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Bidirectional dysregulation of synaptic glutamate signaling after transient metabolic failure

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Abstract

Ischemia leads to a severe dysregulation of glutamate homeostasis and excitotoxic cell damage in the brain. Shorter episodes of energy depletion, for instance during peri-infarct depolarizations, can also acutely perturb glutamate signaling. It is less clear if such episodes of metabolic failure also have persistent effects on glutamate signaling and how the relevant mechanisms such as glutamate release and uptake are differentially affected. We modelled acute and transient metabolic failure by using a chemical ischemia protocol and analyzed its effect on glutamatergic synaptic transmission and extracellular glutamate signals by electrophysiology and multiphoton imaging, respectively, in the hippocampus. Our experiments uncover a duration-dependent bidirectional dysregulation of glutamate signaling. Whereas short chemical ischemia induces a lasting potentiation of presynaptic glutamate release and synaptic transmission, longer episodes result in a persistent postsynaptic failure of synaptic transmission. We also observed unexpected differences in the vulnerability of the investigated cellular mechanisms. Axonal action potential firing and glutamate uptake were unexpectedly resilient compared to postsynaptic cells, which overall were most vulnerable to acute and transient metabolic stress. We conclude that even short perturbations of energy supply lead to a lasting potentiation of synaptic glutamate release, which may increase glutamate excitotoxicity well beyond the metabolic incident.

eLife assessment

The authors show that short bouts of chemical ischemia lead to presynaptic changes in glutamate release and long-term potentiation, whereas longer bouts of chemical ischemia lead to synaptic failure and presumably cell death. This **convincing** work relies on rigorous electrophysiology/imaging experiments and data analysis. It is **important** as it provides new mechanistic details on chemical ischemia, which could offer potential insights into ischemic stroke in vivo.

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Introduction

It is well established that the breakdown of ATP production in the brain in an ischemic stroke, profoundly affects glutamate signaling on various levels. The inability of neurons and other cell types such as astrocytes to maintain transmembrane ionic gradients and their membrane potential leads to increased glutamate release, decreased glutamate clearance, and, thereby, increased glutamate receptor activation and excitotoxicity, which ultimately results in cell death (Dirnagl et al., 1999). The ischemic core is also the source of peri-infarct depolarizations that travel into the primarily undamaged tissue. They are a relatively short-lasting sub-type of spreading depolarizations associated with a significant metabolic burden that can lead to the progressive expansion of tissue damage in the peri-infarct zone (Lauritzen et al., 2011). In animal models of ischemic stroke, they can be detected as transient glutamate increases lasting about a minute using for instance microdialysis and fluorescence imaging (Lauritzen et al., 2011; Rakers and Petzold, 2017). In addition to neuronal glutamate release, glial glutamate release and a transient reduction of glutamate uptake could contribute to such transient glutamate increases (Lauritzen et al., 2011; Rakers and Petzold, 2017; Passlick et al., 2021). Whether peri-infarct depolarizations, and spreading depolarizations in general, acutely trigger lasting changes of glutamate signaling and homeostasis is far less clear.

Spreading depression is accompanied by a transient decrease of ATP levels (Mies and Paschen, 1984). This decrease can be reproduced in vitro by creating anoxic conditions, removal of substrates for ATP production such as glucose, inhibition of metabolic pathways, or combinations of these manipulations. In vitro studies using such approaches to induce a transient metabolic failure have demonstrated for instance that brief periods of anoxia and aglycemia lead to a persistent potentiation of synaptic transmission at CA3-CA1 synapses in the hippocampus, also called ischemic or anoxic long-term potentiation (LTP) (Crepel et al., 1993, 2003; Hsu and Huang, 1997; Stein et al., 2015). This indicates that a transient metabolic failure can persistently alter glutamatergic synaptic transmission. Whether this also applies to glutamate release and glutamate uptake is currently not known but potentially important, because that may increase the likelihood of increased excitability and excitotoxicity in between individual peri-infarct depolarizations.

For these reasons, we explored if and how short and transient periods of metabolic failure affect glutamate release and uptake in parallel to synaptic transmission using combinations of electrophysiology and glutamate imaging in the hippocampus together with a chemical ischemia protocol. We observed a duration-dependent bidirectional dysregulation of glutamate release and synaptic transmission, in which short durations of chemical ischemia led to a persistent potentiation of glutamate release while long durations induced postsynaptic failure. We further uncovered unexpected differences of vulnerability of mechanisms involved in glutamatergic synaptic transmission, including a marked resilience of glutamate uptake to chemical ischemia.

Results

Bidirectional dysregulation of glutamate signaling

For revealing the effect of acute and transient metabolic failure on glutamatergic synaptic transmission, we combined electrophysiological monitoring of glutamatergic synaptic transmission at CA3-CA1 Schaffer collateral synapses in acute hippocampal slices with a chemical ischemia model. Baseline synaptic transmission was monitored for >10 minutes before ATP production was disrupted by switching to a modified extracellular solution lacking glucose and

containing sodium azide and 2-deoxyglucose to inhibit oxidative phosphorylation and glycolysis, respectively (schematic in **Fig. 1A**). This protocol for 'chemical ischemia' has previously been shown to reliably induce a rapid and reversible decline of the intracellular ATP levels (Pape and Rose, 2023). We first tested the effect of acute and transient chemical ischemia lasting for 2 to 5 minutes and observed two qualitatively distinct results. A comparison of recordings with 2 and 5 minutes illustrates both scenarios (**Fig. 1B-F**). Although the postsynaptic response transiently disappeared for both durations, they remained suppressed over the next 50 minutes of recording for 5 min of chemical ischemia but recovered when chemical ischemia lasted only 2 minutes, in which case it showed a significant potentiation at the end of the recording (**Fig. 1B, C, E**). In contrast, the axonal fiber volley, which reflects the axonal firing of action potentials after stimulation, was transiently suppressed and recovered fully for both durations (**Fig. 1D**). Analyzing the entire data set including chemical stress durations of 3 and 4 minutes confirmed that the axonal fiber volley robustly recovered from chemical ischemia for the tested duration (**Fig. 1G**). In contrast, the postsynaptic response was either potentiated for shorter durations or almost completely suppressed for longer durations (**Fig. 1H**). For chemical ischemia with near complete suppression of the postsynaptic response (**Fig. 1H**, orange data points), the average duration of chemical stress was 4.46 ± 0.207 minutes (n = 11) and 2.74 ± 0.20 minutes (n = 19) for all other recordings. Because the cellular depolarization and ensuing increased neuronal synaptic glutamate release should lead to an extracellular potassium increase, we measured extracellular $[K^+]$ in parallel in a subset of experiments (example in **Fig. 1F**). Correlating the maximum increase of the extracellular $[K^+]$ during chemical ischemia revealed that a stronger extracellular $[K^+]$ increase (i.e., a severe metabolic failure) made postsynaptic failure more likely (**Fig. 1I**). Together, these experiments reveal two distinct outcomes of acute chemical ischemia: Either potentiation of the synaptic response or its near-complete suppression, which represents a postsynaptic failure of synaptic transmission as revealed by the following glutamate imaging experiments.

In a subset of the recordings shown in **Fig. 1H** (filled data points), we have combined electrophysiological recordings with monitoring of glutamate release using the fluorescent indicator iGluSnFR (Marvin et al., 2013) (**Fig. 2**). In this subset, iGluSnFR was virally expressed in astrocytes and its fluorescence changes were monitored in response to paired stimuli of CA3-CA1 synapses by line-scanning throughout the experiment as indicated in **Fig. 2A-B**. Additional control experiments confirmed the stability of such combined recordings (**Fig. 2C, F**). We grouped recordings combining iGluSnFR imaging and electrophysiology according to the effect of chemical ischemia on the synaptic response: 'chemical ischemia with postsynaptic failure' (**Fig. 1H**, filled orange data points) if the postsynaptic response did not recover to above 50% of the baseline level and 'chemical ischemia' when it did (**Fig. 1H**, filled dark grey data points). Similar to electrophysiological recordings, two response types could be identified in iGluSnFR recordings. When acute and transient chemical ischemia led to a persistent potentiation of synaptic transmission, this was associated with an increase of the iGluSnFR responses ($\Delta F/F_0$) (**Fig. 2D**). In contrast, if chemical ischemia led to postsynaptic failure, we observed a transient surge of the resting fluorescence of iGluSnFR (F_0) during acute chemical ischemia and reduced and gradually recovering iGluSnFR transients in response to synaptic stimulation after chemical ischemia (**Fig. 2E**). Presented are six out of the nine experiments with postsynaptic failure that were performed. Three recordings were not further analyzed because the iGluSnFR fluorescence became undetectable after chemical ischemia. A more comprehensive statistical analysis of the responses before and after chemical ischemia can be found in **Fig. S1** and **Fig. 2F, G, H**. In a single experiment, we have been able to record the response to two subsequent periods of short chemical ischemia in a > 3-hour recording (**Fig. S2**), which highlights the persistence of the potentiation of both the postsynaptic responses and glutamate transients. In addition to the bidirectional changes of synaptic transmission and glutamate release depending on the severity of chemical ischemia, we also found a consistent decrease of the iGluSnFR resting fluorescence over the duration of the experiments in all conditions. In general, this could be caused by a gradual reduction of the resting extracellular glutamate levels and/or a reduction of fluorescent iGluSnFR,

