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Mild stimulation improves neuronal survival in an *in vitro* model of the ischemic penumbraLorenzo Muzzi^{1,2}, Gerco Hassink¹, Marloes Levers¹, Maikel Jansman¹, Monica Frega¹, Jeannette Hofmeijer^{1,3}, Michel van Putten^{1,4} and Joost le Feber^{1,5,6} ¹ Clinical Neurophysiology, University of Twente, PO Box 217, 7500AE Enschede, The Netherlands² Department of Informatics, Bioengineering, Robotics and System Engineering (DIBRIS), University of Genova, Via All'Opera Pia 13, 16145 Genova, Italy³ Department of Neurology, Rijnstate Hospital, Arnhem, The Netherlands⁴ Department of Clinical Neurophysiology, Medisch Spectrum Twente Hospital, Enschede, The Netherlands⁵ Clinical Neurophysiology, Technohal TL3382, University of Twente, PO Box 217, 7500 AE, Enschede, The Netherlands⁶ Author to whom any correspondence should be addressed.E-mail: j.lefeber@utwente.nl**Keywords:** ischemic penumbra, electrophysiology, optogenetic stimulation, electrical stimulation, activity homeostasis, neuronal survivalRECEIVED
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**Abstract**

Objective. In the core of a brain infarct, characterized by severely reduced blood supply, loss of neuronal function is rapidly followed by neuronal death. In peripheral areas of the infarct, the penumbra, damage is initially reversible, and neuronal activity is typically reduced due to ischemia-induced synaptic failure. There is limited understanding of factors governing neuronal recovery or the transition to irreversible damage. Neuronal activity has been shown to be crucial for survival. Consequently, hypoxia induced neuronal inactivity may contribute to cell death, and activation of penumbral neurons possibly improves survival. Adversely, activation increases ATP demand, and a balance should be found between the available energy and sufficient activity. **Approach.** We monitored activity and viability of neurons in an *in vitro* model of the penumbra, consisting of (rat) neuronal networks on micro electrode arrays (MEAs) under controlled hypoxic conditions. We tested effects of optogenetic and electrical activation during hypoxia. **Main results.** Mild stimulation yielded significantly better recovery of activity immediately after re-oxygenation, compared with no stimulation, and a 60%–70% higher survival rate after 5 d. Stronger stimulation was not associated with better recovery than no stimulation, suggesting that beneficial effects depend on a delicate balance between sufficient activity and available energy. **Significance.** We show that mild activation during hypoxia/ischemia is beneficial for cell survival in an *in vitro* model of the penumbra. This finding opposes the current common belief that suppression of neuronal activity is the cornerstone of neuroprotection during cerebral ischemia, and may open new possibilities for the treatment of secondary brain damage after stroke.

Introduction

In the core of a brain infarct, which is characterized by severely impeded perfusion, loss of neuronal function is followed by neuronal death within minutes. In peripheral areas of the infarct with some remaining perfusion from surrounding arteries, the penumbra, neurons initially remain structurally intact and viable, but their function is significantly compromised. Synaptic failure is one of the early consequences of cerebral ischemia and probably accounts for electric silence in the penumbra [1–3]. Synaptic failure and

neuronal hypo-activity are reversible with timely restoration of blood flow [2–5]. However, in the absence of reperfusion, reversible synaptic failure may be followed by irreversible neuronal damage. Unravelling the mechanisms that govern neurons to recover or die in the ischemic penumbra may lead to identification of treatment targets.

Most evidence supports the notion that ischemic synaptic failure occurs primarily at the presynaptic terminal [5–7], due to reduced endo- and exocytosis [8, 9], decreased phosphorylation of presynaptic proteins [5], and adenosine mediated mechanisms [6, 10]. It has

been hypothesized that suppression of synaptic activity during ischemia is a compensatory mechanism to restore the balance between oxygen supply and energy consumption in favour of maintaining resting potentials [11]. Based on this assumption, many proposed neuroprotective treatments are based on suppression of neuronal activity. However, none has been proven effective in clinical trials [12]. Conversely, persisting synaptic failure has been associated with progression towards irreversible neuronal damage, even in the absence of membrane depolarization. Previous *in vitro* studies showed that the probability of recovery is related to the depth and duration of hypoxia [3]; longer periods or deeper hypoxia are associated with lower probability of recovery. The mechanism, however, that relates hypoxic depth and duration to the probability of recovery is not yet known.

A recent study showed that the density of inhibitory synapses decreased during 6–12 h of severe hypoxia while the excitatory density remained unaffected, thus increasing network excitability [13]. Electrophysiological experiments showed that inhibitory neurons are more vulnerable to hypoxia than excitatory neurons [3], which also increased network excitability. Both findings suggest that networks adapt to hypoxia induced low activity, aiming to re-increase the total amount of activity. The observation that the total amount of activity generally decreased, the notion that neurons must be active to survive [11, 14], and the existence of such adaptive network mechanisms, suggest that insufficient neuronal activity may ultimately contribute to cell death. Supporting this suggestion, pharmacological activation of *in vitro* cultures after severe hypoxia showed a positive effect on culture viability [15]. Beneficial effects of activation during hypoxia, however, have not yet been demonstrated. Neuronal activation increases the demand for ATP, which is scarce under hypoxic conditions, and should be accomplished very carefully.

In the current study, we created a penumbra model using neuronal networks cultured on micro electrode arrays (MEAs) to monitor neuronal viability under controlled hypoxic conditions and following re-oxygenation. We tested different stimulation strategies during hypoxia to validate the hypothesis that prevention of hypo-activity under hypoxic conditions may improve activity recovery after re-oxygenation, as well as survival after several days. Optogenetic activation was used to stimulate all excitatory neurons in the culture, first in a closed-loop protocol and then in a fixed stimulation protocol using settings that evolved from the closed-loop protocol. Activity recovery upon re-oxygenation and neuronal activity after five days were compared to those of cultures that were electrically stimulated only in certain areas (2–3 electrodes), and to unstimulated cultures.

