pulse ratio of evoked excitatory postsynaptic currents in HCA₁R wild-type but not in knockout mice, suggesting it diminished presynaptic release of excitatory neurotransmitters.

Overall, our studies demonstrate that excessive neuronal activity accelerates glycolysis to generate lactate, which translocates to the extracellular space to slow neuronal firing and inhibit excitatory transmission via HCA₁R. These studies may identify novel anticonvulsant target and seizure termination mechanisms.

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Introduction

Increased neuronal firing consumes energy and, in response, there are compensatory changes in the brain vasculature and metabolite delivery to meet energy requirements and feedback mechanisms to reduce firing.^{1,2} There is growing evidence that accelerated glycolysis feeds neuronal demands during high activity states and is vital for sustaining synaptic transmission. Astrocytes are proposed to provide energy substrates for supporting neurons in an activity-

dependent manner. According to Astrocyte to Neuron Lactate Shuttle (ANLS) hypothesis, glutamate uptake into astrocytes stimulates glycolysis and lactate production for the energy needs for neurons.^{3,4} Moreover, glycolytic enzymes localize to the presynaptic compartment to restore the ATP pool under conditions of metabolic stress.⁵ Even at low-frequency transmission, presynaptic glycolysis provides one-third of the energy to support neurotransmission.⁶ Synaptic vesicle refilling, an ATP-dependent step in neurotransmission, is fuelled by glycolysis^{7,8} and inhibition of the presynaptic

glycolysis hinders synaptic vesicle endocytosis.⁹ Reduced synaptic vesicle packaging and recycling would impair neurotransmission.

Under normal conditions, glycolysis generates lactate and pyruvate with an estimated lactate–pyruvate ratio of 10:1.¹⁰ Moreover, lactate concentration increases during physiological conditions, such as stimulation of the human visual cortex with photic stimulation¹¹ or neurological disorders, such as epileptic seizures.^{12–14} The tight relationship between glycolysis and lactate production led us to test whether lactate can modulate neuronal excitability.

G-protein coupled receptor-81 (GPR81) was first cloned in 2001,¹⁵ but its specific agonist, lactate, was identified almost a decade later.^{16,17} The presence of this receptor suggests that lactate can act as a signalling molecule, and the receptor was renamed hydroxycarboxylic acid receptor type 1 (HCA₁R). Initially believed to be restricted to the adipose tissue,¹⁸ later studies found HCA₁R to be widely expressed in the brain.¹⁹ HCA₁R is present in neurons,^{19–21} brain and pial blood vessels²² and the ventricular system.²³ The HCA₁R–lactate signalling pathway promotes neovascularization induced by physical activity,²² ischemia²⁴ and cancer.²⁵ Recent studies also reported HCA₁R-dependent neurogenesis.^{26,27} Previous studies indicate that HCA₁R reduces neuronal excitability *in vitro*.^{20,21} These studies, however, have not shown the effect of acute, fast HCA₁R activation and modulation of neuronal excitability *in vivo*. Here, we show that during increased glycolysis *in vivo*, HCA₁R activation rapidly diminishes neuronal excitability and excitatory synaptic transmission *in vitro*.

Materials and methods

Animals

In this study, we used a total of 45 C57Bl/6 mice 4–8 weeks old of both sexes (Charles River) and 38 HCA₁R KO and 46 HCA₁R littermate wild-type (WT) mice of both genders. For *in vivo* seizure experiments, we used a total of 14 females and 30 males. For calcium imaging studies, we used 7 females and 12 males. The results were similar between male and female mice, and hence the data were pooled. University of Virginia Care and Use Committee approved all experimental protocols. We housed them five per cage, gave them *ad libitum* access to food and water and maintained them on a 12 h light/12 h dark cycle. The HCA₁R knockout (KO) were obtained from the Genetically Engineered Murine Model (GEMM) Core at UVA and were generated using CRISPR–Cas technology in S129 mice. The HCA₁R gene (NM_175520, Ensembl: ENSMUSG00000049241) comprises a single exon of 1118 nucleotides; two targeting RNAs, first directed to a region immediately after the start of the coding sequence and another targeted to the middle of the exon were used. The crRNA1 targeted the antisense strand while crRNA2 targeted the sense strand. Fertilized eggs were injected with cas9 (International DNA technologies, IDT), tracrRNA (IDT), crRNAs (IDT), and then implanted in foster females. The pups were weaned at 21 days old and genotyped (KAPA Biosystems) using primers (Supplementary Table 1) binding to either side of the deleted region. Five animals had a deletion in the coding region; we pursued two lines, 5 and 7, in the subsequent studies. The PCR fragment was sequenced to confirm the deletion of the sequence in the coding region in the two lines. The founders were then backcrossed against C57Bl/6 line once before intercrossing to generate animals for our study. The colony was maintained by breeding heterozygous mice.

Quantitative qRT-PCR

To validate the HCA₁R knockout, we used a qRT-PCR reaction. Brains from 6-week-old HCA₁R WT (*n* = 3) and KO (*n* = 3) mice were isolated, and total RNA isolation and cDNA synthesis were performed as described previously.²⁸ The expression of HCA₁R was determined using sensiFAST SYBR fluorescein kits (BioLine) using the primers to target HCA₁R gene (Supplementary Table 2). The specificity of primers was first tested in a sample PCR assay using cDNA obtained from C57Bl/6 mice, and a single DNA fragment of the predicted size was amplified (data not shown). All samples were run in triplicate, and negative controls (no-RT) were run for each gene to confirm the specificity of primers (data not shown). Relative HCA₁R mRNA expression was quantified using the comparative CT methods and results were expressed as the fold change using the $\Delta\Delta CT$ formula.²⁹

Open-field test

We assessed the general behaviour and locomotor function of 6–8-week-old HCA₁R WT and KO mice by performing an open-field test. We acclimated the mice to the testing room for 30 min then transferred them to an open-field arena with a clean opaque plastic chamber (50 × 50 × 50 cm). Mice explored the arena for 60 min freely. We tracked mouse behaviour and distance travelled using Ethovision 13 software (Noldus, Leesburg, VA, USA).

Lactate concentration measurement during single seizure and prolonged seizures

Lactate concentration during a single seizure and prolonged seizures was measured using a lactate oxidase-based biosensor probe (Pinnacle Technologies) with simultaneous video-EEG monitoring. We custom-built the recording assembly composed of an intracranial guide cannula (for lactate biosensor insertion) with an attached hippocampal electrode with the tip located proximal to tip of the inserted lactate probe, a cortical electrode ipsilateral to the sensor, a reference electrode and a bipolar stimulating electrode (constructed from two twisted insulated stainless-steel wires) contralateral to the probe. We stereotactically implanted 7–8-week-old C57Bl/6 mice with the guide cannula and a hippocampal electrode in the left ventral CA1 hippocampus (−3 mm anteroposterior, −3 mm mediolateral, 3 mm dorsoventral), stimulating electrode in the right ventral CA1 hippocampus (−3 mm anteroposterior, 3 mm mediolateral, 3 mm dorsoventral), unilateral cortical electrode and a cerebellar reference electrode. A week after the surgery, the animals were connected to a video-EEG monitoring system (Lab chart software, ADInstruments) via a flexible cable connected to the amplifier. The lactate biosensor was calibrated according to the manufacturer's manual prior to insertion into the brain. The lactate biosensor was inserted into the guide cannula to reach the left ventral CA1 hippocampus following the calibration. All animals were subjected to single (<60 s) and prolonged (>5 min) seizure induction protocols, described below. To determine the stimulation-induced epileptiform discharge, also referred to as an afterdischarge threshold (ADT), a 0.75-ms biphasic square wave pulse at 50 Hz was applied for 10 s. The initial stimulation was set at 20 μ A. It was increased by 20 μ A in successive stimulation trains, separated by at 60 s until a seizure (ADT) with a duration of at least 10 s was observed for cortical and hippocampal electrodes on the EEG. To induce a single seizure, a current of twice the magnitude of ADT (with a minimum at 100 μ A), 50 Hz stimulation train was applied for 10 s. Prolonged seizures were induced by a

continuous hippocampal stimulation (CHS) protocol.³⁰ Animals were monitored by continuous video-EEG with simultaneous lactate concentration measurements until the end of the seizures.

Electrode implantation, induction of prolonged seizures and rapid kindling

As described above, we induced prolonged seizures in HCA₁R WT and KO mice by CHS. We stereotactically implanted 7–8-week-old HCAR1 WT and KO mice with a stimulating bipolar insulated stainless-steel electrode in the left ventral CA1 hippocampus (3 mm anteroposterior, 3 mm mediolateral, 3 mm dorsoventral), bilateral cortical electrodes and a cerebellar reference electrode. A week after the electrode implantation, the animals were connected to a video-EEG monitoring system (Grass ARUA LTM64 using Twin software, Grass, Warwick, RI, USA) via a flexible cable connected to the amplifier through an electrical swivel. EEG was reviewed as previously described^{30,31} and the end of the seizure was determined based on the drop-off of a spike-wave discharge frequency below 1 Hz, the disappearance of continuous exploration of the cage (thigmotactic behaviour) and the resumption of feeding and grooming.

Behaviour

We analysed seizure severity by scoring behaviour. Behavioural seizures were scored using a modified Racine scale.³² The baseline behaviour of animals during the seizure, which involved thigmotactic behaviour, was classified as Stage 1. Facial twitching, head bobbing or behavioural arrest was classified as Stage 2, unilateral forelimb clonus as Stage 3, bilateral forelimb clonus as Stage 4, rearing and falling as Stage 5 and involuntary jumping in the cage as Stage 6. We observed animals for 2 min and assigned the highest behavioural score. For the rapid kindling protocol, electrical stimulations using a pulse amplitude of 150% ADT were performed four times per day with at least 1 h interval between stimuli until three successive Grade 5 seizures occurred.