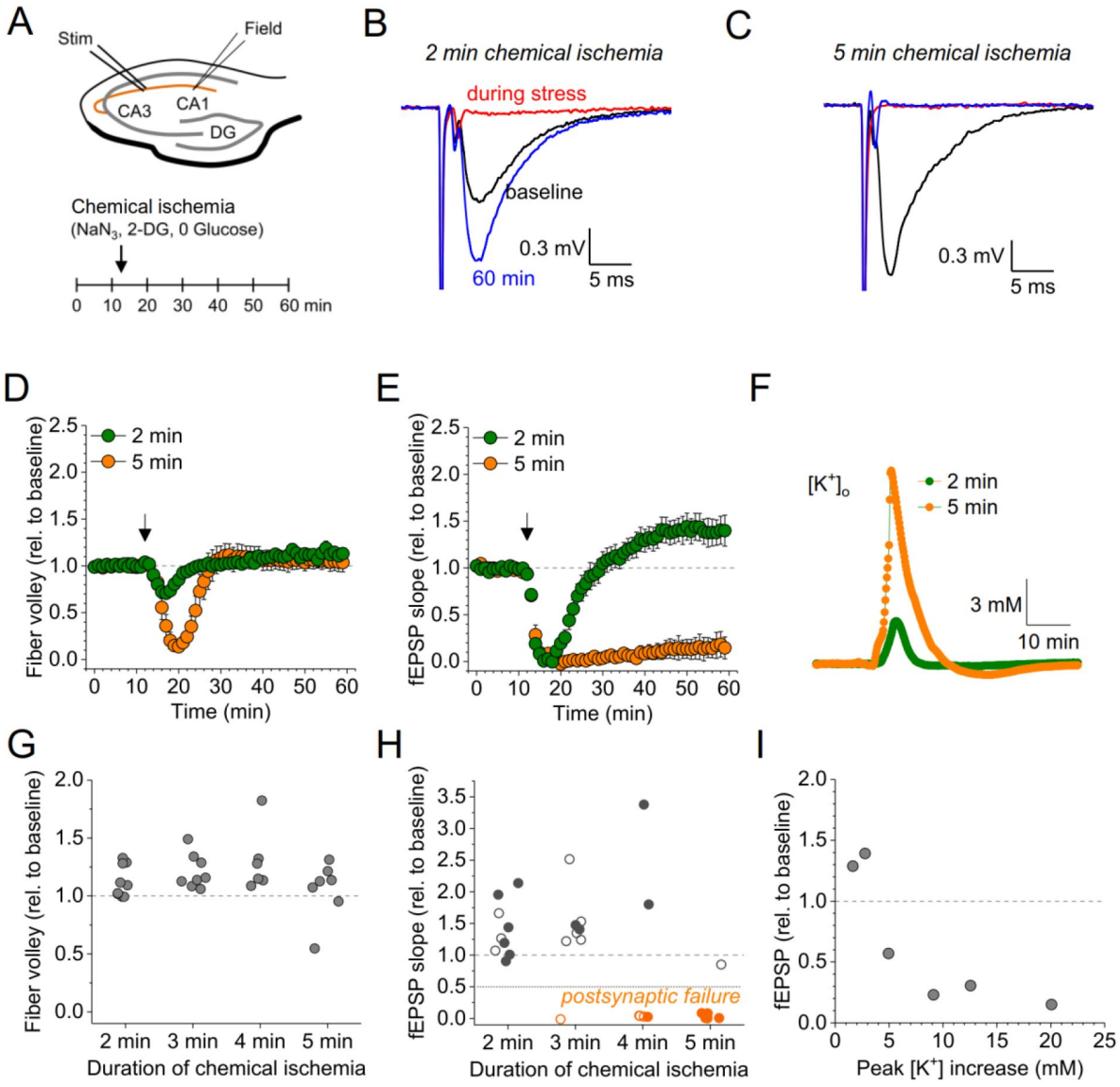

Figure 1

Duration-dependent and bidirectional effect of transient chemical ischemia on synaptic transmission.

(A) Schematic of experimental design. Extracellular field potentials (Field) were recorded in the CA1 region in response to Schaffer collateral stimulation (Stim; paired pulses, 50 ms interstimulus interval, every 20 s). Arrow indicates the time point of application of a modified ACSF, inducing acute chemical ischemia, with 0 mM glucose, 2 mM deoxyglucose (2-DG), 5 mM sodium azide (NaN_3) for 2-5 min. (B-C) Example traces for 2 min chemical ischemia (B) and 5 min chemical ischemia (C) (black, baseline; red, during stress; blue, end of the experiment at 60 min). (D) Relative change of the axonal fiber volley amplitude compared to baseline (0-10 min) for 2 min of chemical ischemia (green) and 5 min of chemical ischemia (orange). Arrow indicates time point of application of the modified ACSF for chemical ischemia induction. (E) Same as in (D) but for the fEPSP slope. (F) Example traces of extracellular $[K^+]$ recordings during for 2 and 5 min of chemical ischemia. (G) Quantification of the relative change of the axonal fiber volley amplitude in the last 10 min of recordings (50-60 min) relative to baseline for 2, 3, 4 and 5 min (n = 9, 8, 6, and 7, respectively) of chemical ischemia. (H) Same experiments as in (G) but analysis of the fEPSP slope. Graphs in H and G include results from recording shown in B-E. Persistent failure of synaptic transmission highlighted in orange. Filled circles correspond to recordings, in which astrocytes expressed iGluSnFR (see **Fig. 2** and text). (I) Change of fEPSP in the last 10 min of recordings (50-60 min) relative to the baseline plotted against maximum $[K^+]$ increase during chemical ischemia. Data are expressed as mean ± s.e.m.

for instance due to bleaching, which appears to be a likely explanation given the repeated imaging over up to 90 minutes. It is important to note that the increase of the iGluSnFR transients ($\Delta F/F_0$) associated with potentiated postsynaptic responses is unlikely due to the reduction of F_0 because the latter is observed in all experimental conditions. Similarly, it is unlikely that the increase of the iGluSnFR transients is because of the increased axonal excitability (increased fiber volley amplitude) because this was also observed in control recordings, where no change of the iGluSnFR response ($\Delta F/F_0$) was detected. The increase of the iGluSnFR response after synaptic activation also points to an increase of extracellular glutamate transients, because the iGluSnFR response to saturating glutamate is unaffected in experiments with chemical ischemia without postsynaptic failure (**Fig. S3**).

Taken together these experiments reveal that longer durations of chemical ischemia result in a persistent postsynaptic failure with slowly recovering glutamate release. A likely reason for the postsynaptic failure is the death or persistent depolarization of CA1 pyramidal cells. Although not quantified, we indeed observed irreversible swelling and/or loss of integrity of CA1 pyramidal cell bodies in parallel to postsynaptic failure. This is in line with previous studies using transient metabolic inhibition with similar durations, which reported irreversible ATP depletion in neocortical neurons after transient chemical ischemia (Pape and Rose, 2023) and persistent depolarization of CA1 pyramidal cells after hypoxia combined with glucose deprivation (Tanaka et al., 1997). The beginning of this postsynaptic failure coincides with a surge of extracellular glutamate (**Fig. 2E**), which we attribute to the increase of synaptic glutamate release at the onset of metabolic stress observed before (Hershkowitz et al., 1993; Tanaka et al., 1997), and of extracellular potassium (**Fig. 1F, I**). Both are likely driven by neuronal depolarization, Ca^{2+} accumulation and the various other contributing mechanisms (Passlick et al., 2021). In contrast, shorter durations of chemical ischemia durations lead to a transient failure of synaptic transmission, possibly due to a transient depolarization of neurons, followed by an unexpected potentiation of synaptically-driven glutamate transients and of postsynaptic responses.

Glutamate uptake is unaffected after acute chemical ischemia

We next asked what the underlying reason for the increase glutamate transients is. One potential explanation is a restriction of extracellular space (ECS): If a given amount of glutamate is released into the ECS leading to a corresponding glutamate concentration, then a reduction of the ECS would increase the glutamate concentration. Indeed, anoxia and ischemia consistently lead to a reduction of the ECS fraction (Syková and Nicholson, 2008). This scenario was investigated using the method of combining TMA-iontophoresis and TMA-selective microelectrode recordings for estimating the ECS fraction and extracellular diffusion (Nicholson, 1993; Hrabětová and Nicholson, 2007; Syková and Nicholson, 2008) in parallel to chemical ischemia induction. We found that chemical ischemia with postsynaptic failure was accompanied by a decreased ECS fraction and a decreased effective diffusion coefficient, which is consistent with previous reports (Syková and Nicholson, 2008) (**Fig. S4**). However, this was not observed for chemical ischemia without postsynaptic failure (**Fig. S4**), in which the increased glutamate transients were observed.

An alternative explanation for the increased glutamate transients is reduced glutamate uptake. To investigate this possibility, we analyzed the decay time constant of the iGluSnFR fluorescence transients after synaptic stimulation, because it is increased when glutamate uptake is decreased (Armbruster et al., 2016; Romanos et al., 2019). However, we did not detect a significant change of the iGluSnFR decay time constant after chemical ischemia (**Fig. 3A, B**). In addition, we tested if a deficit of glutamate uptake could be unmasked by increasing the amount of synaptically released glutamate. This was tested by high-frequency stimulation of CA3-CA1 synapses in the presence of glutamate receptor blockers (to prevent induction of synaptic plasticity, **Fig. 3C**). Again, no differences of the decay time constant were observed between control recordings and recordings with chemical ischemia (**Fig. 3E**). Finally, we verified the sensitivity of our experimental approach by inhibiting glutamate uptake pharmacologically at the end of the experiments. This manipulation indeed increased the decay time constant by a factor of ~ 10-20

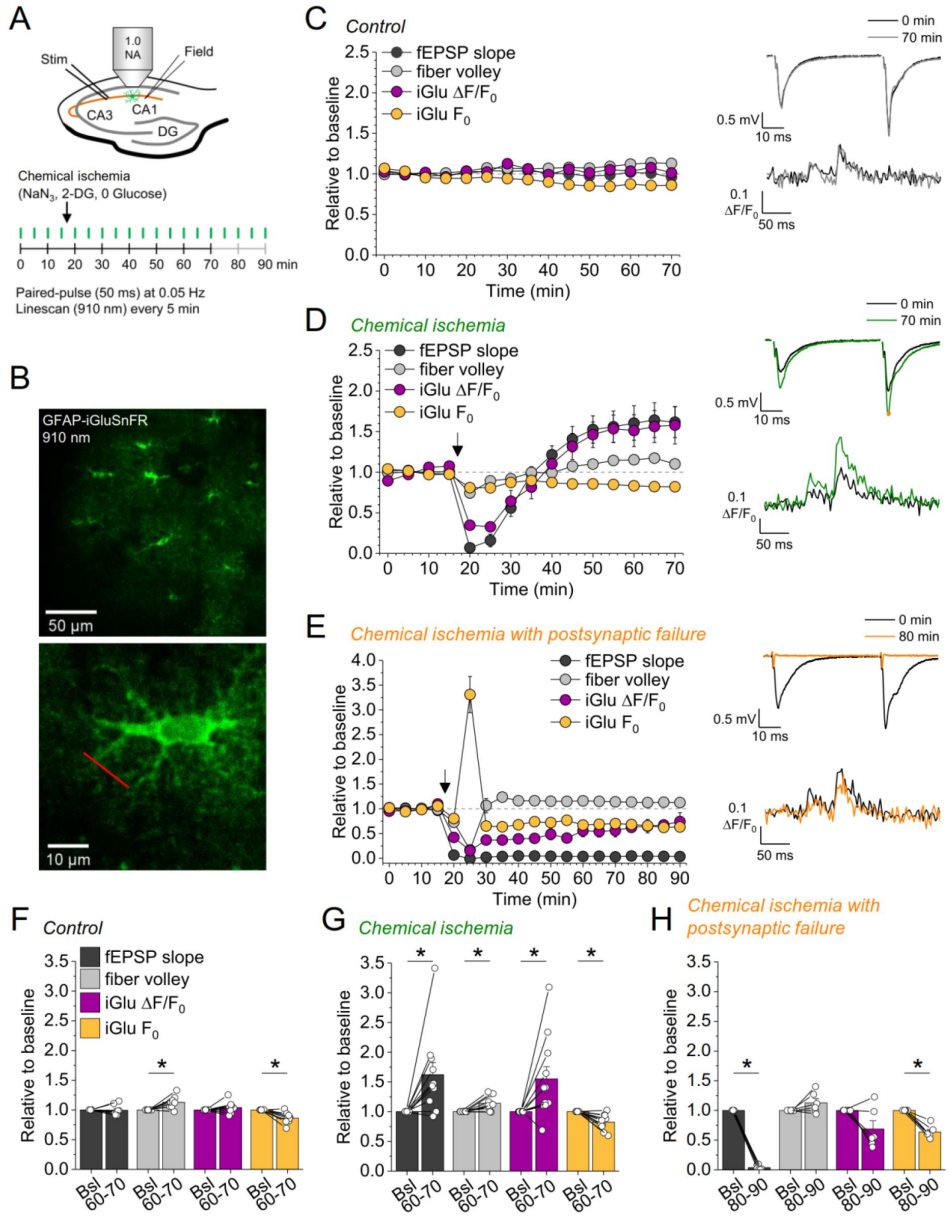

Figure 2

Bidirectional dysregulation of glutamate release by transient chemical ischemia.