Material and methods

Cell cultures

Cells were obtained from brain cortices of newborn Wistar rats (Janvier Labs, France) on the day of the birth. After decapitation, brains were dissected and the cortices isolated (placed in chemically defined R12 medium [16]). After trypsin treatment, cells were dissociated by trituration. We plated ~200 000 neurons (200 μ l suspension) on a micro-electrode array (MEA; Multi Channel Systems, Reutlingen, Germany), precoated with poly-ethylene-imine (PEI). A circular chamber with an inner diameter of 20 mm was glued on top of the MEA to create a culture chamber, which was filled with approximately 700 μ l R12 medium. MEAs were stored in an incubator under standard conditions of 37 °C, high humidity, and 5% CO₂. Twice a week, approximately half of the medium was refreshed. All experiments were conducted according to the Dutch law (Wet op de Dierproeven) and approved by the Dutch committee on animal use (Centrale Commissie Dierproeven). Results are presented in compliance with the ARRIVE guidelines.

Viral transfection

Adeno associated virus (AAV) was obtained from Penn Vector Core, Philadelphia, Pennsylvania, USA. The virus was delivered as serotype 2.1, containing the ChannelRhodopsin-2 gene, fused to mCherry, driven by the CaMKII α promoter, which is found exclusively in excitatory neurons. The ChannelRhodopsin-2 gene contained a mutation (H134R) to make it useable for optogenetic research (making it sensitive for blue light 470 nm [17]). The initial volume of virus with a physical titre of $\sim 1.31 \times 10^{13}$ GC ml⁻¹ was diluted 100 times in DBPS, and cultures were transduced with 50 μ l or 100 μ l at the day after plating or at day *in vitro* 4 (DIV 4). From images taken at DIV1 ($n = 22$) all cells were manually counted in an area of 0.04 mm² in the middle of the electrode zone, which yielded an estimated mean density of 5700 ± 700 cells mm⁻², and a total number of $\sim 171\,000$ cells per MEA. Assuming that the functional titre was ~ 10 times lower than the physical titre, we reached an estimated MOI (Multiplicity of infection) of $4 \cdot 10^3$ or $8 \cdot 10^3$, which is in range with MOIs shown to be effective in recent studies [18–20]. After initial evaluation we chose to transfect cultures at DIV1. Adequate transfection was confirmed by the expression of mCherry fluorescence in the second week post-transfection, using an EVOSTM fluorescence microscope (Thermo Fischer Scientific) and an EVOSTM Light Cube, Texas RedTM (Thermo Fischer Scientific, excitation: 585/29 nm, emission: 628/32 nm).

Data acquisition and hypoxia induction

MEAs (60 TiN electrodes; diameter 30 μm , 200 μm pitch) and measurement setup (MEA1060-BC preamplifier and STG1002 stimulus generator) manufactured by Multi Channel Systems (Reutlingen, Germany) were used to record activity and to electrically stimulate neurons. During measurements, MEAs were sealed with a membrane permeable (Multichannel Systems; ALA Scientific) to O_2 and CO_2 but not to water, and placed under a Plexiglas hood. Under this hood a humidified gas mixture of air and N_2 , complemented with 5% CO_2 , was blown over the setup at a rate of 2 L/min. Mixtures of air and N_2 could be delivered at any ratio and were computer controlled by mass flow controllers (Vögtlin Instruments, Switzerland). Normoxic conditions were realized by setting the flow controllers to 100% air, and hypoxic conditions by setting flow controllers to 10% air and 90% N_2 (yielding $p\text{O}_2 \approx 20$ mmHg [3]). We will refer to these conditions as normoxia and hypoxia, respectively. The temperature at the bottom of the MEA was kept at 36.5 °C. Recordings were obtained at a sample rate of 16 kHz per channel using a custom LabVIEW program (National Instruments, Austin, TX), driving an NI PCI-6071E ADC-board. All analogue signals were band-pass filtered (100–6 kHz) before sampling. Only cultures that showed spontaneous activity were used in this study.

Light sensitivity

Power LEDs on a SinkPAD-II 20 mm Star Base (Blue (470 nm)–74 lm@700 mA, or yellow (591 nm)–75 lm@350 mA, from LuxeonStarLEDs) were placed approximately 7 cm above the top of the MEA. A Faraday cage, created by a stainless steel mesh between the LEDs and the MEA reduced electrically induced artefacts by the LED power cables (figure 1(A)).

Electrical activity was recorded while stimulating with yellow or blue light, and we verified that all cultures were insensitive to the yellow light. Light pulses of varying width (50, 200, 500, or 1000 ms) and intensity (0.19, 2.5, 9.2, or 11.1 klx) were delivered in a pseudo randomized order (30 pulses for each parameter setting) every 10 s. We obtained a stimulus-response curve for every combination of pulse width and light intensity. For visualization, the time of stimulation was set at $t = 0$, the horizontal axis shows the delay after the stimulus (≤ 1000 ms), the vertical axis shows the average spikes counts in 10 ms bins. Tuning curves were constructed to show the dependency of responses on stimulus duration and intensity. Curves of all cultures were normalized to the maximum response recorded in that culture to enable comparison between cultures ($N = 6$).

Experimental protocols

Experiments consisted of a 6 h baseline measurement (normoxia), followed by 24 h of hypoxia and again 6 h after re-oxygenation (see figure 2). The mean

network activity (in spikes/min) during baseline was calculated and an activity threshold was set at 45% of baseline activity, an arbitrary value below baseline, but higher than hypoxic activity when not stimulated [3]. During hypoxia the mean activity was calculated every minute, and optogenetic stimulation was started (1) when the activity dropped below this threshold (closed loop experiments), (2) at fixed time points during the hypoxic period (fixed stimulation experiments), or (3) cultures were left unstimulated, although transfected (control experiments).

In closed loop experiments, the initial number of stimulus pulses per minute was set to zero. The number of pulses per minute increased whenever the recorded activity in the previous minute was below threshold and decreased if the activity was above threshold. Stimuli were evenly distributed, with a maximum of 12/min. When this maximum was reached, stimulus pulses were adjusted following one of two protocols: (1) the cycle was repeated with constant intensity (9.2 klx) and increased pulse duration (10, 50, 100, 200, 500, 600, 700, 800, 900, 1000 ms) and the number of pulses reset to 1 stim/min, or (2) the cycle was repeated with increased light intensity (2.5, 4.3, 6.2, 7.8, 9.2, 10.7 and 11.1 klx), pulse width fixed at 200 ms and the number of pulses was reset to 1 stim/min. When the stimulus reached the maximum intensity, the number of stimulations per minute was set to 1 and, in contrast to protocol 1, were not increased anymore (figure 2). The first protocol resulted in much stronger stimulation than the second (see results), and we will refer to these protocols as strong and mild closed-loop activation, respectively. These names were chosen to qualitatively distinguish between both protocols, and do not represent a quantification of the stimulation strength.