In vitro genetically encoded calcium indicator 7 imaging

To study calcium dynamics in hippocampal slices, we used viral-mediated delivery of genetically encoded calcium indicator 7 (GCaMP7). On post-natal Days 30–40 C57Bl/6, HCA₁R KO and WT mice were stereotactically injected with a mix of pENN.AAV.CamKII 0.4.Cre.SV40 and pGP-AAV-syn-FLEX-jGCaMP7s-WPRE (Addgene, viral titre 1×10^{13} µg/ml) viruses in the ratio 1:1 bilaterally in the ventral and dorsal CA1 region of the hippocampus [anteroposterior (AP), –2.54; mediolateral (ML), –/+2.00; dorsoventral (DV), –1.20 for ventral hippocampus and AP, –3.40; ML, –/+3.35; DV, –2.25 for dorsal hippocampus]. A Hamilton syringe (Hamilton 7000 Glass, 5 µl, 0.3302 mm) was loaded with a virus solution kept on dry ice and mounted in the peristaltic pump holder (P-1500; Harvard Apparatus). The virus was infused at 0.1 µl/min constant flow rate and 0.100 µl per spot. The needle was left in the tissue for 5 min before starting and after the infusion. Mice were euthanized and tissue was processed 10–14 days after the surgery for GCaMP7 imaging.

Preparation of acute hippocampal slices

We sliced the brain into 300 µm coronal sections containing the hippocampus with a vibratome (VT1200S; Leica) in an ice-cold,

oxygenated slicing buffer comprising (mM): 65.5 NaCl, 2 KCl, 5 MgSO₄, 1.1 KH₂PO₄, 1 CaCl₂, 10 dextrose and 113 sucrose (300 mOsm). The slices were then placed in an interface chamber containing oxygenated artificial CSF (aCSF) containing: (in mM) 124 NaCl, 4 KCl, 1 MgCl₂, 25.7 NaHCO₃, 1.1 KH₂PO₄, 10 dextrose and 2.5 CaCl₂ (300 mOsm) at room temperature (25°C) and allowed to equilibrate for 30 min.

In vitro wide-field Ca²⁺ imaging

We imaged the GCaMP7 signal on an inverted epifluorescence microscope (Nikon Eclipse Ti) in combination with a GFP filter set (Nikon, Intensilight C-HGFIE) and build-in NIS-Elements Advanced Research (AR) Software (5.21.03). The wide-field imaging system was equipped with a motorized stage system (OptiScanTMIII, Prior Scientific Instruments Ltd.) and a Z-focus controller (NIKRFK, Prior Scientific Instruments Ltd.). To minimize photo-bleaching of GCaMP7 signal during the imaging and to maintain a high signal-to-noise ratio (SNR), the wide-field imaging system was equipped with an automated light shutter (Prior Scientific Ltd, OptiScan III) and high quantum efficiency camera (ORCA-Fusion BT, Hamamatsu, c15440–20UP). The GCaMP7-expressing hippocampal slices were transferred into the microscope stage chamber (RC-21 B, Warner Instrument Corp.) with continuous perfusion of oxygenated aCSF. CA1 pyramidal cell body layer was first visualized and examined with a 20x lens (Nikon; NA=0.75) using differential interference contrast (DIC) microscopy and the DIC image of each slice was recorded for reference. Light from a LED source was filtered for excitation at 488 and 525 nm for emission collection. Exposure time was adjusted to visualize green fluorescent signals for each slice recorded. Images were acquired at a 0.2 Hz frame rate. Changes in calcium fluorescence were measured in regions of interest comprising the GCaMP7 positive CA1 pyramidal cell bodies. To excite neuronal network, potassium concentration in the aCSF was increased from 2 mM (baseline aCSF) to 5 mM (high K⁺ aCSF). To study the effect of HCA₁R activation, GCaMP7-expressing CA1 principal neurons were perfused with high K⁺ aCSF containing 10 mM L-lactate (Sigma; high K⁺ aCSF + Lac) or high K⁺ aCSF containing 100 µM 3-chloro-5-hydroxybenzoic acid (3Cl-HBA; TCI America; high K⁺ aCSF + 3Cl-HBA). Prior to drug perfusion, brain slices were perfused with high K⁺ aCSF (baseline) and a wash-out step with high K⁺ aCSF was followed by drug perfusion to ensure slice health.

Image analysis

Time-lapse movies were uploaded into ImageJ, and region of interest was drawn based on each slice's DIC image. The multi-measure functionality was used for pixel measurements for all images taken.

All measured fluorescent values were normalized to the mean baseline recording for each individual slice and were expressed as a % change in fluorescence relative to baseline (%ΔF/F). To study phasic calcium activity in individual neurons, we used LabChart software, which includes a Peak Analysis Module for automatic and unbiased detection of signal peaks in recordings. Peak detection function was set to detect events with amplitudes more than twice the standard deviation of the experimental noise. Peak event (1) or no event (0) were then graphically represented on raster plots to examine a frame-by-frame calcium dynamics of individual neurons.

Electrophysiology

In hippocampal slices, CA1 principal neurons were visually identified on an upright microscope (Nikon Eclipse E600FN) with a 40× water-immersion objective (Nikon; NA = 0.8) and a camera (Nikon DS2 Qi). Active and passive neuronal membrane properties were assessed using standard current clamp patch-clamp electrophysiology techniques. In brief, glass microelectrodes were filled with an internal solution containing (in mM): potassium gluconate 120, NaCl 10, MgCl₂ 2, EGTA 0.5, HEPES 10, Na ATP 4, NaGTP 0.3 (pH 7.2, 280–295 mOsm). Input resistance (R_{in}), action potential threshold, amplitude and half width were assessed by applying 300-ms current pulses (from –60 to +80 pA, 5 pA increments) at the end of each 10-min epoch (baseline, lactate, 3Cl-HBA) under current clamp. Input resistance was calculated as the slope of the linear fit of the voltage-current plot. The most negative membrane potential to evoke an action potential was determined as threshold. The time constant was determined by applying 50 ms, 10 pA pre-pulse. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from CA1 principal neurons using standard voltage-clamp patch-clamp electrophysiology techniques at 30°C.³³ D-2-Amino-5-phosphonopentanoic acid (APV; Tocris), 50 μM, was added to prevent plasticity. The GABA_A receptor antagonist, picrotoxin (Sigma-Aldrich), was added (100 μM) to block inhibitory synaptic responses mediated by GABA receptors. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded by blocking excitatory transmission with DNQX/APV (Tocris). Evoked excitatory postsynaptic currents (eEPSCs) were recorded in response to stimulation of Schaffer collateral axons in the presence of 50 μM APV and 100 μM picrotoxin by a bipolar tungsten microelectrode (FHC, Bowdoin) placed in stratum radiatum. Paired-pulse stimulation was applied at different intervals (in ms: 20, 30, 40, 50, 80, 100, 200). The average paired-pulse ratio was measured as the ratio of the amplitude of the second eEPSC to the first eEPSC. Currents were filtered at 2 kHz, digitized using a Digidata 1322 digitizer (Molecular Devices) and acquired using Clampex 10.2 software (Molecular Devices).

Statistical analysis

Statistical analysis was performed in the GraphPad Prism software using t-test, Wilcoxon matched-pairs signed-rank test, Fisher test, Kaplan–Meier survival comparison, Mantel–Cox log-rank test, 2-tailed Mann–Whitney test, Kolmogorov–Smirnov test and one-way ANOVA with *post hoc* Bonferroni correction and P-value < 0.05 was considered significant.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Seizures increase extracellular lactate concentration

In order to determine whether a glycolytic product, lactate, can alter neuronal excitability by acting on HCA₁R, we tested if there is sufficient extracellular lactate to activate the receptor during periods of metabolic stress. The HCA₁R is a membrane receptor with a low affinity for lactate. We determined whether an intense neuronal activity can generate sufficient extracellular lactate concentration to activate the receptor. Metabolic stress elevates brain and CSF lactate production.³⁴ Nonetheless, brain lactate concentration measurements

include both the intra- and extracellular lactate, and they do not reflect extracellular lactate that could activate HCA₁R.

Because excessive neuronal firing associated with epileptic seizures imposes metabolic stress, we tested whether a single seizure can increase extracellular lactate concentration sufficiently to activate HCA₁R. We custom-built a recording assembly (Fig. 1A) to simultaneously record EEG and extracellular lactate concentration in real-time during a single seizure. We placed a recording electrode and a guide cannula for lactate sensor insertion into the left ventral hippocampus and a seizure-inducing electrode into the right ventral hippocampus (Fig. 1B). The probe reported basal lactate concentration stabilized at 0.336 mM (±0.045) 41 min (±5) following its insertion into the hippocampus. Induction of a brief, electrographic seizure not associated with a convulsion caused a rapid increase in extracellular lactate concentration (Fig. 1C) with a peak lactate level of 0.777 mM (±0.407; *n* = 6, *P* < 0.05, paired t-test). Initially, during the seizure we detected a decrease in lactate concentration (Fig. 1D). Following the seizure activity, lactate concentration rapidly increased and remained elevated for several minutes before it slowly declined to the baseline (Fig. 1C and E).

We next investigated the real-time changes in lactate concentration in the hippocampus in response to prolonged (>5 min) seizures. First, we induced prolonged seizures by CHS in mice³⁰ implanted with a lactate probe. Following an hour of electrical stimulation, mice develop self-sustaining prolonged seizures. We detected an increase in extracellular lactate concentration during the self-sustaining phase of the seizure compared to baseline recording (Fig. 1F and G). Lactate concentration persisted elevated throughout the seizure activity (Fig. 1F and G) with a peak lactate level of 0.957 mM (±0.176; *n* = 5, *P* < 0.05, paired t-test). These findings suggested that seizures elevate lactate, which could modulate neuronal activity through HCA₁R.