(A) Schematic of experimental design. Combined recording of extracellular field potentials (Field) in the CA1 region in response to Schaffer collateral stimulation (Stim; paired pulses, interstimulus interval of 50 ms, every 20 s) and two-photon excitation fluorescence line scan imaging (910 nm, 6x every 5 min) of the glutamate indicator iGluSnFR. (B) Top: example of virally induced GFAP-iGluSnFR expression by astrocytes in the *stratum radiatum* of the CA1 region. Bottom: single iGluSnFR-expressing astrocyte and representative location of line scan (red line). (C-E) Left, field excitatory postsynaptic potential (fEPSP) slope, axonal fiber volley, iGluSnFR (iGlu) $\Delta F/F_0$ and iGlu resting fluorescence (F_0) relative to baseline (0-10 min) for control (n = 8) (C), chemical ischemia (n = 11) (D) and chemical ischemia followed by postsynaptic failure of synaptic transmission (n = 6) (E). Arrow indicates start of chemical ischemia. Right, example traces of fEPSP (top) and iGluSnFR $\Delta F/F_0$ (bottom, average of 6 scans) at the beginning and the end of the recording. The electrophysiological results in D and E are a subset from **Fig. 1**. (F-H) Summary of parameters analyzed in C-E in the last 10 min of recording (60-70/80-90 min) compared to baseline (Bsl). (F) Control: fEPSP slope, p = 0.894; fiber volley, p = 0.012; iGlu $\Delta F/F_0$, p = 0.310; iGlu F_0, p = 0.006; n = 8, paired Student's t-test. (G) Chemical ischemia: fEPSP slope, p = 0.013; fiber volley, p = 0.003; iGlu $\Delta F/F_0$, p = 0.024; iGlu F_0, p = 0.001; n = 11, paired Student's t-test. (H) Chemical ischemia with postsynaptic failure: fEPSP slope, p < 0.0001; fiber volley, p = 0.134; iGlu $\Delta F/F_0$, p = 0.073; iGlu F_0, p = 0.0004; n = 6, paired Student's t-test. Data are expressed as mean ± s.e.m.

(**Fig. 3D, F, G**). We therefore conclude that glutamate uptake is surprisingly resilient in the chemical ischemia model and that a reduction of glutamate uptake does not explain the increased extracellular glutamate transients after chemical ischemia without postsynaptic failure.

Increased synaptic glutamate release after chemical ischemia

Our results point towards an increased synaptic glutamate release when chemical ischemia is shorter and followed by postsynaptic potentiation but not when it is associated with postsynaptic failure. Investigating the paired-pulse behavior of synaptic responses is a common approach for probing changes of presynaptic glutamate release (Debanne et al., 1996). Analyzing the paired-pulse ratio (PPR) of postsynaptic response and iGluSnFR transients revealed no consistent changes after chemical ischemia (**Fig. S5**). However, even manifold presynaptically induced increases of synaptic strength were recently shown to result in relatively small changes of the PPR, especially of the iGluSnFR signal at single CA3-CA1 synapses (Dürst et al., 2022). This suggests that a more moderate presynaptic increase of glutamate release may escape its detection using the PPR. Therefore, we used an electrophysiological approach that directly detects changes of the glutamate concentration in the synaptic cleft. γ-D-glutamylglycine (γ-DGG) is a rapidly equilibrating competitive antagonist of AMPA receptors, and its inhibitory effect on AMPA receptor mediated postsynaptic responses depends on the ambient glutamate concentration because glutamate and γ-DGG compete for AMPA receptor binding (Liu et al., 1999; Christie and Jahr, 2006): The lower the glutamate concentration in the synaptic cleft, the stronger the inhibitory effect of γ-DGG, and vice versa. For performing this test after chemical ischemia without postsynaptic failure we combined our previous experimental approach with whole-cell patch clamp recordings from CA1 pyramidal cells starting ~ 45 minutes after induction of chemical ischemia (**Fig. 4A**). Analyzing the effect of γ-DGG application on simultaneously recorded fEPSPs and excitatory postsynaptic currents (EPSCs), we found that the percentages of fEPSP slopes and EPSC amplitudes remaining after γ-DGG were significantly higher in experiments with chemical ischemia compared to control experiments (**Fig. 4B, C**). This indicates that chemical ischemia led to an increased glutamate concentration in the synaptic cleft, which is consistent with an increased synaptic glutamate release. Thus, shorter durations of chemical ischemia led to a potentiation of presynaptic glutamate release and of the postsynaptic response.

We further explored the underlying reason for this increase of glutamate release by computational modelling. First, we tested the hypothesis that presynaptic conditions expected for metabolic failure could increase vesicle loading with glutamate. This was tested using a previously established model for simulating presynaptic vesicle loading (Kolen et al., 2023). As shown in **Fig. S6**, accumulation of glutamate in the synaptic vesicle under control conditions reaches a steady state of close to 3,500 molecules. Cytosolic conditions consistent with metabolic failure (decreasing pH, increasing $[Na^+]$ and $[Cl^-]$, decreasing $[K^+]$) all lead to a smaller number of glutamate molecules in the vesicle. Similarly, a decrease in the extracellular pH and a reduced vesicular ATPase activity (due to reduced ATP levels) also cause a decrease in the luminal glutamate concentration. As a control, we increased the cytosolic pH from 7.2 to 7.4 and observed a significant rise in the number of glutamate molecules. However, we are not aware of any reports regarding a presynaptic alkalinization during ischemia or chemical ischemia. Overall, these simulations show that the conditions that are typically observed during ischemia or metabolic failure tend to lower the vesicular glutamate concentration and therefore cannot explain the observed increased glutamate release. An alternative explanation is an increased probability of presynaptic vesicular release, which could result from increased presynaptic Ca^{2+} influx, for instance, due to a presynaptic broadening of action potentials. For testing this, we analyzed the axonal fiber volley recorded in previous experiments (**Fig. 3C, D**), in which glutamate receptors were blocked and the fiber volley was therefore isolated (**Fig. 4D**). We found that the amplitude of the fiber volley was not different between control recordings and recordings after chemical ischemia without postsynaptic failure (**Fig. 4E**, left panel), which confirms previous conclusions (**Fig. 2**). However, the fiber volley was significantly broadened by ~ 20 % (**Fig. 4E**, middle

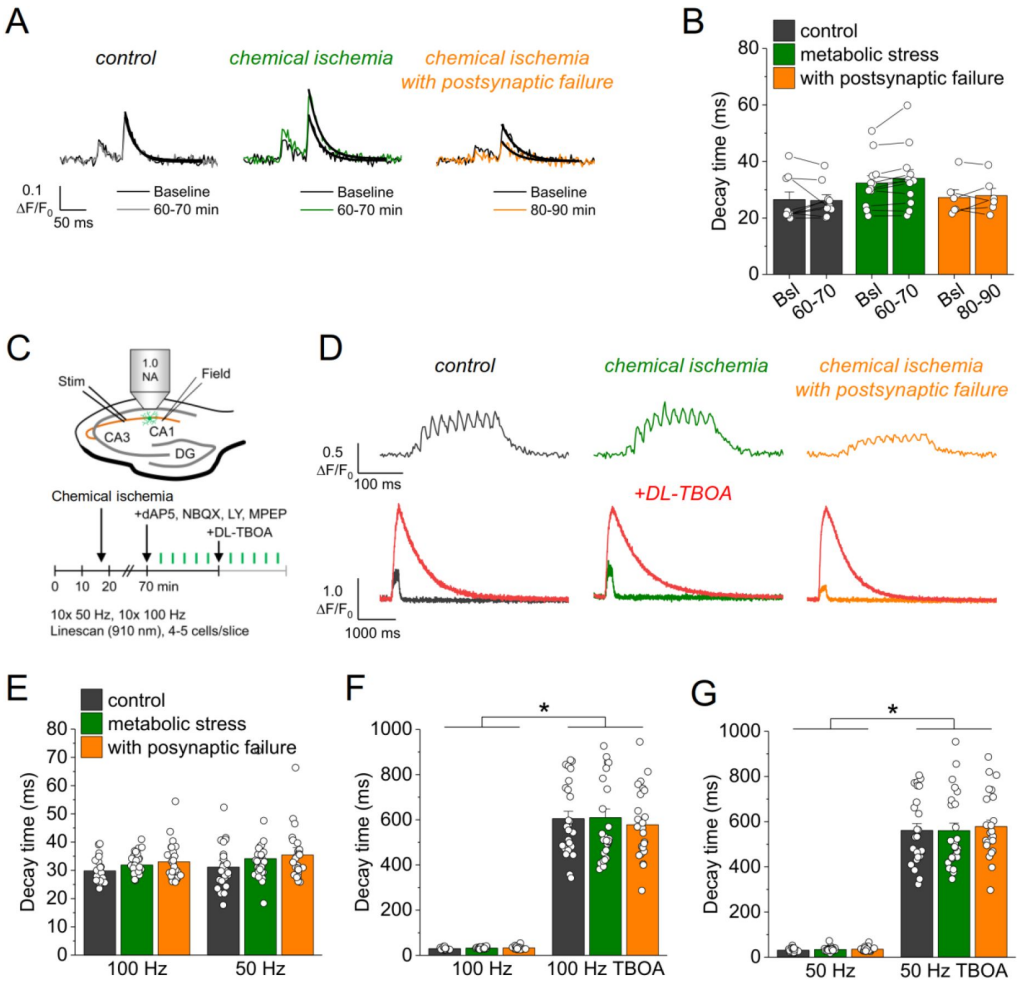

Figure 3

Glutamate clearance is not affected by transient chemical ischemia.

(A) Example traces of iGluSnFR $\Delta F/F_0$ in response to paired-pulse stimulation (interstimulus interval 50 ms) during baseline (black) and last 10 min (60-70/80-90 min) of control (left, gray), chemical ischemia (middle, green) and chemical ischemia with postsynaptic failure (right, orange) recordings. Black line indicates exponential fit for the analysis of iGluSnFR decay time. (B) Quantification of iGluSnFR fluorescence decay time of the 2nd pulse of the paired-pulse stimulation during the last 10 min of recording (60-70/80-90 min) compared to baseline (Bsl) for control, chemical ischemia, and chemical ischemia with postsynaptic failure. Control: p = 0.860, n = 9; chemical ischemia, p = 0.062, n = 12; chemical ischemia with postsynaptic failure, p = 0.648, n = 6; paired Student's t-tests. (C) Schematic of experimental design. Combined recording of extracellular field potentials (Field) in the CA1 region in response to Schaffer collateral stimulation (Stim; paired pulse (50 ms), 0.05 Hz) and two-photon excitation fluorescence line scan imaging (910 nm) of iGluSnFR. After 70 min, D-AP5 (50 µM), NBQX (20 µM), LY341495 (50 µM) and MPEP (10 µM) were added to the recording solution and iGluSnFR fluorescence changes ($\Delta F/F_0$) were recorded in 4-5 cells in response to 10x 50 Hz and 10x 100 Hz stimulation before and after application of DL-TBOA (100 µM). (D) Example traces of iGluSnFR $\Delta F/F_0$ line scan recordings (average of 6 scans) in response to 10x 50 Hz stimulation (top row) after control (left), chemical ischemia (middle) and chemical ischemia with postsynaptic failure recordings (right). The same cells were tested again after block of glutamate transporters by DL-TBOA (bottom row, red traces together with 'before' traces from upper row on different time scale for comparison). (E) Quantification of iGluSnFR fluorescence decay time in response to 10x 100 Hz and 10x 50 Hz stimulation after control, chemical ischemia recording without and with postsynaptic failure. 100 Hz: p = 0.060, 50 Hz: p = 0.149; n = 25, 29, and 25 cells for control/chemical ischemia/chemical ischemia with postsynaptic failure from 5, 6, and 5 independent experiments, respectively; Kruskal-Wallis ANOVA. (F) Quantification of iGluSnFR fluorescence decay time in response to 10x 100 Hz stimulation in the presence and absence of DL-TBOA (same cells as in E). p < 0.0001 for control, chemical ischemia, and chemical ischemia with postsynaptic failure, paired sample Wilcoxon Signed Ranks tests. (G) As in F but for 10x 50 Hz stimulation. p < 0.0001 for control, chemical ischemia, and chemical ischemia with postsynaptic failure, paired sample Wilcoxon Signed Ranks tests. Data are expressed as mean ± s.e.m.