Occasionally one or two neurons showed increased, tonic firing during hypoxia, possibly due to disinhibition [3, 21]. To avoid an unbalanced estimation of the total activity we set a threshold for tonic firing, usually at 120 spikes min^{-1} . If activity recorded at a single electrode exceeded the tonic threshold, or if $\geq 50\%$ of the baseline activity was detected at a single electrode, that electrode was excluded from the activity estimation. In some experiments this threshold was increased because of the presence of one or more tonically firing electrodes during baseline.

For practical application, closed-loop stimulation is rather complicated. To evaluate whether activation could be beneficial without closed-loop control, eleven cultures were stimulated at a fixed frequency, based on the parameter settings that appeared beneficial in the closed-loop protocols (1 pulse min^{-1} , intensity 9.2 klx, and pulse duration 200 ms). Stimulation started 30 min after the onset of hypoxia.

The mean age of the cultures used was 19.7 ± 4.7 DIV. All cultures were returned to the incubator after the 36 h experiments. 5 ± 2 d after the experiment cultures were placed in the recording setup again to check whether they were still active. Cultures were

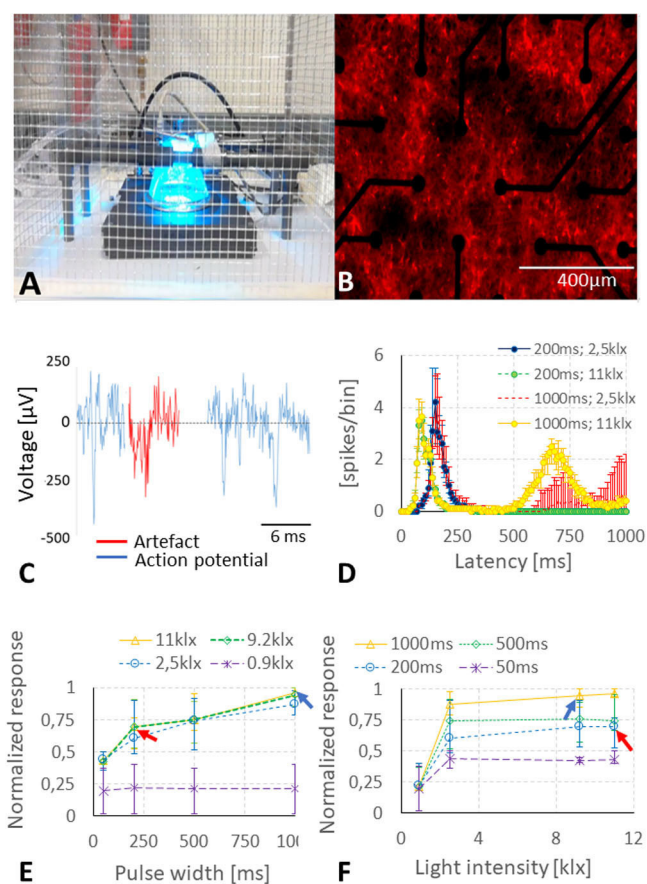


Figure 1. AAV transfection and stimulus responses. (A–B) MEA set-up for light stimulation and a representative fluorescence image of a culture transfected at DIV1 with $50 \mu\text{l}$ of virus (photo obtained at DIV11; scale bar: $400 \mu\text{m}$). (C) Example of raw data recorded by one electrode during baseline under normoxia. Noise levels were estimated for each electrode and continuously updated. Whenever the voltage traces crossed the threshold of 5.5 times (RMS) noise, an event was detected and timestamp, channel number and 6 ms of waveshape was stored. Artefacts were detected offline (see Methods), and are indicated in red. (D) Effect of pulse intensity and duration on stimulus response. Each curve shows the median value of the responses to $n = 30$ equal stimuli in 10 ms bins. Error bars indicate the 25%–75% quartiles. (E–F) Tuning curves obtained from six cultures. For each stimulus parameter setting, we obtained the median number of spikes with latencies ≤ 1 s. Before averaging across cultures, all curves were normalized to their respective maximum values. Error bars indicate the 25%–75% and reflect differences between cultures. Arrows indicate the strongest stimulus reached for protocol 1 (blue) and protocol 2 (red). Responses to maximum stimulation in protocol 2 were $\leq 75\%$ of responses to maximum stimulation in protocol 1, and we will refer to protocol 1 as strong closed loop activation and to protocol 2 as mild closed loop activation.

considered active if it was possible to record activity from at least half of all electrodes that were firing during the baseline recording of that culture.