Generation and characteristics of HCA1R-deficient mice

To investigate the role of lactate action via HCA₁R, we generated the global HCA₁R KO mouse. We determined mRNA expression of HCA₁R on brain samples collected from HCA₁R KO and littermate WT mice by real-time PCR. We detected 80% lower HCA₁R expression in KO versus WT mice (Supplementary Fig. 1; *n* = 3 each, *P* < 0.0001, t-test). Currently available antibodies against HCA₁R are not specific and are unreliable, which we (not shown) and others confirmed.²⁰

We then assessed the general behaviour of mice lacking HCA₁R. As lactate serves as a metabolite, we sought to investigate the potential difference in body weight of HCA₁R-deficient mice. We did not detect any difference between HCA₁R WT and KO mice (Supplementary Fig. 2A; HCA₁R WT females: *n* = 7; males: *n* = 6; HCA₁R KO females: *n* = 6; males: *n* = 7; females HCA₁R WT versus KO: *P* = 0.81, t-test; males HCA₁R WT versus KO: *P* = 0.87, t-test). We then performed an open-field test to examine the locomotor function and did not observe a difference between HCA₁R WT and KO mice (Supplementary Fig. 2B; HCA₁R WT *n* = 10, HCA₁R KO *n* = 9, *P* = 0.86, two-way ANOVA). These findings are similar to those reported by others in a different HCA₁R KO mouse model.²⁰

HCA1R KO mice are more susceptible to developing severe seizures

We investigated whether lactate generated during the metabolic stress of excessive neuronal firing activates HCA₁R to modify

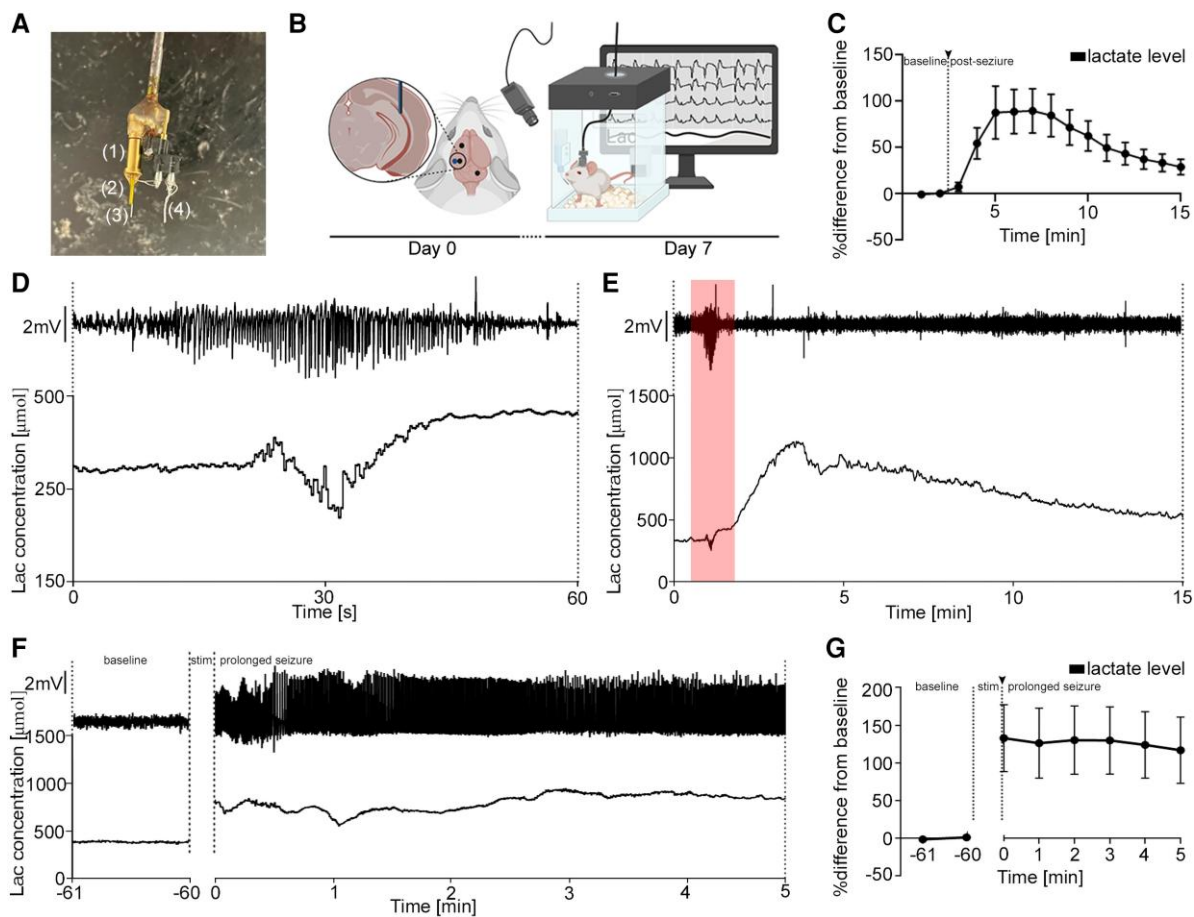


Figure 1 Seizures increase extracellular lactate concentration. (A) A custom-build recording assembly for simultaneous EEG and lactate concentration recordings in real-time during seizures. (1) a guide cannula for lactate biosensor insertion; (2) lactate biosensor; (3) hippocampal EEG recording electrode ipsilateral to the lactate biosensor; (4) hippocampal stimulating electrode contralateral to lactate biosensor. (B) Diagram of study design. Recording assembly was inserted into ventral CA1 hippocampus and a week after the surgery an electrical stimulus induced a brief seizure. A prolonged (>5 min) seizure was induced by stimulating the ventral CA1 hippocampus for an hour. Extracellular lactate concentration and EEGs were recorded simultaneously. (C) Brief seizures increased extracellular lactate concentration in the hippocampus when compared to a baseline lactate. (D) Fast fluctuations of extracellular lactate in response to a brief seizure. Top: A fragment of EEG showing an electrographic seizure, bottom: corresponding lactate concentration. Extracellular lactate concentration decreases during a seizure and quickly increases as the electrographic seizure continues. (E) A brief seizure rapidly increases extracellular concentration. Top: EEG recorded from the ipsilateral to lactate biosensor hippocampal electrode, bottom: corresponding lactate concentration. A seizure event, marked with a box from D. Following a seizure, lactate concentration increases rapidly and peaks within minutes. Elevated lactate level persists in the postictal state. (F) A prolonged seizure elevates extracellular lactate concentration. Top: EEG recorded from the ipsilateral to lactate biosensor hippocampal electrode during a prolonged seizure, bottom: corresponding lactate concentration. During continuous seizure activity, lactate concentration fluctuates but stays elevated above a baseline. High-frequency seizures are associated with decreased lactate concentration, whereas rhythmic spike-wave discharges with increased lactate concentration. (G) Prolonged seizures increase extracellular lactate concentration. Lac = lactate; stim = electrical stimulation.

seizure activity. Even a brief seizure increases extracellular lactate (Fig. 1C–E). We tested whether this was sufficient to activate HCA₁R to terminate the seizure activity or if the basal lactate concentration in the brain can activate HCA₁R to suppress seizures. In the kindling model, the repeated stimulus augments a stimulus-induced seizure duration and behavioural seizure intensity. In that case, the lack of HCA₁R should affect the kindling rate. If lactate acts as a negative feedback loop to fine-tune neuronal excitability, we hypothesized that mice that lack HCA₁R will kindle faster when compared to HCA₁R-expressing mice. Based on an adapted rapid kindling model,³⁵ we implanted HCA₁R WT and KO mice with a stimulating electrode in the left ventral CA1 hippocampus, bilateral cortical electrodes and the cerebellar reference electrode (Fig. 2A). A week after the implantations, mice were connected to a video-EEG monitoring system and the hippocampus was stimulated four times per day until they reached a fully kindled

state (three successive Grade 5 seizures). A significant fraction of HCA₁R WT failed to reach a fully kindled state (HCA₁R WT $n=7$, HCA₁R KO $n=6$, $P<0.05$, Fisher's test; Fig. 2C) and those that did, kindled more slowly than KO mice (HCA₁R WT $n=7$, HCA₁R KO $n=6$, $P<0.001$, Kaplan–Meier survival comparison followed by Mantel–Cox log-rank test; Fig. 2D).

HCA₁R KO mice are susceptible to developing more prolonged and severe seizures

To further confirm the role of HCA₁R in modulating seizure behaviour, we studied prolonged (>5 min) seizures. We induced prolonged seizures in HCA₁R WT and KO mice by CHS.³⁰ In this model, following the electrical stimulation process of the hippocampus, around 85% of mice develop self-sustaining severe seizures,³⁰ but this varies with the mouse's genetic background.