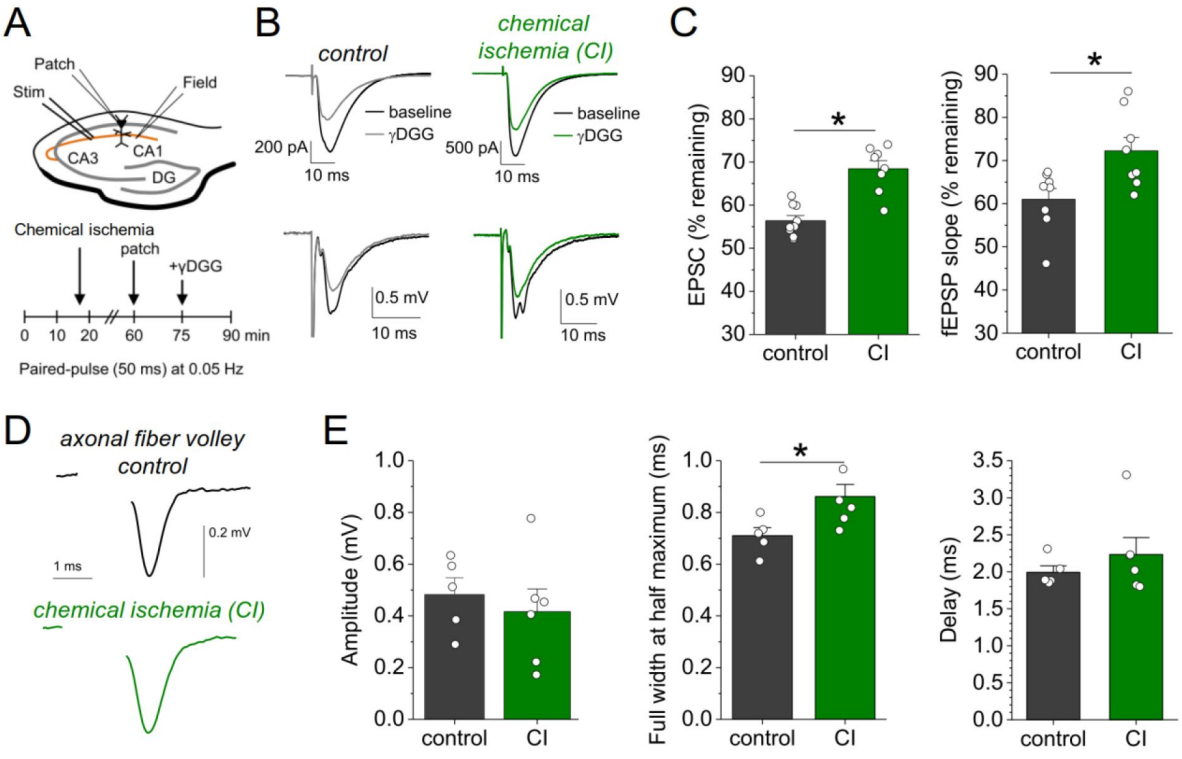

Figure 4

Chemical ischemia increases synaptic glutamate release.

(A) Schematic of experimental design. Extracellular field potentials (Field) were recorded in the CA1 region in response to Schaffer collateral stimulation (Stim; paired pulse (50 ms), 0.05 Hz). After 60 min, a whole-cell patch clamp recording from a CA1 pyramidal neuron was started. After recording baseline excitatory postsynaptic current (EPSC) responses for 15 min, γDGG (1 mM) was added to the extracellular solution. (B) Example traces of EPSCs (top) and fEPSPs (bottom) before (black) and after γDGG application for control (left, gray) and chemical ischemia (right, green) recordings. (C) Quantification of the remaining EPSC amplitude (left) and fEPSP slope (right) (in % of the baseline value) after γDGG application for control (n = 9) and chemical ischemia (n = 8) recordings. EPSC, $p < 0.0001$; fEPSP, $p = 0.014$; paired two-sample Student's t-test. Data are expressed as mean ± s.e.m. (D) Example of axonal fiber volleys from experiments shown in **Fig. 3C-E**. Stimulus artefact removed for clarity. (E) Comparison of the fiber volley amplitude (left, $p = 0.57$), fiber volley half width at half maximum (middle, $p = 0.026$) and fiber volley delay (time between onset of the stimulus and fiber volley peak, right, $p = 0.39$). n = 5 (control) and 6 (chemical ischemia), unpaired Student's t-tests.

panel) without a significant change of its delay after stimulation (**Fig. 4E**, right panel). This could indicate that short transient chemical ischemia broadens the presynaptic action potential, which increases presynaptic Ca^{2+} influx and presynaptic glutamate release and, as a consequence, the postsynaptic response.

Discussion

We modeled pathophysiological scenarios with transiently reduced ATP levels such as peri-infarct depolarizations (PIDs) and spreading depression (Lauritzen et al., 2011) by a chemical ischemia protocol. In our analysis, we focused on the recovery from this acute and transient chemical ischemia and its outcome. Depending on the duration of chemical ischemia, two qualitatively different changes of glutamatergic synaptic transmission became apparent. After prolonged chemical ischemia up to five minutes, the postsynaptic responses did not recover in contrast to presynaptic action potential firing and presynaptic glutamate release, which, however, did not completely return to baseline levels. After shorter chemical ischemia, the postsynaptic responses and presynaptic glutamate release recovered and were potentiated at the end of the recording whereas the axonal fiber volley amplitude was not different from control recordings. Overall, these observations reveal a graded vulnerability of the involved compartments to transient metabolic failure: Least resilient were postsynaptic neurons, followed by presynaptic glutamate release and axonal action potential firing. In contrast, glutamate clearance, which is mostly due to astrocytic glutamate uptake (Danbolt, 2001), was mostly unaffected even after long durations of chemical ischemia. This is in line with the observation from the hippocampus that transient chemical ischemia triggers much smaller ATP decreases and Na^+ increases, a measure of dysregulation of ion homeostasis, in astrocytes compared to neurons (Meyer et al., 2022; Pape and Rose, 2023). This is because of the strong expression of postsynaptic neuronal N-methyl-D-aspartate receptors (NMDARs) and TRPV4 (Pape and Rose, 2023). It is also noteworthy that presynaptic glutamate release recovered to some extent even if the postsynaptic neurons remained unresponsive, thereby masking the recovery of glutamate release. We have not systematically tested durations of chemical ischemia beyond five minutes, because it is safe to assume this would further slow down, and ultimately prevent, the recovery of glutamate release and eventually also impair axonal action potential firing (**Fig. 2E**).

The persistent potentiation of glutamate release after short chemical ischemia was unexpected. It was detected in electrophysiological recording using γ-DGG and iGluSnFR fluorescence imaging and it was not caused by a reduced glutamate uptake or changes of the extracellular space. Instead, a likely explanation is a broadening of presynaptic axonal action potentials, which is strongly suggested by the widened fiber volley in the absence of changes of fiber volley amplitude or delay. Nonetheless, a widened fiber volley could also be explained by, for instance, a desynchronization of presynaptic action potential firing. Presynaptic action potential broadening could explain increased glutamate release, because pharmacological prolongation of presynaptic action potentials by 4-AP was previously shown to increase postsynaptic responses by increasing presynaptic Ca^{2+} influx at these synapses (Wheeler et al., 1996; Qian and Saggau, 1999). A potential reason for the action potential widening is an inactivation of potassium currents (Mitterdorfer and Bean, 2002), because of a lasting depolarization after transient chemical ischemia. Whether this is indeed the case remains to be tested experimentally. Alternatively, short durations of chemical ischemia could increase presynaptic glutamate release by persistently altering the presynaptic resting concentration of Ca^{2+} (Eshra et al., 2021) or presynaptic cAMP signaling (Vaden et al., 2019), which are only two prominent examples among many others.

The time course of the potentiation of glutamate release and of the postsynaptic response were almost identical (**Fig. 2D**), which could suggest that the increased presynaptic release directly leads to the potentiation of the postsynaptic response. It has previously been shown that the potentiation of synaptic transmission (LTP) in ischemia models (ischemic/anoxic LTP) and

physiological LTP can occlude each other (Hsu and Huang, 1997; Stein et al., 2015), which indicates that they can share the mechanisms underlying the potentiation of synaptic transmission. For CA3-CA1 synapses, there is a long-standing debate to what extent and under which conditions postsynaptic mechanisms such as receptor insertion and presynaptic changes of release underlie LTP (Padamsey and Emptage, 2014). Our experiments suggest that the potentiation of postsynaptic responses is at least in part due to increased glutamate release, but they also do not rule out a mix of pre- and postsynaptic changes.

Glutamate uptake can be used as an indirect measure of the astrocytic metabolic status, because it is mostly mediated by astrocytes and driven by their transmembrane Na^+ gradient and membrane potential (Danbolt, 2001; Rossi et al., 2007; Passlick et al., 2021), which are maintained by the Na^+/K^+-ATPase. In experiments with prolonged chemical ischemia with postsynaptic failure, we observed preserved glutamate uptake in two thirds of the experiments whereas in one third iGluSnFR expressed by astrocytes disappeared after chemical ischemia. While we do not know the exact reason for the latter, astrocyte metabolism seems to be overall more resilient to transient ischemia than the postsynaptic cells. In experiments without postsynaptic failure, no lasting reduction of glutamate uptake was detected. Neither observation rules out that glutamate uptake was transiently impaired during or immediately following chemical ischemia, because in this time period astrocytic Na^+ levels were shown to increase reversibly (Gerkau et al., 2018; Pape and Rose, 2023). Similarly, they do not rule out that astrocytes are a transient source of glutamate during chemical ischemia by, for instance, reverse transport and channel-mediated glutamate release (Rossi et al., 2000, 2007; Yang et al., 2019; Passlick et al., 2021). Taken together, lasting effects of chemical ischemia on glutamate uptake were insignificant compared to changes of presynaptic glutamate release and synaptic transmission.