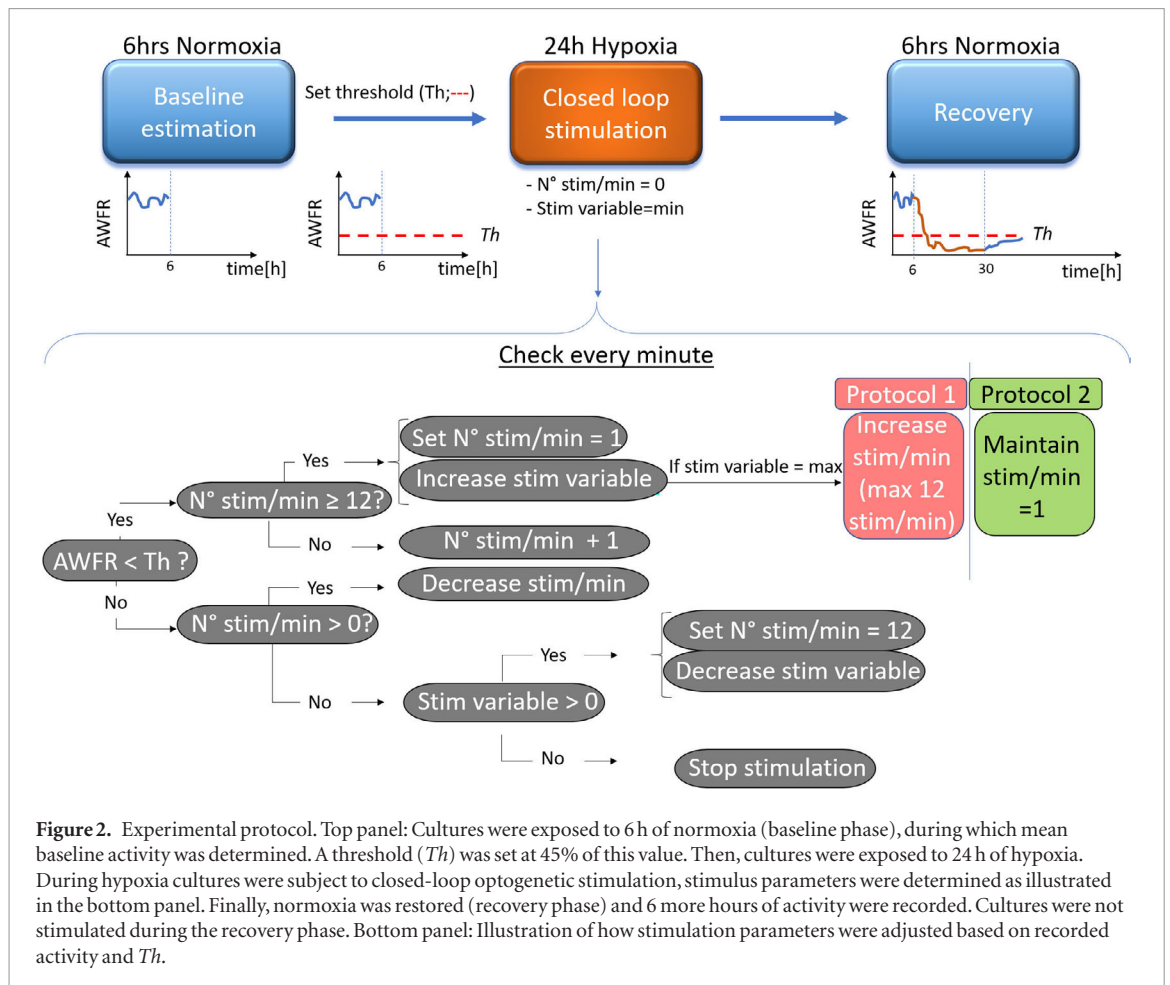
Electrical stimulation

We reanalysed eight experiments from earlier work [3] that were electrically stimulated during hypoxia of the same depth and duration. From these available data, three cultures were excluded; two showed strongly increased activity at 1–2 electrodes during hypoxia, probably due to disinhibition [3], and one had been previously treated with chemical agents that might affect cell viability. At the beginning of each experiment, 2–3 electrodes were selected for stimulation during the first 10 min of every hour with an inter pulse interval of 5–10 s. In these recordings baseline conditions were applied for 2 h and the post-hypoxia period lasted at least 2 h, further details can be found in [3].

Analysis

Spike and artefact detection

A threshold based spike detection algorithm was used that stored timestamps, channel numbers (electrodes), and 6 ms waveshape of detected event. This approach enabled recordings of 36 h while minimizing the required disk space. Noise estimates were obtained for each single channel and updated throughout experiments. Spikes were detected whenever the signal crossed a threshold of 5.5 times the root mean square noise level. Artefacts were detected offline using a wave shape-based algorithm adapted from Wagenaar *et al* [22], and removed. In short, an action potential is considered valid if three criteria are satisfied. First, there should be no peak higher than or equal to the peaks' amplitude within 1 ms around the main peak, in either polarity. Secondly, there should be no peak or line crossing the 90% threshold line. Finally, there



should be no peak or line crossing the 50% outer threshold line (see figure 1(C)).

Effect of activation

To quantify how well cultures survived 24h of hypoxia, we analysed (1) the amount of activity and (2) the number of active electrodes directly after re-oxygenation. Furthermore, (3) ~5 min of spontaneous activity was recorded 5 ± 2 d after the experiments to determine viability. Cultures with no activity were considered dead. These data were not available for the electrically stimulated cultures. For the quantification of the amount of activity, we determined the array-wide firing rate (AWFR) as the summed number of action potentials of all electrodes in 1h time bins. To compare experiments, all curves were normalized to the average baseline AWFR and aligned at the onset and cessation of the hypoxic period. Similar to AWFR, the number of active electrodes (NAE) was determined as the summed number of active electrodes in 1h time bins. An electrode was considered active if it recorded at least 60 spikes in that bin.

Sensitivity to stimulation per electrode

The sensitivity to stimulation is a measure to quantify the propensity of neurons in contact with a specific electrode to respond to light stimulation at a certain time. We monitored these values to verify that cultures continued to be optogenetically activated, even under

severe hypoxic conditions. We considered only those electrodes in which at least 100 spikes were recorded during the entire hypoxic phase. This allowed for inclusion of electrodes that were not spontaneously active, or that became inactive rapidly after the onset of hypoxia. For each of these electrodes we considered the entire spike train, divided in 10 min bins.

In each bin, i , all stimulations were counted ($N_{stim}[i]$), and all spikes that were recorded within 1 s after a stimulus were summed ($N_{response}[i]$). To estimate the spontaneous background activity ($N_{spontaneous}[i]$) in each bin, $N_{response}[i]$ was subtracted from the total number of spikes detected in the bin ($N_{total}[i]$). Mean response firing rate was determined as $FR_{response}[i] = N_{response}[i]/N_{stim}[i]$, and mean spontaneous firing rate was determined as $FR_{spontaneous}[i] = N_{spontaneous}[i]/(600 - N_{stim}[i])$.

The sensitivity to stimulation per electrode in bin i ($SSE[i]$) was calculated as $SSE[i] = FR_{response}[i]/FR_{spontaneous}[i]$. $SSE > 1$ was interpreted as a measurable response to stimulation.