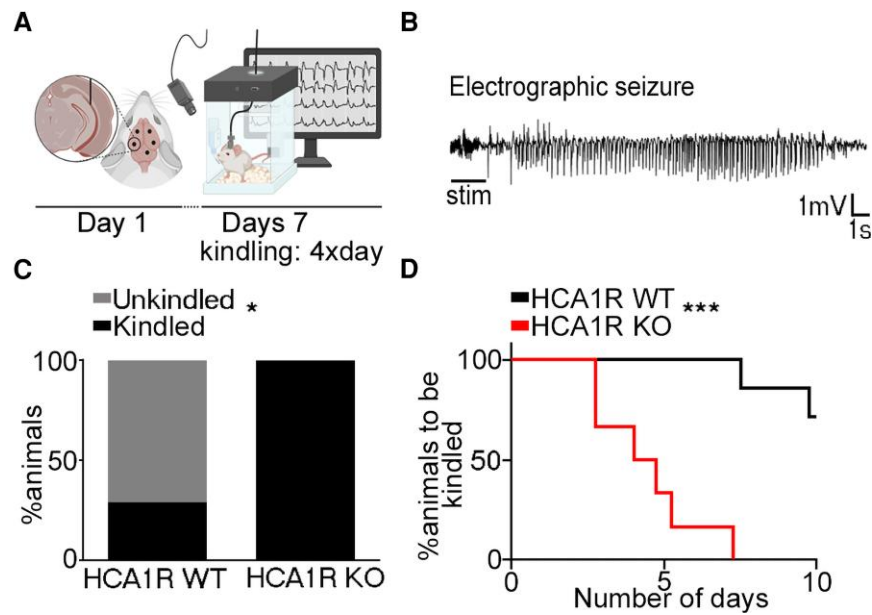


Figure 2 HCA₁R decreases susceptibility to seizures. (A) Diagram of study design. A stimulating electrode was implanted in the ventral CA1 hippocampus and recording electrodes in the cortex bilaterally. A week following the surgery, mice were kindled ('Materials and methods' section). Mice were video- and EEG-monitored. (B) A representative electrographic seizure. (C) HCA₁R KO mice are more susceptible to developing behavioural seizures. Number of HCA₁R WT mice failed to kindle fully, whereas all HCA₁R KO mice were fully kindled (HCA₁R WT $n=7$, HCA₁R KO $n=6$, $*P < 0.05$, Fisher's test). (D) HCA₁R impacts the kindling rate. HCA₁R KO mice kindled faster than WT mice (HCA₁R WT $n=7$, HCA₁R KO $n=6$, $***P < 0.001$, Kaplan–Meier survival comparison followed by Mantel–Cox log-rank test).

Surprisingly, all HCA₁R KO mice developed prolonged seizures independently of electrical stimuli, whereas a significant number of HCA₁R WT mice did not experience prolonged seizures (HCA₁R WT $n=16$, HCA₁R KO $n=9$, $P < 0.05$, Fisher's test; Fig. 3A). Moreover, raw EEG analysis revealed that HCA₁R KO developed longer seizures as opposed to WT mice that did experience self-sustaining seizures ($n=9$ each, $P < 0.05$, Kaplan–Meier survival comparison followed by Mantel–Cox log-rank test; Fig. 3B). Visual examination of raw EEGs revealed more high-power periods that were associated with low-amplitude, high-frequency spike-wave discharges in HCA₁R-deficient mice with a fewer in WT mice. Figure 3C illustrates representative power spectrograms generated from representative HCA₁R WT (top) and KO (bottom) mice. Stimulated and autonomous seizures were associated with periods of high-frequency discharges that are more apparent in HCA₁R KO than in WT mice (Fig. 3D). These high-frequency discharges are often associated with more severe behavioural seizures.³⁰ Therefore, we analysed behaviour during stimulation and self-sustaining seizure to investigate the severity of seizures in HCA₁R WT and KO mice. We randomly selected six video recordings from each genotype and blindly scored behaviour on a modified Racine scale³² (Fig. 3E). We detected more severe, convulsive behavioural seizures (Grades 4, 5 or 6) in HCA₁R KO than in WT mice ($n=6$ each, $P < 0.0001$, 2-tailed Mann–Whitney test; Fig. 3F).

HCA₁R activation decreases network excitability

Animal seizure studies suggested that seizure-generated lactate reduced network excitability by activating HCA₁R. We tested the role of HCA₁R on network excitability in the CA1 region of the hippocampus. Previous studies have demonstrated extensive neuronal activity in hippocampal–parahippocampal circuitry, including the CA1 hippocampus, during prolonged seizures.³¹ In addition, CA1

is also involved in encoding memory, which is modulated by lactate.³⁶ Therefore, CA1 principal neurons represent an ideal target population to study the effects of lactate activation of HCA₁R. GCaMP is widely used to study neuronal excitability in vivo and in vitro. We used viral-mediated delivery of GCaMP7 and CaMKII-Cre transgenes to dorsal and ventral CA1 hippocampus (Fig. 4A) to target CA1 principal neurons (Fig. 4B and C). We prepared acute hippocampal slices 10–14 days following viral injections and studied those expressing GCaMP7 (Fig. 4A).

First, we developed an *in vitro* model of heightened network activity to study the effect of HCA₁R activation on the excited population of CA1 principal neurons. Small increases in K⁺ concentration can affect hippocampal neuronal excitability.^{37,38} We increased the extracellular solution potassium concentration from 2 to 5 mM K⁺, following which we quickly detected more GCaMP7-expressing neurons with sustained (tonic) increase in bright fluorescence and induced phasic GCaMP7 signal. Phasic activity decreased and overall GCaMP7 fluorescence became dimmer following reperfusion with aCSF containing the basal K⁺ level (Fig. 4D). We detected bright, saturated fluorescence in some neurons that did not change during the experiment and assumed that those represented injured or dead neurons. We then performed a quantitative analysis in ImageJ using multimeasure functionality on a CA1 cell body layer and found that elevated K⁺ increased GCaMP7 fluorescence [Fig. 4E; $\Delta F/F$ baseline 2 mM versus $\Delta F/F$ 5 mM K⁺ aCSF: $9.16\% \pm 0.64$ (mean diff., SE of diff), $P < 0.0001$, one-way ANOVA, Bonferroni correction, $\Delta F/F$ 5 mM versus $\Delta F/F$ wash-out 2 mM K⁺ aCSF: $10.67\% \pm 0.28$ (mean diff., SE of diff), $P < 0.0001$, one-way ANOVA, Bonferroni correction, $\Delta F/F$ baseline 2 mM versus $\Delta F/F$ wash-out 2 mM K⁺ aCSF: ns, $P = 0.059$, one-way ANOVA, Bonferroni correction, $n=5$ animals, 7 slices], which returned to the baseline when the slices were perfused with aCSF containing 2 mM K⁺ (Fig. 4E).

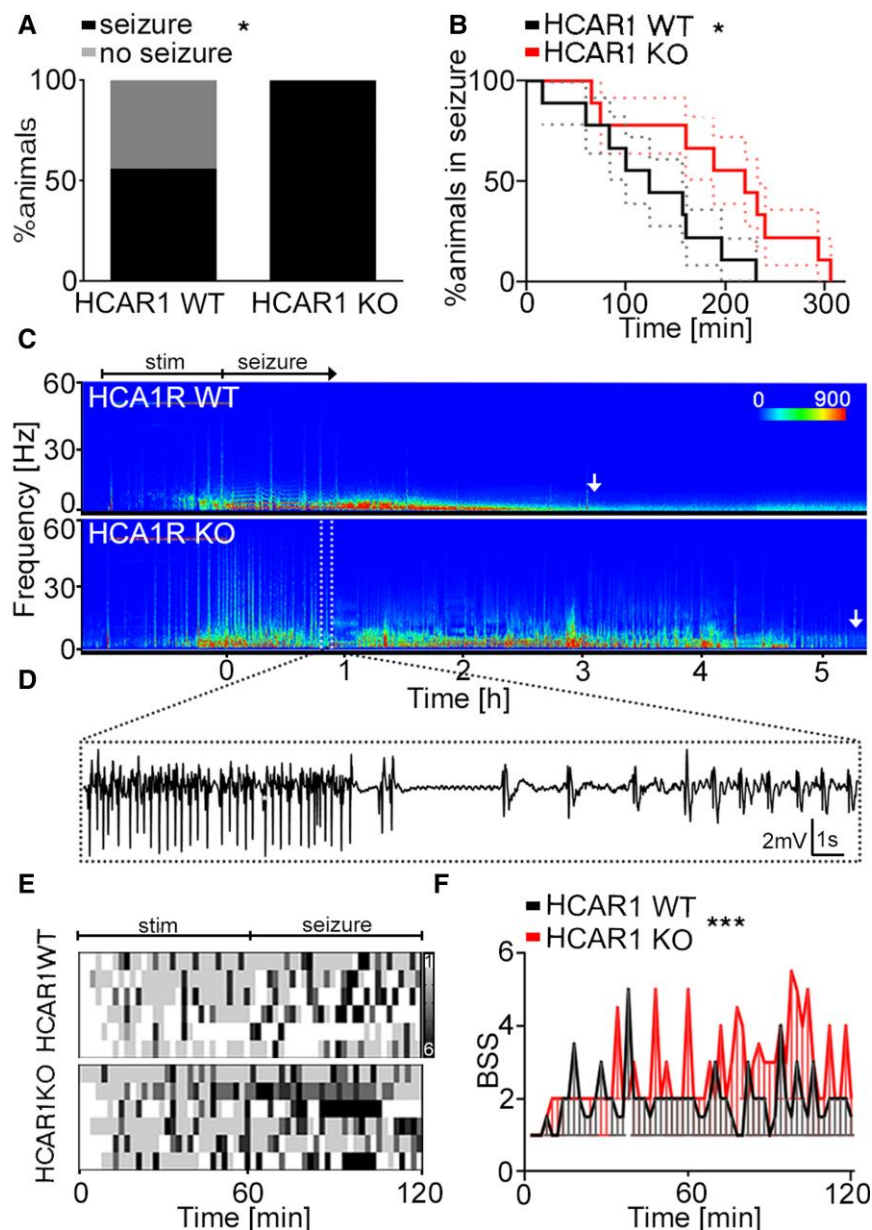


Figure 3 HCA₁R decreases susceptibility to developing more prolonged and severe seizures. (A) Susceptibility to developing self-sustaining seizure following electrical stimulation in HCA₁R KO and WT mice. Many HCA₁R WT mice did not develop autonomous seizures, whereas all HCA₁R KO experienced seizures (HCA₁R WT $n=16$, HCA₁R KO $n=9$, $*P < 0.05$, Fisher's test). (B) Seizure duration in HCA₁R WT and KO mice. HCA₁R KO mice developed significantly longer seizures when compared to HCA₁R WT mice ($n=9$ each, $*P < 0.05$, Kaplan–Meier survival comparison followed by Mantel–Cox log-rank test). (C) Spectrograms illustrating the power of EEGs recorded from the cortex during stimulation and a self-sustaining seizure from a representative HCA₁R WT (top) and KO (bottom) mouse. Arrows indicate seizure termination, note longer seizures in HCA₁R KO mouse compared to WT mouse. Note more streaks of increased power in HCA₁R KO versus HCA₁R WT. (D) An EEG trace representing high-frequency spike-wave discharge in the HCA₁R KO mouse. High-frequency discharges were more frequent in HCA₁R KO than in WT mice. These events are associated with severe behavioural seizures. (E) Heat maps represent seizure severity in six randomly selected HCA₁R WT (top) and KO (bottom) mice. Rows signify individual animals. Seizures were scored every 2 min during electrical stimulation and an hour of self-sustaining seizure on a Racine scale. (F) The median behavioural seizure score (BSS) along with 95% CI in the HCA₁R WT and KO mice illustrated in E. HCA₁R KO mice received significantly higher scores when compared to WT mice ($n=6$ each, $***P < 0.0001$, 2-tailed Mann–Whitney test), which indicates that seizures in HCA₁R KO were more severe.