In summary, our experiments reveal a bidirectional dysregulation of glutamate signaling after transient metabolic failure. Especially the lasting potentiation of synaptic glutamate release after short periods of energy depletion could be an important new aspect in the disease context. If individual PIDs lead to a similar potentiation of glutamate release, the latter could promote excitotoxic damage in the peri-infarct tissue. However, our experiments using chemical ischemia in acute hippocampal slices do not fully reproduce the complex situation in the brain during stroke or PIDs, of course. While our approach allowed us to investigate the effect of variable degrees of cellular ATP depletion (Pape and Rose, 2023) on glutamatergic synapse function and to dissect the relevant cellular and synaptic mechanisms in detail, it investigates cellular changes independent of, for instance, blood circulation and the constraints imposed by a fixed skull volume. Another variable that could be of interest is sex, because many aspects of glutamate signaling such as receptor and transporter expression can depend on sex and/or estrous cycle (Giacometti and Barker, 2020). In the present study, we have performed experiment exclusively on male mice, leaving the question of a sex-dependence of our observation unanswered. It will be interesting to understand, if such factors modify the bidirectional dysregulation of glutamate signaling observed here.

Acknowledgements

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Author contributions

S.P. performed and analyzed all electrophysiological and imaging experiments. Modelling of glutamate transport was done by G.U. C.H. conceived the study, analyzed data, and wrote the initial version of the manuscript, to which all authors subsequently contributed.

Competing Interest Statement

There are no competing interests.

Materials and Methods

Animals

Experiments were performed using 2-4-months-old male C57BL/6N mice (Charles River) that were kept under 12 h light/dark conditions and had *ad libitum* access to food and water. All animal procedures were conducted in accordance with the regulations of the European Commission Directive 2010/63/EU and all relevant national and institutional guidelines and requirements. Procedures were further approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV, Recklinghausen, Germany) where required.

Stereotactic injections

In order to express the glutamate sensor iGluSnFR (Marvin et al., 2013) on the surface of astrocytes, 4-weeks-old C57BL/6N mice were injected bilaterally with an AAV expressing iGluSnFR under control of the GFAP promoter (AAV1.GFAP.iGluSnFr.WPRE.SV40, PennCore) into the CA1 region of the dorsal hippocampus. Mice were deeply anesthetized by intraperitoneal injection (i.p.) of Fentanyl/Midazolam/Medetomidin (0.05/5.0/0.5 mg/kg bodyweight, injection volume 0.1 ml/10 g bodyweight). After deep anesthesia was confirmed, the head was shaved, the skin disinfected, and the head fixed in a stereotactic frame (Model 901, David Kopf Instruments). An incision was made, bregma localized, and a small hole was drilled with a dental drill (coordinates for the dorsal hippocampus, relative to bregma: anterior −1.8 mm, lateral +/− 1.6 mm, ventral −1.6 mm). Next, a beveled needle nanosyringe (nanofil 34 G BVLD, WPI) was slowly inserted into the brain and 0.5 to 1 µl of viral particles were injected into the hippocampus using a microinjection pump (50 nl/min, WPI). The injection needle was left in place for about 3 minutes and then slowly retracted. After repeating the procedure for the other hemisphere, the incision was sutured using absorbable thread (Ethicon) and an antibiotic was applied (Refobacin 1 mg/g, Gentamicin). Finally, anesthesia was terminated by i.p. injection of Naloxon/Flumazenil/Atipamezol (1.2/0.5/2.5 mg/kg bodyweight, injection volume 0.1 ml/10 g bodyweight). Analgesia was applied 30 min before terminating the anesthesia and 24 h after surgery by subcutaneous injection of carprofen (5 mg/kg bodyweight, injection volume 0.1 ml/20 g bodyweight). Animals were used for experiments 2–5 weeks after virus injection.

Hippocampal slice preparation

Electrophysiology and two-photon excitation fluorescence microscopy in acute hippocampal slices were combined as previously described (Minge et al., 2017). Briefly, coronal slices were obtained from mice and virus-injected mice (see above) with a thickness of 300 µm. Slices were prepared in an ice-cold slicing solution containing (in mM): NaCl 60, sucrose 105, KCl 2.5, $MgCl_2$ 7, NaH_2PO_4 1.25, ascorbic acid 1.3, sodium pyruvate 3, $NaHCO_3$ 26, $CaCl_2$ 0.5, and glucose 10

(osmolarity 300-310 mOsm/l), and kept in the slicing solution at 34°C for 15 minutes before being stored at room temperature (21-23 °C) in an artificial cerebral spinal fluid (ACSF) containing (in mM): NaCl 131, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 21, and glucose 10 (osmolarity adjusted to 295-305 mOsm/l). Slices were allowed to rest for at least 60 minutes. For recordings, slices were transferred to a submersion-type recording chamber and perfused with extracellular solution (ACSF). All recordings were performed at 33-35 °C. All solutions were continuously bubbled with 95% O_2/ 5% CO_2. The flow rate of the extracellular solution was kept constant at 6-7 ml/min throughout all experiments.

Electrophysiology

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed using patch pipettes filled with extracellular solution placed in the stratum radiatum of the CA1 region. For stimulation of CA3-CA1 Schaffer collateral axonal connections, a bipolar concentric stimulation electrode was placed in the stratum radiatum at the border between CA2/3 and CA1. Stimulation intensities (DS3, Digitimer Ltd., UK) were set to obtain ~ 50% of the maximum fEPSP amplitude. The duration of individual stimuli was 100 µs. In some experiments, field recordings were combined with whole-cell patch clamp recordings from CA1 pyramidal cells using standard patch pipettes (3-4 MΩ) filled with an intracellular solution containing (in mM): KCH_3O_3S 135, HEPES 10, di-Tris-Phosphocreatine 10, $MgCl_2$ 4, Na_2-ATP 4, Na-GTP 0.4, 0.2 BAPTA, 5 QX314-Cl (pH adjusted to 7.2, osmolarity 290-295 mOsm/l). CA1 pyramidal cells were identified using infrared differential interference contrast or Dodt contrast optics by their typical morphology and location. Data were recorded using MultiClamp 700B (Molecular Devices) amplifiers, digitized (10 kHz) and stored for offline analysis. Whole-cell patch clamp recordings were rejected if at any time the access resistance exceeded 25 MΩ or changed by more than 20%.

Acute chemical ischemia was induced by switching from normal ACSF to a modified ACSF with 0 mM glucose, 2 mM deoxyglucose (2-DG) to inhibit ATP production through glycolysis, and 5 mM sodium azide (NaN_3) to inhibit ATP production through oxidative phosphorylation. The modified ACSF was applied for variable durations ranging from 2 to 5 minutes. In some experiments, the inhibitors D-AP5 (dAP5 in figures, 50 µM, Abcam), NBQX disodium salt (10 µM, Abcam), DL-TBOA (100 µM, Tocris), LY341495 (50 µM, Tocris), MPEP (10 µM, Tocris), and/or γ-DGG (1 mM, Tocris) were added to the extracellular solution as indicated.

Glutamate imaging using iGluSnFR

Glutamate imaging using iGluSnFR (Marvin et al., 2013) in combination with electrophysiology was performed similarly as previously described (Herde et al., 2020). Slices with astrocytes expressing iGluSnFR after viral injection (see above) were transferred to a submersion-type recording chamber mounted on a Scientifica two-photon excitation fluorescence microscope with a 60x/1.0 NA objective (Olympus). iGluSnFR was excited using a femtosecond pulse laser (Vision S, Coherent, excitation wavelength of 910 nm). During imaging, line scans of iGluSnFR fluorescence were repeatedly obtained. The scanned line was positioned in the periphery of an iGluSnFR-expressing astrocytes (for example **Fig. 2B**). The line was scanned at a frequency of 378.8 Hz to obtain a fluorescence profile over time. Line scans were repeated 4-6 times for each individual time point and experiment and averaged before the background fluorescence was subtracted to obtain an average iGluSnFR fluorescence time course. The resting iGluSnFR fluorescence before synaptic stimulation (F_0), the maximum change of iGluSnFR fluorescence (ΔF_{MAX}) and the normalized iGluSnFR time course ($\Delta F/F_0$) were determined. iGluSnFR imaging was combined with paired synaptic stimuli (**Fig. 2**), high-frequency synaptic stimulation (**Fig. 3**), and iontophoretic glutamate application (Suppl. Fig. 2). For the latter, an iontophoretic application system (MVCS-02C-150, NPI) was used with iontophoretic pipettes (60-80 MΩ resistance) filled with 150 mM glutamic acid (pH adjusted to 7.0 with NaOH) and 40 µM Alexa Fluor 633 to localize the pipette.

Measurements of extracellular space fraction and diffusivity and extracellular K^+

Extracellular [K^+] was recorded using K^+-sensitive microelectrodes as previously described (Breithausen et al., 2020). Briefly, these microelectrodes were built using theta-glass capillaries and pulled with a horizontal pipette puller. The reference and the K^+-sensitive barrel were filled with a NaCl (154 mM) and KCl (150 mM) solution, respectively. The tip of the K^+-sensitive barrel was filled with a valinomycin-based K^+ ionophore (K^+ ionophore 1, cocktail B, Sigma-Aldrich). The K^+-sensitive microelectrodes were calibrated before each experiment with solutions containing 154 mM NaCl and 3 or 30 mM KCl. Electrodes were used if they responded to this 10x increase of K+ with a voltage response > 50 mV. Measurements of the relative changes of the extracellular space (ECS) fraction and of extracellular diffusion during experiments with acute chemical ischemia were obtained using the method of combining tetramethylammonium (TMA) iontophoresis and TMA-sensitive electrodes recordings (Nicholson, 1993; Hrabětová and Nicholson, 2007; Syková and Nicholson, 2008). TMA-sensitive, double-barrel microelectrodes were built like K^+-sensitive microelectrodes except that the TMA-sensitive ionophore solution (IE190, WPI) and 150 mM TMA chloride, as the intrapipette solution, were used for the TMA-sensitive barrel. The reference barrel was filled with 154 mM NaCl and 60 µM Alexa Fluor 594 to visualize the electrode. Recordings were performed using an ION-01M amplifier (NPI, Germany). TMA-sensitive microelectrodes were tested for their sensitivity to TMA before each experiment by two calibration solutions containing either (in mM) TMA 1, KCl 3, NaCl 150 or TMA 10, KCl 3, NaCl 141. Only electrodes that responded to this 10-fold increase of TMA with a potential increase of > 50 mV were used for the experiments. A subset of electrodes was also tested for their sensitivity over a larger range of TMA concentrations, which revealed Nernst-like behavior over higher concentrations and only a departure from that in the low sub-millimolar range. To correct for that, the calibration curve was fitted with the Nicolsky-Eisenman equation and the conversion of the TMA-electrode potential into a TMA concentration was adapted accordingly (Nicholson, 1993). For recordings in chemical ischemia experiments (Suppl. Fig. 3), the TMA-sensitive microelectrode was placed into the CA1 stratum radiatum and an iontophoresis pipette filled with 100 mM TMA and 60 µM Alexa Fluor 594 was placed at distance of 100-200 µm from the TMA-sensitive microelectrode. At each time point during the recording, TMA was iontophoretically injected into the tissue (100 nA, 30 s) and the corresponding [TMA] time course was calculated. For examples, see Suppl. Fig. 3. Each time course was fitted using equations 10.2 to 10.4 from (Hrabětová and Nicholson, 2007) using a MATLAB script to obtain the extracellular space (ECS) fraction and the effective TMA diffusion coefficient (D*). D* deviates from the free diffusion coefficient of TMA (D) because the tortuosity of the ECS reduces TMA diffusion over the distances investigated here (Hrabětová and Nicholson, 2007). Therefore, changes of D* can indicate a change of ECS tortuosity. In these experiments, the extracellular solution always contained 1 mM TMACl to provide a known basal extracellular TMA concentration for the conversion of the recorded microelectrode potential into [TMA]. The reference barrel of the TMA-sensitive microelectrode was also used for recording fEPSPs in response to axonal stimulation. We have verified these recordings to report accurate values by recording [TMA] transients in agarose gel (ECS fraction ~ 1, tortuosity ~ 1) (Nicholson, 1993) and in acute brain slices, in which we obtained an ECS fraction of 0.19 ± 0.023 (n = 5) and a tortuosity of 1.54 ± 0.046 (n = 5) and which matches previous measurements in this region (Nicholson, 1993; Syková and Nicholson, 2008)