Statistical analysis

Main readouts were the mean array wide firing rate (AWFR) and the mean number of active electrodes (NAE) after reoxygenation. Differences between groups of treated and untreated cultures were statistically evaluated using the two-tailed Mann-Whitney U-test. $P < 0.05$ was considered significant.

Results

Efficacy of transfection

We observed a diverse expression of mCherry across samples transfected under equal conditions. In general, cultures transduced at DIV1 tended to show more efficient mCherry expression than cultures transduced at DIV4, while higher virus concentrations showed more rapid expression. At the concentrations used, mCherry was generally well expressed after ~11 d, and cultures responded to light stimulation. Almost all cultures >14DIV and >10 d post transfection showed a network burst when illuminated with blue light, while some showed only few activated electrodes. None of the samples responded to yellow light. The shape of stimulus response curves differed between cultures and depended on pulse width and intensity. Figure 1(D) shows a representative example of responses to stimulation with varying intensity and duration. Despite the varying timing of responses, tuning curves were comparable across cultures (figures 1(E) and (F)). Cultures were more sensitive to an increase of the light pulse duration than to an increase of light intensity. In fact, beyond 4.3 klx network responses saturated, whereas increasing pulse width yielded increasingly larger responses (figures 1(E) and (F)). We determined the sensitivity to stimulation (SSE) from light stimulated experiments. Figure 3 shows three examples of stimulus responses in cultures exposed to strong (figure 3(A)) or mild closed loop activation (figure 3(B)), or fixed stimulation (figure 3(C)). On average, stimulus responses decreased during hypoxia, but remained measurable, confirming that optogenetic stimulation was able to elicit a response even after a long period of hypoxia. (figure 3).

Transfected controls

Seven transfected cultures were exposed to hypoxia, but not stimulated with light. In all of these cultures AWRFR dropped to almost zero during hypoxia and the number of active electrodes dropped to ~20% of baseline. Both parameters showed no recovery ($p > 0.95$) after return to normoxia (figures 4(A) and (C)). None of them showed any activity when checked 5 ± 2 d after the experiments (figure 5(E)).

Closed-loop stimulation

Four cultures were subjected to strong closed-loop activation (increasing pulse duration, with constant intensity at 9.2 klx). All four cultures showed little activity and very few active electrodes during hypoxia, and no recovery after return to normoxia (figures 4(A) and (C)). None of these cultures showed any activity when checked 5 ± 2 d after the experiments (figure 5(E)).

Six other cultures were subjected to mild closed-loop activation (increasing light intensity, with constant pulse duration at 200 ms). In these experiments, the activity during hypoxia remained around 10% of

baseline activity, and partially recovered after return to normoxia to ~20% of baseline activity (figure 4(A)). Recovery as assessed by post-hypoxia AWRFR was significantly better than in control and strong closed-loop activation experiments ($p < 0.01$; figure 4(B)). Also, the NAE was higher than in control/strong closed-loop activation experiments and showed better recovery after re-oxygenation (~50% of baseline NAE; $p < 0.03$). In addition, four cultures (67%) were still active when checked 5 ± 2 d after the experiments (figure 5(E)).

Mild fixed stimulation

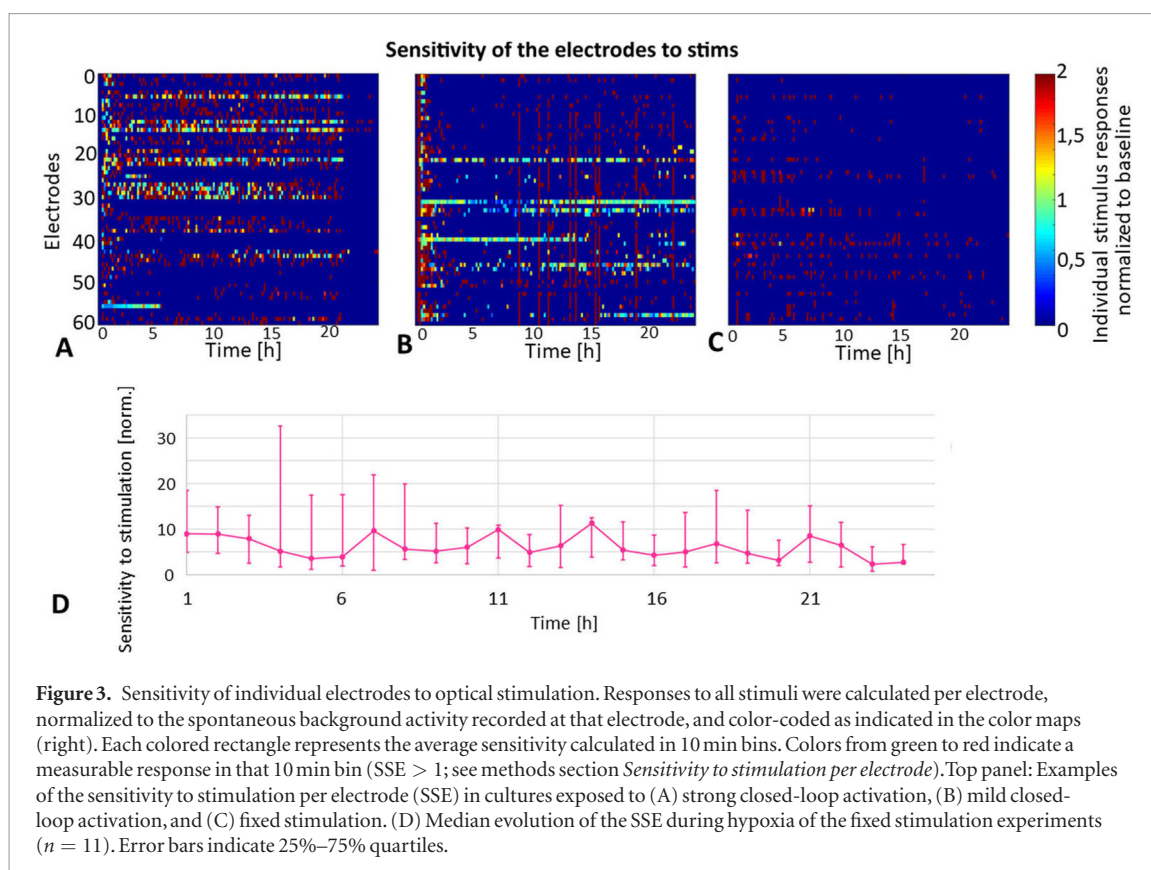
Eleven cultures were stimulated at a constant rate of 1 pulse min^{-1} (200 ms; 9.2 klx) during hypoxia. Activity during hypoxia was around 10%–15% of baseline activity, slightly higher than samples treated with mild closed-loop activation. After re-oxygenation median activity remained around 10% of baseline (figure 5, purple line), but the range of the 75% quartile strongly increased. The high 75% quartiles in this curve reflect that median values were biased by the four cultures that did not survive hypoxia. Mean activity after re-oxygenation was substantially higher than median activity (~25% of baseline activity, which tended to be better than controls ($p < 0.07$)). Significant recovery after re-oxygenation in surviving cultures was confirmed when the curve was redrawn for the seven cultures that survived (figure 5, dashed pink line). The number of active electrodes tended to be higher than in control cultures, but differences were not significant (figure 5(C)). Seven cultures (63%) remained active 5 ± 2 d after the experiments, similar to the fraction achieved with mild closed-loop activation (figure 5(E)).