We employed this paradigm to investigate the effect of HCA₁R activation on the excited population of CA1 pyramidal neurons. In this approach, high K⁺ aCSF perfusion was followed by aCSF with high K⁺ and 10 mM lactate and then returned to high K⁺ aCSF. We detected a gradual decrease in fluorescence and phasic GCaMP7 signals that persisted during lactate perfusion. Lactate wash-out with aCSF with high K⁺ resulted in an abrupt neuronal response characterized by increased in bright vibrant phasic GCaMP7 signal [$\Delta F/F$ 5 mM versus

$\Delta F/F$ 5 mM K⁺ + 10 mM lactate: 4.95 ± 0.41 (mean diff., SE of diff.), $P < 0.0001$, one-way ANOVA, Bonferroni correction, $\Delta F/F$ wash-out 5 mM versus $\Delta F/F$ 5 mM K⁺ + 10 mM lactate: 10.95 ± 0.37 (mean diff., SE of diff.), $P < 0.0001$, one-way ANOVA, Bonferroni correction, $n=4$ animals, 8 slices; Fig. 4F and G]. Figure 4G depicts this rebound firing. These findings suggested that lactate reduced neuronal network excitability.

Next, to focus on HCA₁R, we studied the effect of a non-metabolized HCA₁R agonist, 3Cl-HBA, on CA1 neuronal excitability

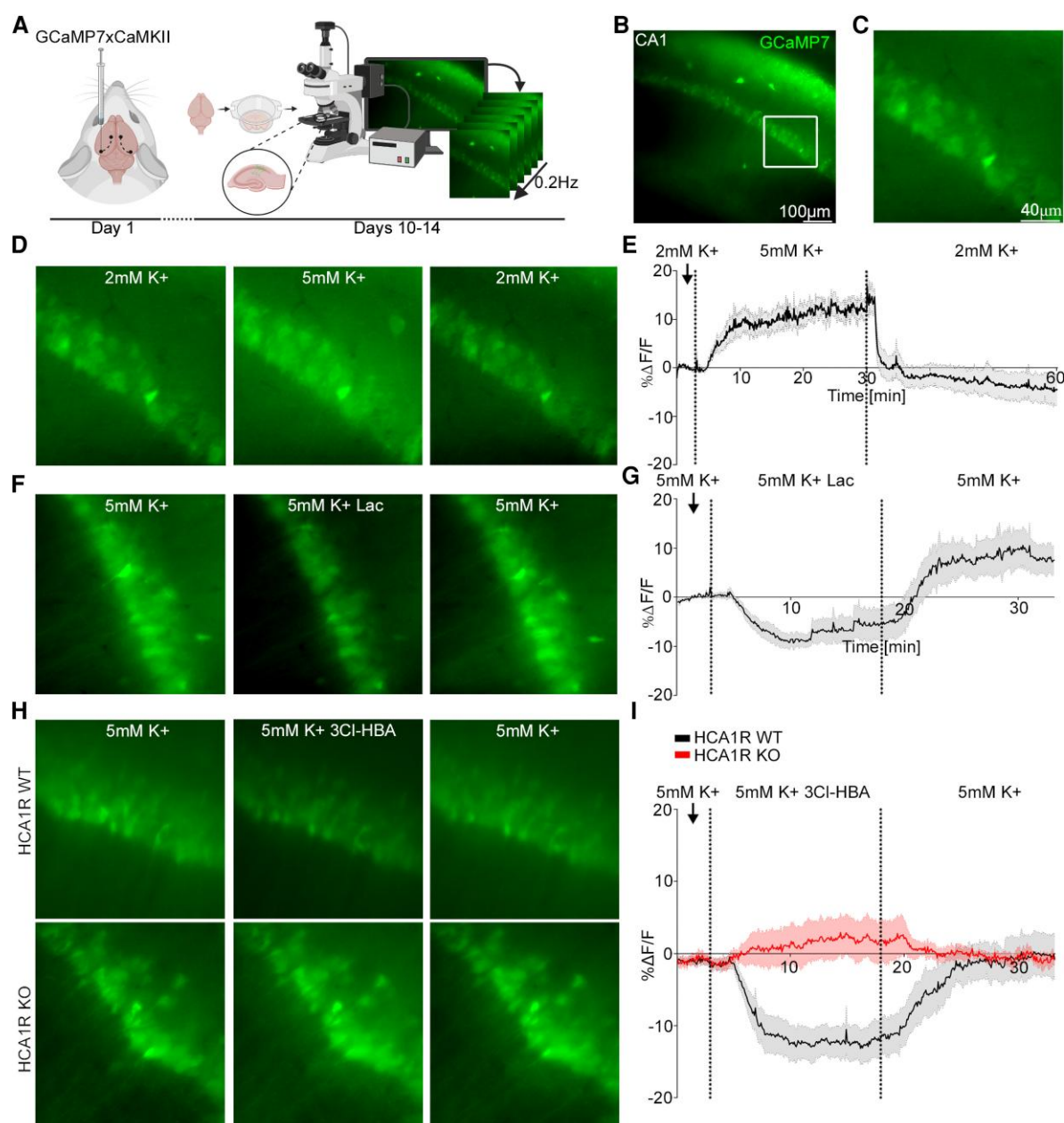


Figure 4 HCA₁R decreases CA1 neuronal network excitability. (A) Diagram of study design. A mix of GCaMP7 and CaMKII Cre was injected into ventral and dorsal CA1. Ten to fourteen days following viral injection, mice were sacrificed and acute hippocampal brain sections were prepared and studied under the wide-field scope. (B) CA1 principal neurons targeted with GCaMP7. (C) A magnified picture from B. (D) Elevated potassium concentration increases GCaMP7 signal in CA1 neuronal network. A representative image from a brain slice following perfusion with aCSF containing normal concentration of K⁺ (2 mM) (left), elevated K⁺ (5 mM) (middle) and 2 mM K⁺ ('wash-out' step) (right). (E) GCaMP7 fluorescence increases in response to 5 mM K⁺ aCSF [*n* = 5 animals, 7 slices, %ΔF/F baseline 2 mM versus ΔF/F 5 mM K⁺ aCSF: $9.16\% \pm 0.64$ (mean diff., SE of diff.), *P* < 0.0001, one-way ANOVA, Bonferroni correction, ΔF/F 5 mM versus ΔF/F wash-out 2 mM K⁺ aCSF: $10.67\% \pm 0.28$ (mean diff., SE of diff.), *P* < 0.0001, one-way ANOVA, Bonferroni correction, ΔF/F baseline 2 mM versus ΔF/F wash-out 2 mM K⁺ aCSF: ns, *P* = 0.059, one-way ANOVA, Bonferroni correction] and it returns to baseline following perfusion with 2 mM K⁺ aCSF. (F) Lactate decreases GCaMP7 fluorescence. A representative image from a brain slice following perfusion with 5 mM K⁺ aCSF (left), 5 mM K⁺ aCSF + 10 mM lactate (middle) and 5 mM K⁺ aCSF ('wash-out' step) (right). (G) GCaMP7 fluorescence decreases in response to 10 mM lactate [*n* = 4 animals, 8 slices, ΔF/F 5 mM versus ΔF/F 5 mM K⁺ + 10 mM lactate: $4.95\% \pm 0.41$ (mean diff., SE of diff.), *P* < 0.0001, one-way ANOVA, Bonferroni correction, ΔF/F wash-out 5 mM versus ΔF/F 5 mM K⁺ + 10 mM lactate: $10.95\% \pm 0.37$ (mean diff., SE of diff.), *P* < 0.0001, one-way ANOVA, Bonferroni correction] and then increases after the lactate wash-out with 5 mM aCSF. (H) Activation of HCA₁R with a specific agonist decreases GCaMP7 fluorescence in HCA₁R-dependent fashion. Representative images from a brain slice following perfusion with 5 mM K⁺ aCSF (left), 5 mM K⁺ aCSF + 100µM 3Cl-HBA (middle) and 5 mM K⁺ aCSF ('wash-out' step) (right) from HCA₁R WT (top) and KO (bottom) mice. GCaMP7 fluorescence decreases in response to 100µM 3Cl-HBA in HCA₁R WT but not in KO mice (HCA₁R WT *n* = 4 animals, 9 slices, HCA₁R KO *n* = 4 animals, 8 slices, *P* < 0.0001, *t*-test with Welch's correction).

in HCA₁R WT and KO mice. Like lactate, we found that 3Cl-HBA decreased GCaMP7 fluorescence in the HCA₁R WT mice. We detected increased GCaMP7 signal, reflected by abundant phasic activity and

more GCaMP7-expressing neurons during the wash-out, in brain slices generated from HCA₁R WT mice. We further confirmed that effect of 3Cl-HBA was HCA₁R-mediated. In slices from KO mice,

3Cl-HBA did not suppress neuronal activity (Fig. 4H and I) and there was no rebound firing effect (HCA₁R WT $n=4$ animals, 9 slices, HCA₁R KO $n=4$ animals, 8 slices, $P<0.0001$, t-test with Welch's correction).