Glutamate accumulation in synaptic vesicles

The accumulation of glutamate in synaptic vesicles is modeled as previously described (Kolen et al., 2023) and the code for the model is archived along with it. Briefly, the temporal evolution of luminal glutamate molecule number, $[H^+]$, pH, and $[Cl^-]$ are modeled by the following rate equations:

$$\frac{d[Glut]_L}{dt} = J_{VGLUT}, \quad (1)$$

$$\frac{d[H^+]_L}{dt} = J_{H^+} + J_{V_{ATPase}} - J_{VGLUT}, \quad (2)$$

$$\frac{dpH_L}{dt} = -\frac{1}{N_A}\left(\frac{dH_L^+}{dt}\frac{1}{V}\right)\left(\frac{1}{\beta_{pH}}\right), \quad (3)$$

$$\frac{d[Cl^-]_L}{dt} = J_{VGLUT_{Cl}}. \quad (4)$$

Where N_A, β_{pH} $\left(= \frac{40\ mM}{pH}\right)$, , and V represent Avogadro's number, the pH-buffering capacity of the vesicle, and the vesicle volume assuming a radius of 20 nm. The equations for glutamate/H^+ flux through glutamate transporters (J_{VGLUT}), Cl^- flux through glutamate transporters ($J_{VGLUT_{Cl}}$), proton leak (J_{H^+}), , and V-type ATPase ($J_{V_{ATPase}}$) are described in (Kolen et al., 2023).

The equation for the membrane potential of the vesicle ($\Delta\psi$) is,

$$\Delta\psi = \frac{F\times V}{C}([H^+]_L + [K^+]_L + [Na^+]_L - [Cl^-]_L - [Glut]_L - B) \quad (5)$$

with $C = C_0 \times S$ being the total capacitance of the vesicle with C_0 = 1 µF/cm^2, S the surface area of the vesicle, and F Faraday's constant. Luminal K^+ ($[K^+]_L$) and Na^+ ($[Na^+]_L$) concentrations were fixed to extracellular values, i.e. to 5 mM and 145 mM, respectively. B is the luminal concentration of impermeant charges given by the conservation of charge in the vesicle under initial conditions (indicated by the subscript 0).

$$B = [H^+]_{L,0} + [K^+]_{L,0} + [Na^+]_{L,0} - [Cl^-]_{L,0} - [Glut]_{L,0} - \frac{C}{F\times V}(\psi_{in} - \psi_{out}) \quad (6)$$

Finally, the effect of surface charge on various ion concentrations at the membrane was also taken into account, with the inner (ψ_{in}) and outer (ψ_{out}) leaflets potentials set to 0 mV and −50 mV, respectively.

Starting conditions for the mathematical modeling reflect the ion concentrations immediately after endocytosis of synaptic vesicles: the vesicular lumen contained solutions resembling the external solutions, with high $[Cl^-]$, neutral pH and negligible glutamate concentration ($[K^+]_{L,0}$ = 5 mM, $[Na^+]_{Lm,0}$ = 145 mM, $[Cl^-]_{L,0}$ = 110 mM, $[Glut]_{L,0}$ = 1 µM, and $pH_{L,0}$ = 7.2); cytoplasmic pH, Cl^-, K^+, Na^+, and glutamate concentration was set to 7.2, 10 mM, 140 mM, 10 mM, and 10 mM, respectively. We also incorporated changes in luminal Na^+ and K^+ concentrations, however, both did not affect our results.

Statistics

Analyses were performed using ImageJ (NIH), Origin (OriginLab) and Matlab (MathWorks). Numerical data are reported as mean ± s.e.m. with n being the number of samples. In all electrophysiological and imaging experiments in acute hippocampal slices, n refers to the number of recordings. was typically performed per acute hippocampal slice. Because the success rate of experiments combining electrophysiology, two-photon excitation imaging and pharmacology is

low, a single successful experiment was typically performed per day and animal. For all other experimental designs, detailed information can be found in the figure legends. The Shapiro-Wilk test was used to establish if data were normally distributed. Comparisons were then performed using the appropriate parametric and non-parametric tests (e.g., Student's t-test or Mann-Whitney U-test). The statistical tests used are indicated throughout. In figures, asterisks indicate significance levels where *** indicated $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$, error bars represent s.e.m. and dots represent individual data points, which are connected by lines for paired data points.

References

Armbruster M., Hanson E., Dulla C. G. (2016) **Glutamate Clearance Is Locally Modulated by Presynaptic Neuronal Activity in the Cerebral Cortex** *J. Neurosci* **36**:10404–10415 https://doi.org/10.1523/JNEUROSCI.2066-16.2016

Breithausen B., Kautzmann S., Boehlen A., Steinhäuser C., Henneberger C. (2020) **Limited contribution of astroglial gap junction coupling to buffering of extracellular K+ in CA1 stratum radiatum** *Glia* **68**:918–931 https://doi.org/10.1002/glia.23751

Christie J. M., Jahr C. E. (2006) **Multivesicular Release at Schaffer Collateral–CA1 Hippocampal Synapses** *J Neurosci* **26**:210–216 https://doi.org/10.1523/JNEUROSCI.4307-05.2006

Crepel V., Epsztein J., Ben-Ari Y. (2003) **Ischemia induces short- and long-term remodeling of synaptic activity in the hippocampus** *J. Cell. Mol. Med* **7**:401–407 https://doi.org/10.1111/j.1582-4934.2003.tb00242.x

Crepel V., Hammond C., Chinestra P., Diabira D., Ben-Ari Y. (1993) **A selective LTP of NMDA receptor-mediated currents induced by anoxia in CA1 hippocampal neurons** *J. Neurophysiol* **70**:2045–2055 https://doi.org/10.1152/jn.1993.70.5.2045

Danbolt N. C. (2001) **Glutamate uptake** *Prog. Neurobiol* **65**:1–105

Debanne D., Guérineau N. C., Gähwiler B. H., Thompson S. M. (1996) **Paired-pulse facilitation and depression at unitary synapses in rat hippocampus: quantal fluctuation affects subsequent release** *J. Physiol* **491**:163–176 https://doi.org/10.1113/jphysiol.1996.sp021204

Dirnagl U., Iadecola C., Moskowitz M. A. (1999) **Pathobiology of ischaemic stroke: an integrated view** *Trends Neurosci* **22**:391–397 https://doi.org/10.1016/S0166-2236(99)01401-0

Dürst C. D., Wiegert J. S., Schulze C., Helassa N., Török K., Oertner T. G. (2022) **Vesicular release probability sets the strength of individual Schaffer collateral synapses** *Nat Commun* **13** https://doi.org/10.1038/s41467-022-33565-6

Eshra A., Schmidt H., Eilers J., Hallermann S. (2021) **Calcium dependence of neurotransmitter release at a high fidelity synapse** *eLife* **10** https://doi.org/10.7554/eLife.70408

Gerkau N. J., Rakers C., Durry S., Petzold G. C., Rose C. R. (2018) **Reverse NCX Attenuates Cellular Sodium Loading in Metabolically Compromised Cortex** *Cereb Cortex* **28**:4264–4280 https://doi.org/10.1093/cercor/bhx280

Giacometti L., Barker J. (2020) **Sex differences in the glutamate system: Implications for addiction** *Neuroscience & Biobehavioral Reviews* **113**:157–168 https://doi.org/10.1016/j.neubiorev.2020.03.010

Herde M. K., Bohmbach K., Domingos C., Vana N., Komorowska-Müller J. A., Passlick S., et al. (2020) **Local Efficacy of Glutamate Uptake Decreases with Synapse Size** *Cell Rep* **32** https://doi.org/10.1016/j.celrep.2020.108182

Hershkowitz N., Katchman A. N., Veregge S. (1993) **Site of synaptic depression during hypoxia: a patch-clamp analysis** *Journal of Neurophysiology* **69**:432–441 https://doi.org/10.1152/jn.1993.69.2.432

Hrabětová S., Nicholson C., Michael A. C., Borland L. M. (2007) **Biophysical Properties of Brain Extracellular Space Explored with Ion-Selective Microelectrodes, Integrative Optical Imaging and Related Techniques** *Electrochemical Methods for Neuroscience*

Hsu K.-S., Huang C.-C. (1997) **Characterization of the anoxia-induced long-term synaptic potentiation in area CA1 of the rat hippocampus** *British Journal of Pharmacology* **122**:671–681 https://doi.org/10.1038/sj.bjp.0701409

Kolen B., Borghans B., Kortzak D., Lugo V., Hannack C., Guzman R. E., et al. (2023) **Vesicular glutamate transporters are H+-anion exchangers that operate at variable stoichiometry** *Nat Commun* **14** https://doi.org/10.1038/s41467-023-38340-9

Lauritzen M., Dreier J. P., Fabricius M., Hartings J. A., Graf R., Strong A. J. (2011) **Clinical Relevance of Cortical Spreading Depression in Neurological Disorders: Migraine, Malignant Stroke, Subarachnoid and Intracranial Hemorrhage, and Traumatic Brain Injury** *J Cereb Blood Flow Metab* **31**:17–35 https://doi.org/10.1038/jcbfm.2010.191

Liu G., Choi S., Tsien R. W. (1999) **Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices** *Neuron* **22**:395–409

Marvin J. S., Borghuis B. G., Tian L., Cichon J., Harnett M. T., Akerboom J., et al. (2013) **An optimized fluorescent probe for visualizing glutamate neurotransmission** *Nat. Methods* **10**:162–170 https://doi.org/10.1038/nmeth.2333

Meyer J., Gerkau N. J., Kafitz K. W., Patting M., Jolmes F., Henneberger C., et al. (2022) **Rapid Fluorescence Lifetime Imaging Reveals That TRPV4 Channels Promote Dysregulation of Neuronal Na+ in Ischemia** *J. Neurosci* **42**:552–566 https://doi.org/10.1523/JNEUROSCI.0819-21.2021

Mies G., Paschen W. (1984) **Regional changes of blood flow, glucose, and ATP content determined on brain sections during a single passage of spreading depression in rat brain cortex** *Experimental Neurology* **84**:249–258 https://doi.org/10.1016/0014-4886(84)90222-X