Electrical stimulation

Five cultures were included for analysis. Activity during hypoxia fluctuated between 15% and 20% of baseline activity and remained in this range after re-oxygenation (figure 5(A), yellow line). Post-hypoxia activity following electrical stimulation was significantly higher than in control cultures ($p < 0.003$). During hypoxia, NAE initially remained around 30%–40% of baseline, slightly decreased to ~20% in the last ten hours of hypoxia, and remained around this level after re-oxygenation (figure 5(C)), comparable to control cultures ($p > 0.2$).

Discussion

With this study, we assessed the effects of neuronal stimulation during hypoxia/ischemia in cultured neuronal networks, which served as a model system for the ischemic penumbra. We show that moderate to severe hypoxia leads to a considerable decrease of neuronal activity. We further show that mild stimulation during 24 h of hypoxia enhances activity recovery immediately after re-oxygenation, as well as



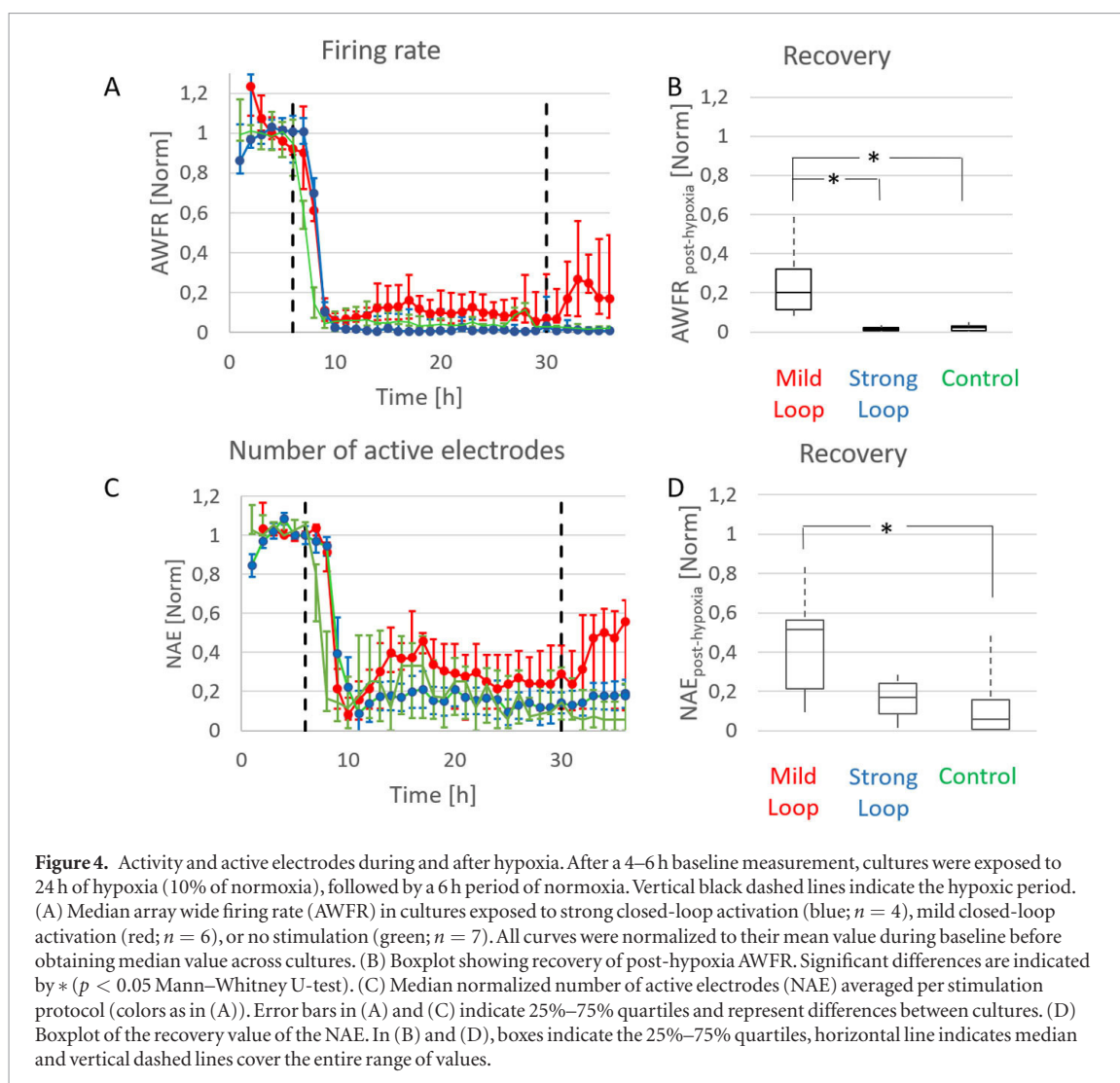
neuronal survival after five days. This finding stands perpendicular to the current common belief that suppression of neuronal activity is the cornerstone of neuroprotection during cerebral ischemia [12]. Apparently, both remaining ATP and remaining activity levels are crucial for neuronal survival. The notion that activity is vital for neuronal survival is not new [14, 23–26], but here we show for the first time that activity is crucial for cell survival, even during hypoxia-induced energy scarcity.