To further understand the effect of lactate on neuronal excitability we evaluated the behaviour of individual neurons. Elevated potassium concentration in the recording solution can induce epileptiform activity, spontaneous bursts and synchronous firing of interconnected CA1 pyramidal neurons.³⁹ When neurons fire action potentials there is a time-locked increase in GCaMP7 signal that gradually returns to baseline. Therefore, the frequency and duration of phasic events provide information about the bursting activity of individual neurons and the network. We randomly selected five time-lapse movies and we selected individual GCaMP7-expressing neurons to study their behaviour following lactate perfusion (Fig. 5A). We observed a similar trend for all neurons analysed, i.e. lactate reduced fluorescence peaks and the rebounded during the wash-out step with high K⁺ (Fig. 5B). We then studied calcium spikes in individual neurons. Peak event (1) or no event (0) were then graphically represented on raster plots to examine a frame-by-frame calcium dynamics of individual neurons in response to high K⁺, high K⁺ + 10 mM lactate and lactate wash-out with high K⁺ (Fig. 5B and C). Figure 5B depicts a representative raster plot (top) and fluorescent traces (bottom) of four individual neurons (neuron 1 to neuron 4). During perfusion with high K⁺, we detected synchronous and non-synchronous phasic firing of neurons (marked with dotted lines). Interestingly, the synchronous, phasic neuronal firing was reduced by lactate perfusion. Moreover, we observed significantly fewer calcium spike events when lactate was present in the recording solution (Fig. 5D, left, $n=20$ neurons, $P<0.05$, Kolmogorov–Smirnov test). During lactate wash-out, phasic and synchronous activity resumed and became more apparent and frequent (Fig. 5D, right, $n=20$ neurons, $P<0.0001$, Kolmogorov–Smirnov test).

HCA₁R activation reduces neuronal excitability

Previous experiments showed that lactate reduces network excitability via HCA₁R. Next, we investigated the cellular basis of this effect using the whole-cell patch-clamp technique to study single CA1 pyramidal neurons. First, we investigated the passive and active membrane properties of CA1 pyramidal neurons following HCA₁R activation with lactate in current-clamp conditions. We recorded resting membrane potentials (RMPs) from CA1 pyramidal cells soon after break in under control aCSF and then after perfusion with 3 mM lactate in aCSF (Fig. 6). Lactate hyperpolarized the resting membrane potential (Fig. 6A). This effect was observed consistently in $n=9$ neurons (Fig. 6B; $n=9$, $P<0.05$, paired t-test). In addition to lactate, we tested a HCA₁R-specific agonist, 3Cl-HBA. Similarly, we found 3Cl-HBA hyperpolarized RMP in CA1 neurons (Fig. 6C; $n=12$ cells, $P<0.05$, paired t-test). Moreover, we detected that 3Cl-HBA perfusion increased the action potential threshold (Fig. 6D; $n=8$, $P<0.01$, paired t-test), whereas we did not observe changes in action potential threshold following lactate perfusion. We did not observe changes in action potential frequency after treatment with either lactate or 3Cl-HBA.

Activation of HCA₁R reduces sEPSC frequency

We recorded sEPSC to understand the lactate effect on excitatory transmission (Fig. 7A). HCA₁R is a G-protein coupled receptor (GPCR). Lactate binding to this receptor initiates an effector pathway downstream of the GPCR. This response peaks within minutes,

depending on the GPCR.⁴⁰ Upon lactate application, sEPSC frequency diminished over 10 min, then stabilized (Fig. 7B). We found that activation of HCA₁R with lactate significantly reduced the sEPSC frequency demonstrated by a rightward shift in the inter-event interval cumulative fraction probability distribution (Fig. 7C; $n=5$ cells, $P<0.0001$, Kolmogorov–Smirnov test). We also detected change in the sEPSC amplitude (Supplementary Fig. 3A; $n=5$ cells, $P<0.0001$, Kolmogorov–Smirnov test). In addition to lactate, we tested a HCA₁R-specific agonist, 3Cl-HBA. Similarly, we found 3Cl-HBA reduced sEPSC frequency in CA1 neurons (Fig. 7D; $n=10$ cells, $P<0.0001$, Kolmogorov–Smirnov test). Moreover, we detected small, although significant change in sEPSC amplitude following 3Cl-HBA application (Supplementary Fig. 3B; $n=10$ cells, $P<0.05$, Kolmogorov–Smirnov test).

We then investigated whether the lactate effect is HCA₁R-mediated. We recorded sEPSCs in HCA₁R WT and KO mice before and after lactate perfusion. Lactate perfusion reduced sEPSC frequency in CA1 neurons from HCA₁R WT but not in KO mice (Fig. 7E; HCA₁R WT $n=6$ cells, $P<0.0001$, Kolmogorov–Smirnov test; HCA₁R KO $n=7$ cells, $P=0.242$, Kolmogorov–Smirnov test). We did not detect changes in sEPSC amplitude in either HCA₁R WT or KO mice (Supplementary Fig. 3C; HCA₁R WT $n=6$ cells, $P=0.847$, Kolmogorov–Smirnov test; HCA₁R KO $n=7$ cells, $P=0.975$, Kolmogorov–Smirnov test). We then tested the effect of HCA₁R activation on GABAergic neurotransmission by recording sIPSC before and after the perfusion of lactate. The sIPSC frequency and amplitude did not change following HCA₁R activation with lactate (Supplementary Fig. 4; $n=8$ cells, $P=0.3221$, Kolmogorov–Smirnov test; $n=8$ cells, $P=0.149$, Kolmogorov–Smirnov test).

The altered frequency of sEPSC could be an effect on presynaptic glutamatergic neurotransmission and action potential generation in the presynaptic neuron. We measured paired-pulse responses of eEPSC in CA1 principal neurons at different intervals before and after lactate perfusion (Fig. 8A). We then calculated the ratio of the second eEPSC to the first eEPSC amplitudes (Fig. 8B) and found that lactate treatment altered the paired-pulse ratio compared to baseline (aCSF) in HCA₁R WT but not in KO mice (Fig. 8C; time constant τ for HCA₁R WT: control 47.76 ms versus lactate treatment 31.2 ms and for HCA₁R KO: control 29.37 ms versus lactate treatment 33.3 ms; HCA₁R WT: $P<0.0001$, unpaired t-test; HCA₁R KO: $P=0.098$, unpaired t-test). We found that paired-pulse ratio at 20 ms was increased in HCA₁R WT mice but not in KO mice (HCA₁R WT $n=5$ pairs, $P<0.01$, paired t-test; HCA₁R KO $n=5$, $P=0.94$, paired t-test). The remaining intervals were no different in either HCA₁R WT or KO mice. These findings are identical to those reported by others on cultured cortical neurons²⁰ and granule cells²¹ in a different HCA₁R KO mouse.

Discussion

Here we demonstrate for the first time the rapid action of metabolically generated lactate to reduce neuronal excitability through HCA₁R *in vivo* and *in vitro*. We propose that excessive neuronal activity accelerates glycolysis to generate lactate, which translocates to the extracellular space to slow neuronal firing in a HCA₁R-dependent fashion. This is the first study demonstrating the fast, acute action of HCA₁R-dependent lactate function in the brain in response to metabolic stress.

Metabolic intermediates can alter neuronal firing properties by directly activating ligand-gated ion channels, such as the K_{ATP} channel or G-protein coupled purinergic receptors. ATP and