Minge D., Senkov O., Kaushik R., Herde M. K., Tikhobrazova O., Wulff A. B., et al. (2017) **Heparan Sulfates Support Pyramidal Cell Excitability, Synaptic Plasticity, and Context Discrimination** *Cereb. Cortex* **27**:903–918 https://doi.org/10.1093/cercor/bhx003

Mitterdorfer J., Bean B. P. (2002) **Potassium Currents during the Action Potential of Hippocampal CA3 Neurons** *J. Neurosci* **22**:10106–10115

Nicholson C. (1993) **Ion-selective microelectrodes and diffusion measurements as tools to explore the brain cell microenvironment** *J. Neurosci. Methods* **48**:199–213 https://doi.org/10.1016/0165-0270(93)90092-6

Padamsey Z., Emptage N. (2014) **Two sides to long-term potentiation: a view towards reconciliation** *Philosophical Transactions of the Royal Society B: Biological Sciences* **369** https://doi.org/10.1098/rstb.2013.0154

Pape N., Rose C. R. (2023) **Activation of TRPV4 channels promotes the loss of cellular ATP in organotypic slices of the mouse neocortex exposed to chemical ischemia** *The Journal of Physiology* **601**:2975–2990 https://doi.org/10.1113/JP284430

Passlick S., Rose C. R., Petzold G. C., Henneberger C. (2021) **Disruption of Glutamate Transport and Homeostasis by Acute Metabolic Stress** *Front Cell Neurosci* **15** https://doi.org/10.3389/fncel.2021.637784

Qian J., Saggau P. (1999) **Modulation of Transmitter Release by Action Potential Duration at the Hippocampal CA3-CA1 Synapse** *J. Neurophysiol* **81**:288–298 https://doi.org/10.1152/jn.1999.81.1.288

Rakers C., Petzold G. C. (2017) **Astrocytic calcium release mediates peri-infarct depolarizations in a rodent stroke model** *J Clin Invest* **127**:511–516 https://doi.org/10.1172/JCI89354

Romanos J., Benke D., Saab A. S., Zeilhofer H. U., Santello M. (2019) **Differences in glutamate uptake between cortical regions impact neuronal NMDA receptor activation** *Commun Biol* **2**:1–15 https://doi.org/10.1038/s42003-019-0367-9

Rossi D. J., Brady J. D., Mohr C. (2007) **Astrocyte metabolism and signaling during brain ischemia** *Nat. Neurosci* **10**:1377–1386 https://doi.org/10.1038/nn2004

Rossi D. J., Oshima T., Attwell D. (2000) **Glutamate release in severe brain ischaemia is mainly by reversed uptake** *Nature* **403**:316–321 https://doi.org/10.1038/35002090

Stein E. S., Itsekson-Hayosh Z., Aronovich A., Reisner Y., Bushi D., Pick C. G., et al. (2015) **Thrombin induces ischemic LTP (iLTP): implications for synaptic plasticity in the acute phase of ischemic stroke** *Sci. Rep* **5** https://doi.org/10.1038/srep07912

Syková E., Nicholson C. (2008) **Diffusion in Brain Extracellular Space** *Physiol. Rev* **88**:1277–1340 https://doi.org/10.1152/physrev.00027.2007

Tanaka E., Yamamoto S., Kudo Y., Mihara S., Higashi H. (1997) **Mechanisms Underlying the Rapid Depolarization Produced by Deprivation of Oxygen and Glucose in Rat Hippocampal CA1 Neurons In Vitro** *Journal of Neurophysiology* **78**:891–902 https://doi.org/10.1152/jn.1997.78.2.891

Vaden J. H., Banumurthy G., Gusarevich E. S., Overstreet-Wadiche L., Wadiche J. I. (2019) **The readily-releasable pool dynamically regulates multivesicular release** *eLife* **8** https://doi.org/10.7554/eLife.47434

Wheeler D. B., Randall A., Tsien R. W. (1996) **Changes in action potential duration alter reliance of excitatory synaptic transmission on multiple types of Ca2+ channels in rat hippocampus** *J. Neurosci* **16**:2226–2237 https://doi.org/10.1523/JNEUROSCI.16-07-02226.1996

Yang J., Vitery M. del C., Chen J., Osei-Owusu J., Chu J., Qiu Z. (2019) **Glutamate-Releasing SWELL1 Channel in Astrocytes Modulates Synaptic Transmission and Promotes Brain Damage in Stroke** *Neuron* **102**:813–827 https://doi.org/10.1016/j.neuron.2019.03.029

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Reviewer #1 (Public Review):

Summary:

This work by Passlick and colleagues set out to reveal the mechanism by which short bouts of ischemia perturb glutamate signalling. This manuscript builds upon previous work in the field that reported a paradoxical increase in synaptic transmission following acute, transient ischemia termed ischemic or anoxic long-term potentiation. Despite these observations how this occurs and the involvement of glutamate release and uptake mechanisms remained unanswered.

Here the authors employed two distinct chemical ischemia models, one lasting 2-minutes, the other 5-minutes. Recording evoked field excitatory postsynaptic potentials in acute brain slices, the authors revealed that shorter bouts of ischemia resulted in a transient decrease in postsynaptic responses followed by an overshoot and long-term potentiation. Longer bouts of chemical ischemia (5-minutes), however, resulted in synaptic failure that did not return to baseline levels over 50-minutes of recording (Figure 1).

Two-photon Imaging of fluorescent glutamate sensor iGluSnFR expressed in astrocytes matched postsynaptic responses with shorter ischemia resulting in a transient dip before increase in extracellular glutamate which was not the case with prolonged ischemia (Figure 2).

Mechanistically, the authors show that this increased glutamate levels and postsynaptic responses were not due to changes in glutamate clearance (Figure 3). Next using a competitive antagonist for postsynaptic AMPA receptors the authors show that synaptic glutamate release was enhanced by 2-minute chemical ischemia.

Taken together, these data reveal the underlying mechanism regarding ischemic long-term potentiation, highlighting presynaptic release as the primary culprit. Additionally, the authors show relative insensitivity of glutamate uptake mechanisms during ischemia, highlighting the resilience of astrocytes to this metabolic challenge.

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Reviewer #2 (Public Review):

Summary:

To investigate the impact of chemical ischemia induced by blocking mitochondrial function and glycolysis, the authors measured extracellular field potentials, performed whole-cell patch-clamp recordings, and measured glutamate release with optical techniques. They found that shorter two-minutes-lasting blockade of energy production initially blocked synaptic transmission but subsequently caused a potentiation of synaptic transmission due to increased glutamate release. In contrast, longer five-minutes-lasting blockage of energy production caused a sustained decrease of synaptic transmission. A correlation between the increase of extracellular potassium concentration and the response upon chemical ischemia indicates that the severity of the ischemia determines whether synapses potentiate or depress upon chemical ischemia. A subsequent mechanistic analysis revealed that the speed of uptake of glutamate is unchanged. An increase in the duration of the fiber volley reflecting the extracellular voltage of the action potentials of the axon bundle was interpreted as an action potential broadening, which could provide mechanistic explanation. In summary, the data convincingly demonstrate that synaptic potentiation induced by chemical ischemia is caused by increased glutamate release.

Strengths:

The manuscript is well written, and the experiments are carefully designed. The results are exciting, novel, and important for the field. The main strength of the manuscript is the combination of electrophysiological recordings and optical glutamate imaging. The main conclusion of increased glutamate release was furthermore supported with an independent approach relying on a low-affinity competitive antagonist of glutamate receptors. The data are of exceptional quality. Several important controls were carefully performed, such as the stability of the recordings and the size of the extracellular space. The number of experiments are sufficient for the conclusions. The careful data analysis justifies the classification of two types of responses, namely synaptic potentiation and depression after chemical ischemia. The data are carefully discussed and the conclusions are justified.

Weaknesses:

The weaknesses are minor. The authors measured the fiber volley, which reflects the extracellular voltage of the compound action potential of the fiber bundle. The half-duration of the fiber volley was increased. These results are consistent with action potential broadening in the axons but the action potential broadening was not experimentally demonstrated. However, these results are carefully discussed.

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Reviewer #3 (Public Review):

Summary:

This valuable study shows that shorter episodes (2min duration) of energy depletion, as it occurs in ischemia, could lead to long lasting dysregulation of synaptic transmission with presynaptic alterations of glutamate release at the CA3-CA1 synapses. A longer duration of chemical ischemia (5 min) permanently suppresses synaptic transmission. By using electrophysiological approaches, including field and patch clamp recordings, combined to imaging studies, the authors demonstrated that 2 min of chemical ischemia leads to a prolonged potentiation of synaptic activity with a long lasting increase of glutamate release from presynaptic terminals. This was observed as an increase in iGluSnFR fluorescence, a sensor for glutamate expressed selectively on hippocampal astrocytes by viral injection. The increase in iGluSnFR fluorescence upon 2 min chemical ischemia could not be ascribed to an altered glutamate uptake, which is unaffected by both 2 min and 5 min chemical ischemia.

The presynaptic increase in glutamate release upon short episodes of chemical ischemia is confirmed by a reduced inhibitory effect of the competitive antagonist gamma-D-glutamylglycine on AMPA receptor mediated postsynaptic responses. Fiber volley durations in field recording are prolonged in slices exposed to 2 min chemical ischemia. The authors interpret this data as an indication that the increase in glutamate release could be ascribed to a prolongation of the presynaptic action potential possibly due to inactivation of voltage-dependent K+ channels. However, more direct evidence are needed to fully support this hypothesis. This research highlights an important mechanism by which altered ionic homeostasis underlying metabolic failure can impact on neuronal activity. Moreover, it also showed a different vulnerability of mechanisms involved in glutamatergic transmission with a marked resilience of glutamate uptake to chemical ischemia.

Strengths:

(1) The authors use a variety of experimental techniques ranging from electrophysiology to imaging to study the contribution of several mechanisms underlying the effect of chemical ischemia on synaptic transmission.
(2) The experiments are appropriately designed and clearly described in the figures and in the text.
(3) The controls are appropriate

Weaknesses:
- The results are obtained in an ex-vivo preparation

Impact:

This study provides a more comprehensive view of the long term effects of energy depletion during short episodes of experimental ischemia leading to the notion that not only post-synaptic changes, as reported by others, but also presynaptic changes are responsible for long-lasting modification of synaptic transmission. Interestingly, the direction of synaptic changes is bidirectional and dependent on the duration of chemical ischemia, indicating that different mechanisms involved in synaptic transmission are differently affected by energy depletion.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
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> ***Reviewer #1 (Public Review):***
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> *[...]*
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> *Weaknesses:*
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> *The question of the physiological relevance of short bouts of ischemia remains.*

The chemical ischemia protocol induces a duration-dependent ATP depletion in acute slices on a time scale of minutes (Pape and Rose 2023). This is about the same time scale as the peri-infarct depolarisation (Lauritzen et al. 2011) that the protocol attempts to model. Of course, such models do not completely replicate the complex situation in vivo. However, the presented analyses of synapse function cannot be performed in vivo. We discuss this now in the manuscript.

> *The precise mechanisms underlying the shift between ischemia-induced long-term potentiation and long-term failure of synaptic responses were not addressed. Could this be cell death?*

Thank you for the comment. Yes, we indeed believe that the persistent failure of synaptic transmission is because of neuronal cell death (i.e., of CA1 pyramidal cells) or at least persistent depolarisation. We did not explicitly state that in the original submission but do so in the revised manuscript. It is supported by the unquantified observation of swelling and/or loss of integrity of CA1 pyramidal cell bodies in parallel to postsynaptic failure. It is also in line with many reports from the literature, of which we now cite two (lines 186-198).