Although stimulus responses tended to decrease with increasing hypoxia duration, in general, activation under low oxygen conditions remained possible (figure 3). Under normoxic conditions, stimuli with pulse width ≥ 500 ms often induced two response peaks (particularly in highly active cultures), separated by 300–400 ms, reflecting a sort of response refractory period during which light could not activate the network. Similar results were obtained in a recent *in vivo* study [27]. Increasing pulse width induced stronger responses. In contrast, responses rapidly saturated with increasing light intensity. Thus, under control conditions protocol 1 (increasing pulse width) provided a stronger stimulus. Remarkably, it resulted in less activity during hypoxia and less recovery after re-oxygenation. Possibly, cultures were overstimulated during hypoxia in these experiments, causing an aggravation of the cells' condition. While activity in all stimulated cultures was higher than in unstimulated cultures during the first two hours of hypoxia, it tended to be highest in cultures exposed to strong closed-loop activation, especially during the first hour.

Particularly under hypoxic conditions, cells may be sensitive to overstimulation as action potential firing consumes energy that would otherwise be available to preserve the membrane potential. Cultures exposed to mild closed-loop activation received milder stimulation, as increasing intensity hardly induced larger responses, and the number of pulses per minute at maximum intensity was limited to one in this protocol. This yielded significantly better recovery of activity immediately after re-oxygenation, and four out of six cultures exposed to this milder regime were still spontaneously active a few days after the experiment. These findings confirm that mild stimulation during severe hypoxia can be beneficial to the neurons if overstimulation can be avoided.

If beneficial effects depend on the delicate balance between activity and available energy (ATP), it should be possible to obtain better results in a closed-loop protocol. The set threshold of 45% of baseline activity was not achieved during hypoxia in any of the closed-loop experiments, and, consequently, fixed optogenetic stimulation was able to yield the same activity recovery and survival. Determination of the optimum balance between activity and energy is essential to maximize the neuroprotective effect of activation during hypoxia.

Similar to transfected cultures exposed to mild closed-loop activation, cultures that were electrically stimulated were more active during hypoxia than unstimulated cultures. Recovery after re-oxygenation was also significantly better than recovery in unstimulated cultures ($p < 0.003$). The number of active

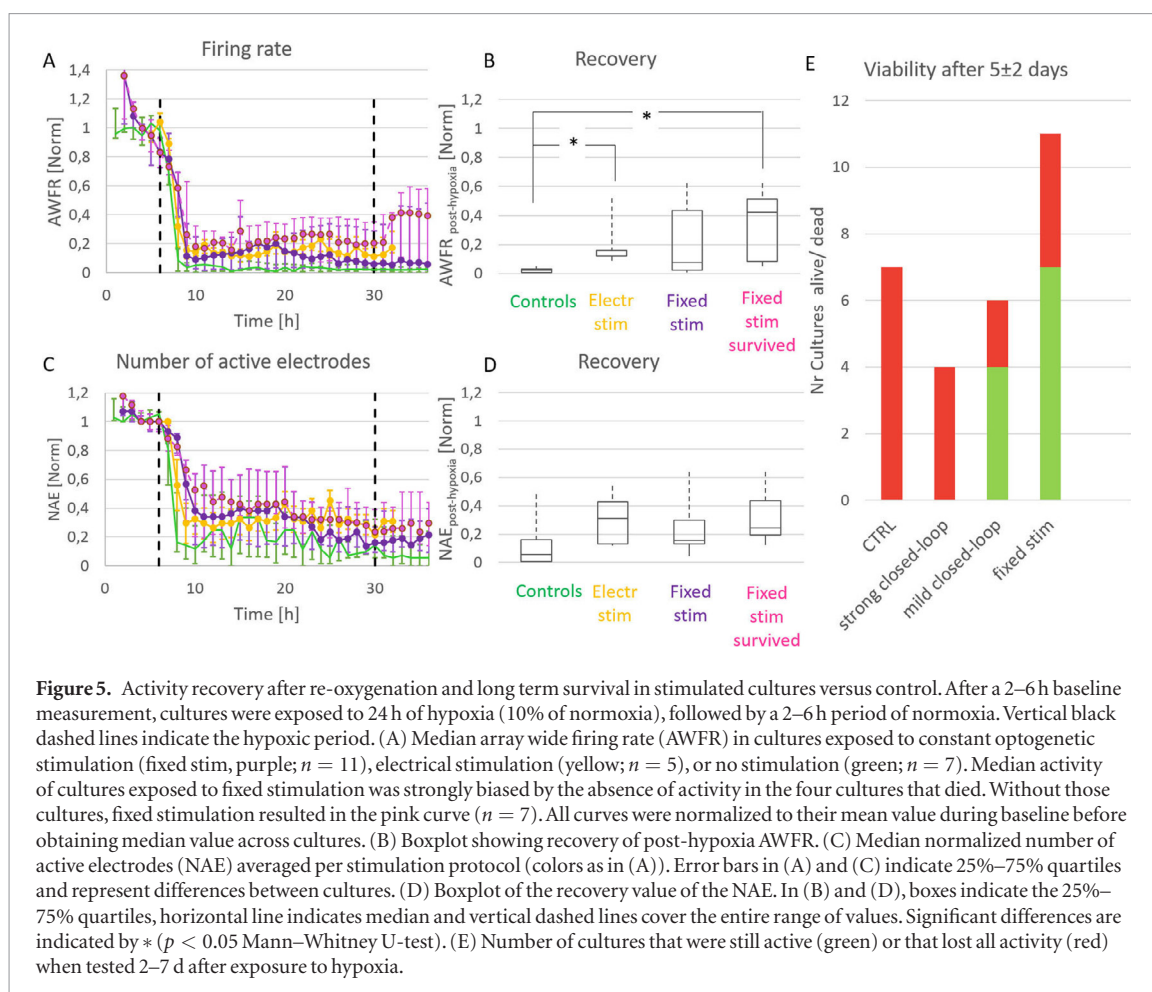


electrodes, however, was less than in light stimulated cultures exposed to protocol, possibly reflecting the different nature of both stimulation techniques. Electrical stimulation will directly activate only a subset of neurons that are in the near vicinity of the stimulated electrode, or with an axon running near that electrode. Under control conditions, this initial response usually triggers a network response through synaptic propagation [9, 28], but under hypoxic condition, this second phase is absent due to wide spread synaptic failure [2, 3, 29]. In the transfected cultures, all excitatory neurons hit by the light were stimulated, resulting in more efficient stimulation. These differences may explain why electrical stimulation yielded favourable results at much higher stimulation frequency than fixed optogenetic stimulation and why the NAE during recovery was higher in optogenetically stimulated cultures.