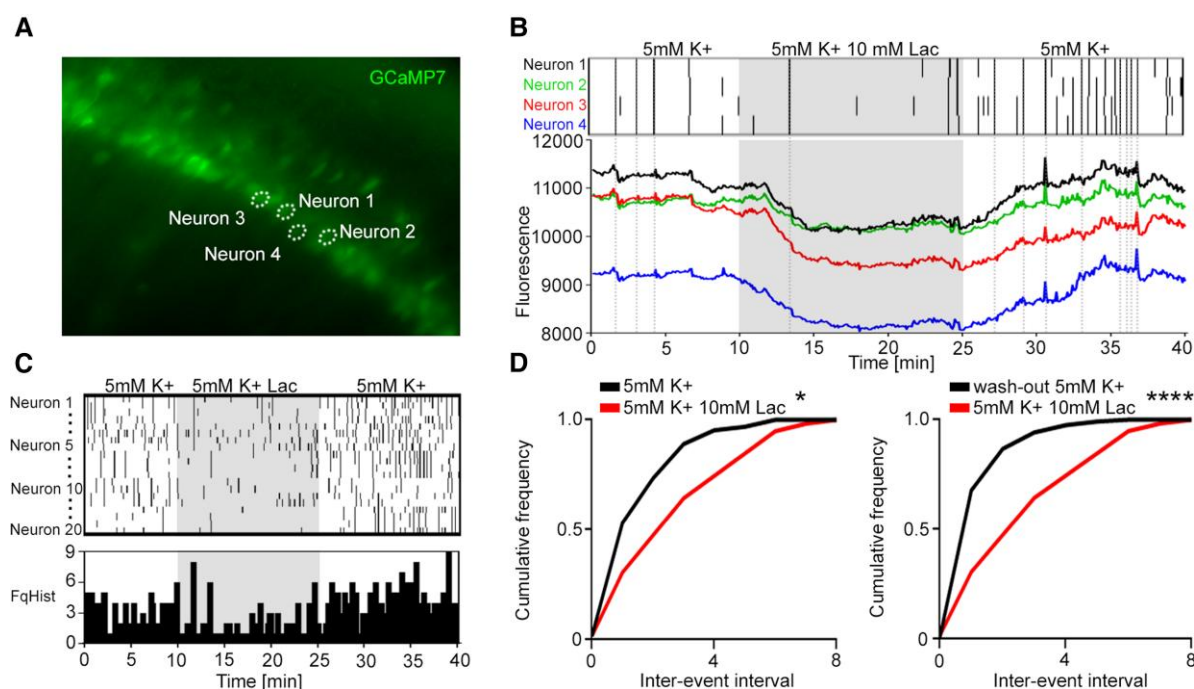


Figure 5 Lactate decreases calcium peaks in individual neurons and decreases synchronous neuronal activity. (A) GCaMP7-expressing CA1 pyramidal neurons. Individual neurons were selected randomly to study the phasic calcium events. (B) Lactate suppressed phasic GCaMP7 fluorescence in CA1 pyramidal neurons. Representative raster plot (top) and fluorescent traces (bottom) of four individual neurons (Neuron 1 to Neuron 4) from A. High K⁺-induced synchronous and non-synchronous phasic activity. (C) Lactate decreased calcium peaks in CA1 principal neurons. Raster plot (top) and frequency histogram (bottom) of the calcium peaks recorded from 20 individual neurons in response to 5 mM K⁺ aCSF, 5 mM K⁺ aCSF + 10 mM lactate and wash-out with 5 mM K⁺ aCSF. Note reduced frequency of calcium peaks during perfusion with lactate and rebound during wash-out with 5 mM K⁺ aCSF. (D) A cumulative frequency distribution demonstrating significant decrease in calcium events frequency during lactate perfusion. Left: Baseline 5 mM K⁺ aCSF versus lactate: $n = 20$ neurons, $*P < 0.05$, Kolmogorov–Smirnov test. Right: Wash-out 5 mM K⁺ aCSF versus lactate: $n = 20$ neurons, $****P < 0.0001$, Kolmogorov–Smirnov test).

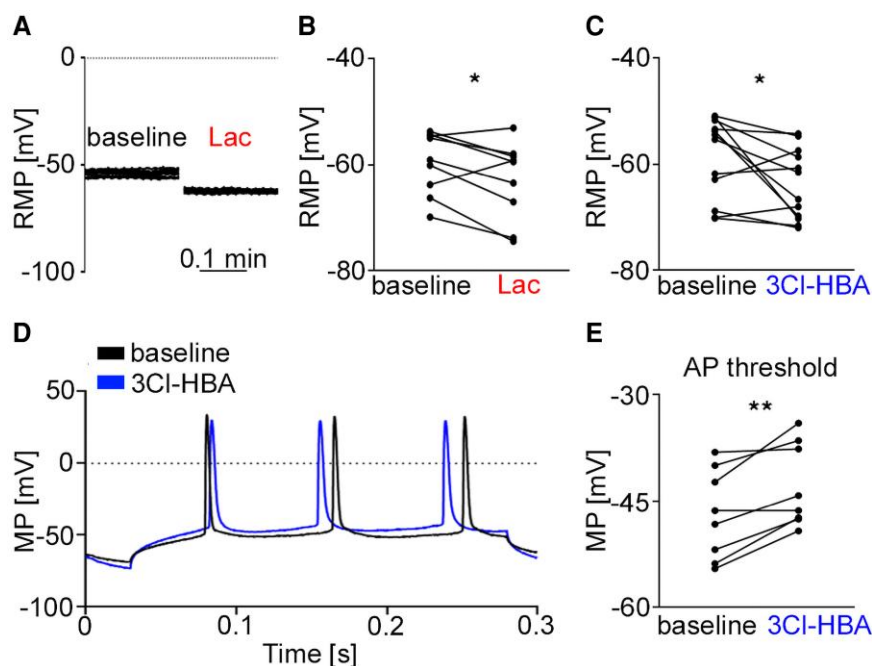


Figure 6 HCA₁R activation reduces neuronal excitability. (A) Trace recorded from CA1 excitatory neuron before (baseline) and during 10 min perfusion with 3 mM lactate. (B) Lactate hyperpolarized membrane potential of CA1 principal neurons; 3 mM lactate perfusion decreased the resting membrane potential of CA1 excitatory neurons ($n = 9$ cells, $*P < 0.05$, paired t-test). (C) Activation of HCA₁R hyperpolarized membrane potential of CA1 principal neurons. Application of a specific HCA₁R agonist, 3CI-HBA, decreased the resting membrane potential of excitatory neurons ($n = 12$ cells, $*P < 0.05$, paired t-test). (D) Traces illustrating action potentials evoked for a CA1 principal neuron before and after application of 3CI-HBA. The current injected for both traces was 60 pA. (E) Action potential threshold for CA1 neurons before and after treatment with 3CI-HBA ($n = 8$ cells each, $**P < 0.01$, paired t-test). Lac = lactate.

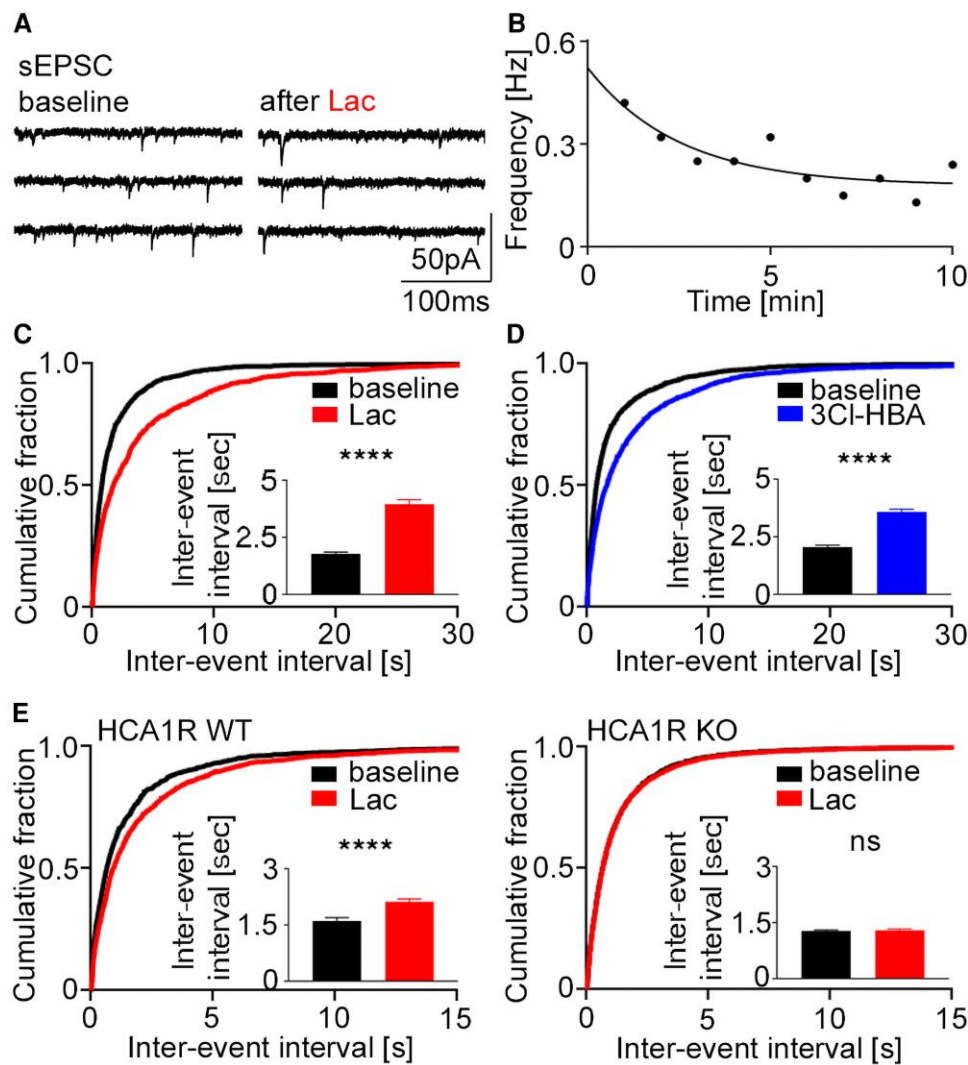


Figure 7 Activation of HCA₁R reduces spontaneous excitatory postsynaptic current frequency. (A) A representative sEPSC recorded from CA1 principal neurons from HCA₁R WT before (left) and 10 min after (right) lactate perfusion. (B) Kinetic of lactate-mediated reduction in sEPSC frequency fitted in one phase decay model. (C) Application of lactate ($n = 5$ cells, **** $P < 0.0001$, Kolmogorov–Smirnov test) reduced frequency of sEPSCs recorded in CA1 pyramidal neurons. (D) Specific HCA₁R agonist, 3Cl-HBA, reduced sEPSC frequency recorded in CA1 pyramidal neurons ($n = 10$ cells, **** $P < 0.0001$, Kolmogorov–Smirnov test). (E) Lactate reduces frequency of sEPSCs in HCA₁R-dependent fashion. Lactate treatment reduced frequency of sEPSCs recorded in CA1 pyramidal neurons from HCA₁R WT ($n = 6$ cells, **** $P < 0.0001$, Kolmogorov–Smirnov test) but not in HCA₁R KO ($n = 7$ cells, $P = 0.242$, Kolmogorov–Smirnov test) mice.

adenosine are classical modulators of neuronal excitability. They are synthesized locally in the neurons and glia and are found in the extra- and intracellular space. Adenosine elicits modulatory function by activation of adenosine receptors (A_1 , A_{2A} , A_{2B} and A_3). A_1 receptor prominent function involves inhibition of excitatory glutamatergic system,⁴¹ and adenosine-dependent activation of A_1 was found to decrease the frequency of miniature excitatory synaptic currents.⁴² We found that activation of HCA₁R by lactate inhibits the release of glutamate in CA1 pyramidal cells on hippocampal slices. This was also demonstrated for cultured cortical neurons²⁰ and granule cells on hippocampal slices.²¹ Our studies indicate that HCA₁R activation did not affect GABA-ergic neurotransmission in CA1 principal neurons.