> *Sex differences are not addressed or considered.*

We have performed all experiments on male mice, as indicated in Material and Methods. We have indeed not addressed sex differences of the observed effects. We consider this, and many other important factors, to be interesting topics for follow-up studies. This is now discussed (lines 413-424).

> ***Reviewer #2 (Public Review):***
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> *[…]*
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> *Weaknesses:*
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> *The weaknesses are minor and only relate to the interpretation of some of the data regarding the presynaptic mechanisms causing the potentiation of release. The authors measured the fiber volley, which reflects the extracellular voltage of the compound action potential of the fiber bundle. The half-duration of the fiber volley was increased, which could be due to the action potential broadening of the individual axons but could also be due to differences in conduction velocity. We are therefore skeptical whether the conclusion of action broadening is justified.*

These are excellent points. We have added an analysis demonstrating that axonal conduction velocity is unlikely to be affected. Nonetheless, the fiber volley is indeed an indirect measure of what happens in individual axons. We have adjusted our interpretation accordingly and now also discuss alternative explanations of our findings (lines 363-379).

> ***Reviewer #3 (Public Review):***
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> *[…]*
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> *Weaknesses:*
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> *The data on fiber volley duration should be supported by more direct measurements to prove that chemical ischemia increases presynaptic Ca2+ influx due to a presynaptic broadening of action potentials. Given the influence that positioning of the stimulating and recording electrode can have on the fiber volley properties, I found this data insufficient to support the assumption of a relationship between increased iGluSnFR fluorescence, action potential broadening, and increased presynaptic Ca2+ levels.*

We have added a new analysis showing that the latency of the fiber volley is unaffected and relatively constant, which strengthens our conclusion. But the fiber volley is indeed an indirect measure of action potential firing in individual axons. The suggested experiment, which would require simultaneous recording of Ca2+ and action potentials in single axons in combination with chemical ischemia, is extremely difficult, if possible at all. Instead, we have

extended the discussion and include now further alternative mechanistic explanations (lines 363-379).

> *The results are obtained in an ex-vivo preparation, it would be interesting to assess if they could be replicated in vivo models of cerebral ischemia.*

This would certainly be very interesting but also extremely challenging technically. For a detailed analysis of synaptic changes as presented here, the main difficulty will be to stimulate and visualise glutamate release exclusively in an isolated population of synapses while recording postsynaptic responses in a stroke model.

> ***Recommendations For The Authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *[…]*
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> *Labelling of experimental groups of 2-minute and 5-minute chemical ischemia is more accurate than "metabolic stress" and "with postsynaptic failure". The critical difference between these two conditions is lost with this nomenclature. The reader could be misled to believe that the two groups form a heterogenous population of responses from the same experimental manipulation which is incorrect.*

We had stated in the manuscript that we ' … grouped combined iGluSnFR and electrophysiological recordings according to the effect of chemical ischemia on the synaptic response: 'chemical ischemia with postsynaptic failure' if the postsynaptic response did not recover to above 50% of the baseline level and 'chemical ischemia' when it did (as indicated in Fig. 1H). …'. The recordings were not grouped according to chemical stress duration but according to the effect on the postsynaptic response. We have revised the text explaining this (lines 125-135) and illustrate that now also in Fig. 1H. We hope this is easier to follow now.

> *More details on the long-term impact of 5-minute ischemia on cell viability would be enlightening regarding the specific mechanism separating these two conditions. With 2 minutes it would appear that cells remain alive (i.e. intact post-synaptic responses), 5 minutes however, inducing cell death.*

Yes, our observations, although not quantified, are in line with cell death as CA1 pyramidal cell bodies appeared swollen and/or lost their integrity when chemical ischemia was followed by postsynaptic failure. This is also in line with reports from the literature. We have revised the results section accordingly (lines 186-201).

> *In the paragraph titled "glutamate uptake is unaffected after acute chemical ischemia", there are two erroneous citations of Figure S3 that should be Figure S4.*

Thank you. We corrected this mistake.

> *The sex of animals is not given. This is essential information.*

We used male mice as indicated in the initial version of the manuscript (Material and Methods). We have added a statement regarding the role of sex to the final section of the Discussion.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *We propose addressing the weaknesses mentioned in the public review. As said, the fibre volley is a very indirect measure of action potential broadening. Based on the iGluSnFR data, the authors predict that the potentiation is mediated by depolarization, action potential broadening, and increased presynaptic calcium influx. The latter could be tested experimentally, but this does not seem necessary if the data are interpreted more cautiously. For example, other explanations for the broadened fiber volley could be mentioned, such as a slowing and/or dispersion of the action potential propagation speed. Furthermore, depolarization could cause elevated resting calcium concentrations, which could potentiate release independently of action potential broadening. Finally, classical forms of presynaptic potentiation of the release machinery that occur during homeostatic plasticity or Hebbian plasticity may operate independently of calcium dynamics.*

Thank you for this comment. The discussion of the mechanism was indeed too short. We have added an analysis of the fiber volley delay after stimulation, which was not affected. Presynaptic action potential broadening is, in our opinion, a very likely explanation for our observations but we did not perform direct experiments. Directly recording presynaptic action potentials and Ca2+ transients in the chemical ischemia model over extended periods of time is a major technical challenge and certainly of interest in the future. As suggested, we have expanded the discussion section and now mention various alternative explanations (lines 363-379).

> *There are the following minor suggestions:*
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> *Add line numbers.*

We have added line numbers.

> *We would suggest providing exact P values instead of asterisks in the figures.*

We agree that having exact P values in the figure panels can be very helpful. However, in the present figures they are hard to integrate without overcrowding the already complex panels and thereby obscuring other important details. All p-values are included in the figure legends and/or main text.

> *Abstract: "We also observed an unexpected hierarchy of vulnerability of the involved mechanisms and cell types." This sentence is hard to understand and cell types were not directly compared (i.e. axons of CA3 and axons of CA1 neurons were not compared).*

We have revised this statement and removed the reference to cell types.

> *In Figure 1G there seems to be an increase in the fiber volley. Is this significant? Could this be due to swelling of the slice during chemical ischemia? Or an increase in excitability? Maybe this could be discussed.*

The effect was analysed in the context of Fig. 2. A significant increase of the fiber volley amplitude was detected in chemical ischemia (Fig. 2H) but also under control conditions (Fig. 2F). We therefore consider this a change that is detectable but not related to chemical ischemia and not a potential explanation for increased glutamate release (lines 157-160). Also, no significant fiber volley increase was detected in chemical ischemia with postsynaptic failure (Fig. 2H) and in the experiments illustrated in Fig. 4E. Our interpretation is that the

fiber volley unspecifically increases in some experiments over the time course of the experiment (~ 60 min) but this is unrelated to chemical ischemia.

> *In the results: "A fully separate set of experiments..." Please explain better what this means.*

We have revised the entire section to explain more clearly how recordings were grouped (lines 125135).

> *In the results: "...(Syková and Nicholson, 2008) (Figure S3). However, this was not observed for chemical ischemia without postsynaptic failure (Figure S3), in which the increased glutamate transients were observed." This should probably refer to Figure S4.*

Thank you for spotting this mistake. We corrected it.

> *The last sentence in the results "... most likely by increased presynaptic Ca2+ influx, and, at the same time, the postsynaptic response." This is difficult to understand. Does "at the same time" refer to another mechanism or the consequence of more Ca2+?*

We revised this part of the results section to improve clarity and toned down our conclusions (lines 328-335 and 363-379).

> ***Reviewer #3 (Recommendations For The Authors):***
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> *There are a few points that the author needs to clarify:*
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> *The authors do not discuss the different behaviour of iGlu F0 during chemical ischemia and chemical ischemia with postsynaptic failure shown in Figure 2, panels D and E. In the first case, during the application of the solution to induce ischemia, iGluF0 decreases while in the other case, it strongly increases before falling down. In both cases, the fEPSP slope is decreased. How does the author explain this observation?*

We attribute the transient increase of extracellular glutamate during prolonged chemical ischemia to the increase of synaptic glutamate release observed previously under such conditions (Hershkowitz et al. 1993; Tanaka et al. 1997) and other mechanisms reviewed by us (Passlick et al. 2021) (e.g., glial glutamate release, transiently reduced glutamate uptake), which we could not detect during shorter chemical ischemia. The initial drop of the fEPSP slope is most likely due to postsynaptic depolarisation, which is followed by a repolarisation if the chemical stress duration is short. We now explain this in more detail in lines 185-200 of the revised manuscript. Although we focussed on the bi-directional effect on longer timescales in this manuscript, this transient phase during chemical ischemia is very interesting for further investigations.

> *On page 8, first line, I think that the authors meant Figure S4, not Figure S3 when they mentioned results on ECS diffusivity and ECS fraction.*

Yes, thank you for spotting this. We corrected the mistake.

> *In Supplementary Figure 5 panel B It seems that PPR is significantly reduced upon chemical ischemia (asterisk on columns green) but the authors claimed in the paper at page 10 that "Analysing the paired-pulse ratio (PPR) of postsynaptic response and iGluSnFR transients revealed no consistent changes after chemical ischemia (Figure S5).". Did the authors refer to the data normalized in panel D? In this case, I do not see the need to normalize raw data that have been already shown in a previous panel and that*

> *give different statistical results, probably due to the different tests used (paired in panel B and not paired in panel D).*

We have clarified this point in the supplementary material (Figure S5, legend). There is a relevant difference between the analyses presented in panel B and D. The paired test presented in B analyses the change of the electrophysiological PPR in response to chemical ischemia. The test in D on the electrophysiologically PPR asks if the reduction in B is significantly different from the changes seen under control conditions. Because it is not, we conclude that chemical ischemia has no relevant effect on the electrophysiological PPR and, in combination with the results on the iGluSnFR PPR, also not on short-term plasticity, as tested here.

References

Hershkowitz N, Katchman AN, Veregge S. Site of synaptic depression during hypoxia: a patch-clamp analysis. Journal of Neurophysiology 69: 432–441, 1993.

Lauritzen M, Dreier JP, Fabricius M, Hartings JA, Graf R, Strong AJ. Clinical Relevance of Cortical Spreading Depression in Neurological Disorders: Migraine, Malignant Stroke, Subarachnoid and Intracranial Hemorrhage, and Traumatic Brain Injury. J Cereb Blood Flow Metab 31: 17–35, 2011.

Pape N, Rose CR. Activation of TRPV4 channels promotes the loss of cellular ATP in organotypic slices of the mouse neocortex exposed to chemical ischemia. The Journal of Physiology 601: 2975–2990, 2023.

Passlick S, Rose CR, Petzold GC, Henneberger C. Disruption of Glutamate Transport and Homeostasis by Acute Metabolic Stress. Front Cell Neurosci 15: 637784, 2021.

Tanaka E, Yamamoto S, Kudo Y, Mihara S, Higashi H. Mechanisms Underlying the Rapid

Depolarization Produced by Deprivation of Oxygen and Glucose in Rat Hippocampal CA1 Neurons In Vitro. Journal of Neurophysiology 78: 891–902, 1997.

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