Although optogenetic stimulation during hypoxia did improve activity recovery immediately after reoxygenation and longer-term survival, the biological mechanisms that lead to improved recovery remain unclear. Several findings support the view that insufficient activation may lead to neuronal death. Heck *et al* found a significant increase in the number of apoptotic neurons in organotypic cortical slices as early as 6 h

after tetrodotoxin (ttx)—induced silencing [24], while the condition of neurons is not negatively affected if ttx is washed out within a few hours [30]. Fishbein *et al* also found apoptotic cell death in ttx-silenced cultures, which was attributed to impeded calcium clearance and upscaled mEPSCs. Interestingly, inhibitory neurons generally survived ttx-induced silencing better than excitatory neurons. Removal of ttx did not reverse the death process, once initiated [25]. In a later study also the overall inhibitory influence of high Mg^{2+} was shown to negatively affect neuronal survival [26], further supporting the notion that neurons must be active to survive. Conversely, environmental stimuli aiming to increase the level of brain activity, decreased naturally occurring apoptosis by 45% in the rat hippocampus [31]. Evidence obtained from cultured rat cortical neurons suggests that activity dependent cell survival is related to the expression of brain-derived neurotrophic factor (BDNF) [14]. BDNF levels depend on (regularity of) neuronal activation and may form the link between inactivity and (apoptotic) neuronal death.

If apoptosis is triggered by low BDNF concentrations, avoiding extended periods without activity may be more relevant than maintaining average activity



above a certain minimum. Recent studies [32, 33] postulate that not only the mere levels of electrical activity but also specific activity patterns are essential in counterbalancing default pro-apoptotic signal. In addition, it may be beneficial to postpone stimulation to 3–6 h after the onset of hypoxia. Earlier research showed that activity after 3–6 h of hypoxia usually recovers to baseline values [3], indicating that the decreased activity during hypoxia is initially mediated by a reversible mechanism, possibly aiming to preserve energy. This mechanism alone may be insufficient to ensure recovery after longer hypoxic periods, when stimulation would become beneficial.

Possible limitations of the penumbra model used here include the interpretation of oxygen levels and the unrestricted availability of glucose. In the normoxic *in vivo* rat brain, $pO_2 \approx 30\text{--}35$ mmHg [34, 35]. Dissociated cortical neurons are usually cultured under atmospheric pO_2 , which is much higher than physiological oxygen pressure *in vivo*. The observation that all cultures responded immediately to decreasing pO_2 , far before it dropped below 30–35 mmHg, suggests that cultures are adapted to atmospheric pO_2 , and that $pO_2 \approx 20$ mmHg should be interpreted as severe hypoxia. In the ischemic penumbra, the availability of both glucose and oxygen are restricted. *In vivo*, additional processes, possibly related to the limited avail-

ability of glucose may occur in parallel to the processes observed in our model system.

Sensitivity to hypoxia increases with maturation of the cortex [21]. This phenomenon also occurs in cultures: Di Loretto and Balestrino showed that very young cultures (up to 6 DIV) were highly resistant against hypoxia. In older cultures, sensitivity to hypoxia increased until ~ 18 DIV [36]. Similar observations were obtained in cortical slices, where sensitivity to hypoxia was significantly less in young (p5–8) or juvenile (p14–18) than in adult cortex ($p > 28$). Thus, obtained results might in principle contain age dependent confounders. However, cultures used in this study were grown for 20 ± 5 d *in vitro*. Cultures are generally considered as mature beyond this age [37–41], and results represent here most likely reflect vulnerability to hypoxia of adult cortex. Large differences in vulnerability to hypoxia have been found between various cell types [42], and the optimum balance between activity and energy may depend on the location of the ischemic penumbra. Other possible differences may result from environmental factors like the suddenly vanished input from the infarct core, which may also affect the level of spontaneous activity. Furthermore, the neuronal cultures used here lack the *in vivo* brain architecture. Consequently, any optimum found for rat cortical neurons cannot be directly trans-

lated to clinical applications. In addition, optogenetic activation may not be the most applicable technique for therapeutic purposes, despite recent developments in gene therapies. Focus of the current study, however, was on general synaptic and neuronal functioning during hypoxia, and it provides a proof of principle that neuronal activation during energy depletion is possible and may be beneficial for cell survival, provided that a good balance can be achieved. A closed-loop optogenetic approach is very suitable to further explore optimum activation criteria.

In conclusion, mild activation during severe hypoxia enhances activity recovery immediately after re-oxygenation and improves culture survival after five days. These findings emphasize the necessity of activity for neuronal survival, even under harsh hypoxic conditions. Stronger stimulation, however, may harm the cells, probably reflecting the delicate balance between activity homeostasis and energy depletion. Current results provide a proof of principle that mild activation during severe hypoxia may be beneficial for cell survival. Whereas possible treatment has focused on energy saving, the notion of activity dependent cell survival has thus far been largely unexplored. Further research is needed to shed light on the underlying biological mechanism.

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Author contribution statement

LM Acquired and analysed data, drafted the manuscript, approved of the final version.

GH Contributed to conception of study, revised the manuscript, and approved of the final version.

ML Acquired data, revised the manuscript, and approved of the final version.

MJ Acquired data and analysed data, approved of the final version.

JH Contributed to conception of study, revised the manuscript, and approved of the final version.

MvP Contributed to conception of study, and approved of final version.

JIF Contributed to the conception and design of study, analysis and interpretation of data, drafted and revised the manuscript, approved of the final version.

Conflict of interest

The Authors declare that there is no conflict of interest.

ORCID iDs

Joost le Feber  <https://orcid.org/0000-0002-0605-1437>

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