Inhibition of glutamatergic neurotransmission may limit excitotoxicity. Glutamatergic neurotoxicity associated with excessive neuronal firing can cause neuronal damage and degradation.⁴³ Neuronal vulnerability to excitotoxicity is not uniform; CA1

neurons that are especially rich in NMDA receptors⁴⁴ are particularly susceptible. Lactate generated during metabolic stress can act as a negative feedback loop to fine-tune the excitability of CA1 principal neurons by decreasing the glutamate pool, which may be neuroprotective by reducing energy failure and glutamate neurotoxicity. In fact, lactate can act as a neuroprotective agent in several neurological conditions associated with excitotoxicity. In the *in vitro* model of oxygen and glucose deprivation, lactate⁴⁵ and HCA₁R agonist⁴⁶ administration attenuated neuronal death. Lactate was also shown to exert a neuroprotective effect in the *in vivo* model of neonatal stroke.^{47,48} Our findings explain the neuroprotective action of lactate documented in these studies.

In addition to the purinergic system, ATP couples the cellular metabolic status to resting membrane potential via ATP-sensitive K^+ channels (K_{ATP} channels).^{49,50} In the time of elevated intracellular ATP/ADP ratio, K_{ATP} channels close and depolarize the neuronal membrane. Conversely, when ATP/ADP ratio decreases, K_{ATP}

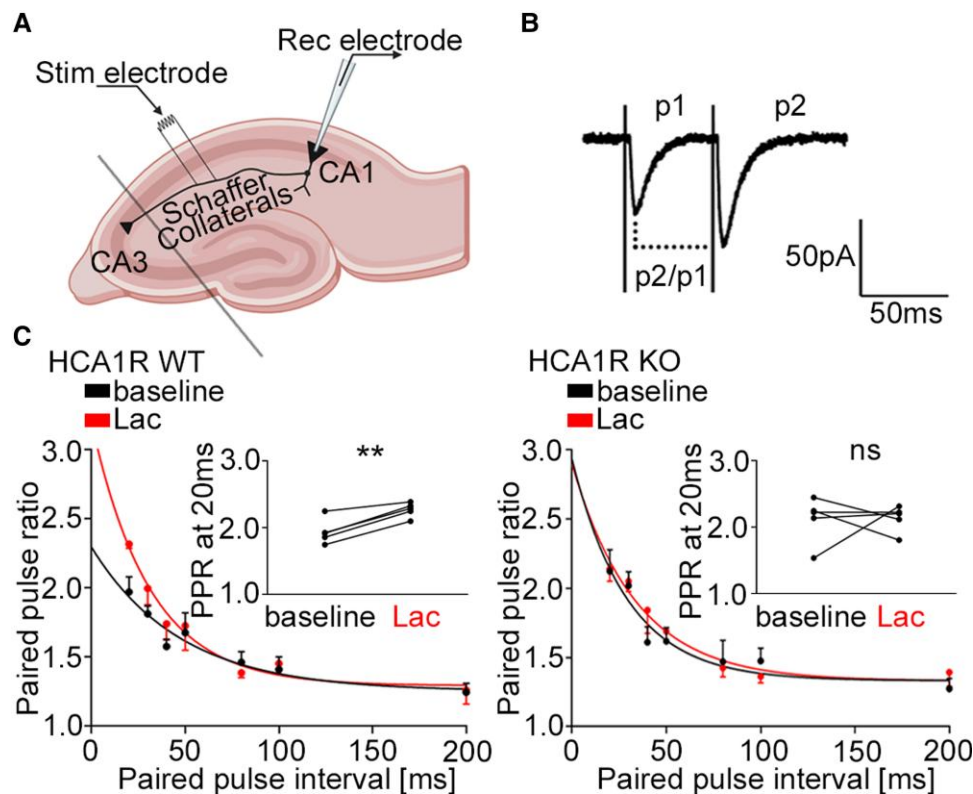


Figure 8 HCA₁R activation alters Schaffer collateral-CA1 paired-pulse ratio. (A) Evoked EPSCs were recorded in CA1 principal neurons in response to stimulation of Schaffer collateral axons. (B) Representative eEPSCs in CA1 in response to Schaffer collateral paired-pulse stimuli at a 50 ms interstimulus interval recorded from HCA₁R WT mouse. Paired pulse ratio (PPR) was calculated as the ratio of the second (p2) eEPSC to the first (p1) eEPSC (p2/p1). (C) Lactate alters paired-pulse ratio in HCA₁R WT but not in KO mice. The decay of facilitation was fit in one phase decay model with time constant τ for HCA₁R WT: control 47.43 ms versus lactate treatment 31.2 ms and for HCA₁R KO: control 29.37 ms versus lactate treatment 33.8 ms, which are statistically different in HCA₁R WT ($P < 0.0001$, unpaired *t*-test) but not in KO mice ($P = 0.098$, unpaired *t*-test). PPR at 20 ms was increased in HCA₁R WT mice but not in KO mice (HCA₁R WT $n = 5$ pairs, $P < 0.01$, paired *t*-test; HCA₁R KO $n = 5$, $P = 0.94$, paired *t*-test).

channels open, leading to hyperpolarization. Glycolytic enzymes are associated with cell membrane proteins⁵¹; therefore, glycolysis-generated ATP molecules may reside near the cell membrane and constitute a portion of ATP sensed by K_{ATP} channels. Thus, local energy production from glycolysis in the form of intracellular ATP links to neuronal excitability via K_{ATP} channels.⁵² K_{ATP} channels are expressed predominantly in GABAergic interneurons,^{53,54} whereas HCA₁R-dependent lactate action is selective to excitatory neurons, at least in CA1. Thus depolarization through the closure of K_{ATP} channels in interneurons would enhance the inhibition of principal neurons, at the same time lactate-HCA₁R mediated hyperpolarization and reduced excitatory transmission would inhibit the principal neurons. Therefore, accelerated glycolysis would limit the firing of principal neurons via HCA₁R and enhance firing of inhibitory interneurons.

Neuronal excitability and brain metabolism are also linked through synaptic transmission^{55–57} and the potential membrane maintenance through ion transporters⁵⁸ are metabolically expensive. ATP, the main cellular energy currency, is synthesized in the brain to supply energy in a state-dependent manner. Failure to meet metabolic demand can alter synaptic transmission and ionic balance, leading to altered neuronal excitability.

Finally, lactate acts as a pleiotropic molecule; therefore, it contributes to neuronal excitability through multiple mechanisms. For example, lactic acid acidifies the extracellular space⁵⁹ and acid-sensing channels⁶⁰ and overall decreases neuronal excitability. In

some studies, however, lactate appears to increase the activity of neurons. Astrocyte-derived lactate was shown to activate orexin neurons in the lateral hypothalamic area⁶¹ and neurons in locus coeruleus.⁶² Similar to other GPCRs, HCA₁R may couple with more than one G protein and produce a diverse response in distinct brain areas. Alternatively, the excitatory effect of lactate in some brain areas may depend on the unidentified lactate receptor. Activation of locus coeruleus neurons with D-lactate, a weak HCA₁R agonist, acted as an antagonist, and the downstream molecular modulation involved the G_s signalling pathway rather than G_i .⁶² Moreover, expression of HCA₁R in locus coeruleus neurons was not detected by RNA sequencing.⁶³

Seizures rapidly accelerate glycolysis to generate sufficient extracellular lactate. While oxidative phosphorylation provides more ATP per glucose molecule than glycolysis, 2 ATP molecules generated by the glycolytic pathway alone are faster accessible.⁶⁴ Thus, to quickly regenerate the pool of ATP molecules consumed by the seizures, the glycolytic rate increases and lactate is being produced. Therefore, seizure-born lactate can activate HCA₁R to modulate neuronal excitability. In previously reported literature, lactate concentration measurements during seizures lack spatial-temporal resolution and do not reflect the extracellular lactate concentration, i.e. the portion of HCA₁R-activating lactate molecules. Moreover, ligand and binding assays performed by several laboratories reported that HCA₁R has a relatively low affinity for lactate (EC₅₀ of 1.2 mM⁶⁵ to 5

mM¹⁷) when compared to other GPCRs for their ligands (low to high micromolar range). Given the potentially high EC50 values for HCA₁R, we first intended to investigate real-time fluctuations in extracellular lactate concentration during brief (<60 s and prolonged (>5 min) seizures with simultaneous video-EEG monitoring. We detected rapid changes in extracellular lactate concentration during seizures, which is likely to cause HCA₁R activation.

In two animal models, we found that HCA₁R KO mice are more susceptible to developing severe behavioural epileptic seizures and more prolonged electrographic seizures. Activation of HCA₁R decreases network excitability of CA1 principal excitatory neurons. HCA₁R alters neuronal excitability by hyperpolarizing the resting membrane potential of excitatory CA1 principal and reducing glutamatergic neurotransmission at synapses.

The ketogenic diet (KD)—the high-fat, low-carbohydrate diet—and other dietary modifications represent promising remedies for patients suffering from various neurological diseases, including epilepsy,⁶⁶ relapsing–remitting multiple sclerosis⁶⁷ and Alzheimer's disease.⁶⁸ Interestingly, studies in human subjects show that during ketosis, a hallmark of KD, CSF lactate levels rises.⁶⁹ The gluconeogenesis intermediate oxaloacetate can generate lactate. During gluconeogenesis, oxaloacetate converts to phosphoenolpyruvate in the enzymatic reaction catalysed by phosphoenolpyruvate carboxykinase, followed by an enolase-catalysed bidirectional response to 2-phosphoglycerate. Alternatively, oxaloacetate-derived phosphoenolpyruvate can be converted to pyruvate by pyruvate kinase. Lactate-mediated HCA₁R activation may be at least partially responsible for the beneficial effect of KD and KD could protect from seizures by increased activation of HCA₁R.

Excessive neuronal activity accelerates glycolysis to generate lactate, which translocates to the extracellular space to slow neuronal firing and excitatory transmission via HCA₁R. Lactate mediates metabolic feedback inhibition of excitatory neurons through HAC₁R.

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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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