Gamma oscillations in the hippocampus require high complex I gene expression and strong functional performance of mitochondria

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Fast neuronal network oscillations in the gamma range (30–90 Hz) have been implicated in complex brain functions such as sensory processing, memory formation and, perhaps, consciousness, and appear to be exceptionally vulnerable to various pathologies. However, both energy demand and mitochondrial performance underlying gamma oscillations are unknown. We investigated the fundamental relationship between acetylcholine-induced gamma oscillations, mitochondrial gene expression and oxidative metabolism in hippocampal slice preparations of mouse and rat by applying electrophysiology, in situ hybridization, quantitative polymerase chain reaction, oxygen sensor microelectrode (interstitial partial oxygen pressure) and imaging of mitochondrial redox state [nicotinamide adenine dinucleotide (phosphate) and flavin adenine dinucleotide fluorescence]. We show that (i) gamma oscillation power, oxygen consumption and expression of complex I (nicotinamide adenine dinucleotide:ubiquinone oxidoreductase) subunits are higher in hippocampal subfield CA3 than in CA1 and dentate gyrus; (ii) the amount of oxygen consumption of gamma oscillations reaches that of seizure-like events; (iii) gamma oscillations are exquisitely sensitive to pharmacological complex I inhibition; and (iv) gamma oscillations utilize mitochondrial oxidative capacity near limit. These data suggest that gamma oscillations are especially energy demanding and require both high complex I expression and strong functional performance of mitochondria. Our study helps to explain the exceptional vulnerability of complex brain functions in ischaemia as well as in neurodegenerative and psychiatric disorders that are associated with mitochondrial dysfunction.

Keywords: gamma oscillations; hippocampus; mitochondrial DNA; oxidative metabolism; seizure
Abbreviations: FAD = flavin adenine dinucleotide; LFP = local field potential; NAD(P)H = nicotinamide adenine dinucleotide (phosphate); pO2 = partial oxygen pressure

Introduction

It is well known that complex brain functions are much more vulnerable to various pathological processes than evoked neuronal responses and ion distributions, a prime example being rapid loss of consciousness and occurrence of electroencephalographic slow-wave activity during brain ischaemia (Hansen, 1985; Verweij et al., 2007). However, despite major relevance for clinical medicine, the underlying mechanisms are hardly understood.
Fast neuronal network oscillations in the gamma range (~30–90 Hz) are thought to provide a temporal matrix for complex brain functions such as sensory processing, memory formation and perhaps consciousness (Bartos et al., 2007; Melloni et al., 2007; Axmacher et al., 2008). For example, gamma oscillations are prominent in the hippocampus, where they might participate in encoding and retrieval of memory traces (Montgomery and Buzsáki, 2007; van Vugt et al., 2010). Gamma oscillations arise from the precise interplay of action potential firing of excitatory glutamatergic pyramidal neurons and fast inhibitory GABAergic interneurons (Hájos et al., 2004; Bartos et al., 2007). As a consequence, alternating pairs of current sinks and sources occur in the tissue (Csicsvari et al., 2003; Mann et al., 2005), which require enhanced Na⁺/K⁺-ATPase activity to restore ionic gradients and to maintain excitability (Attwell and Iadecola, 2002).

Such local ATP consumption in neurons is rapidly counterbalanced by mitochondria via oxidative phosphorylation, mainly in response to changes in substrates and intracellular Ca²⁺ (Kann and Kovács, 2007). The process requires sufficient glucose and O₂ supply (Gjedde et al., 2002; Verweij et al., 2007) as well as proper activities of mitochondrial enzymes such as complex I (NADH:ubiquinone oxidoreductase; Zickermann et al., 2008). Interestingly, this multi-protein complex appears to exert major control over oxidative phosphorylation and to play a crucial role in neurodegenerative diseases (DiMauro and Schon, 2008; Pathak and Davey, 2008).

Based on these clinical and experimental observations, we hypothesized that gamma oscillations might be associated with high energy demand and enhanced mitochondrial oxidative metabolism. This hypothesis was indirectly supported by two reports showing that gamma oscillations correlated tightly with haemodynamic signals in vivo (Niessing et al., 2005) and were highly sensitive to decreases in interstitial partial oxygen pressure (pO₂) in vitro (Huchzermeyer et al., 2008). Nevertheless, the fundamental relationship between gamma oscillations, O₂ consumption, mitochondrial gene expression and mitochondrial functional performance has never been addressed directly.

The present study was designed to explore this relationship in acute hippocampal slices from mouse and in organotypic hippocampal slice cultures from rat. Gamma oscillations were induced by bath application of acetylcholine, which mimics cholinergic input from the septum. Gamma oscillations in vitro share many features with gamma oscillations in vivo and require both neuronal excitation and fast inhibition (Traub et al., 1996; Csicsvari et al., 2003; Bartos et al., 2007).

## Materials and methods

Further details are provided in the ‘Materials and methods’ section of the Supplementary Material.

### Slice preparations

Organotypic hippocampal slice cultures were prepared as described (Kann et al., 2003; Huchzermeyer et al., 2008). Slice cultures were used after 7–12 days in vitro (residual thickness of 180–210 μm). Acute hippocampal slices (400 μm) were prepared using a vibratome (VT 1000 S, Leica, Bensheim, Germany) from brains of C57BL/6 mice that had been decapitated under isoflurane anaesthesia (Kann et al., 2005). Experiments were performed in a custom-built interface recording chamber (95% O₂, 5% CO₂) or under submerged recording condition. For in situ hybridization, mouse brains were rinsed in phosphate buffered saline and immediately stored at −80°C. Brain slices (20 μm) were prepared using a cryostat, thaw-mounted on Superfrost™ slides and kept at −20°C. Animals were housed, cared and sacrificed in accordance with the recommendations of the European Commission and the Berlin Animal Ethics Committee (FE32/08).

### Bath solutions and drugs

Slice preparations were maintained in recording chambers in carbogen (95% O₂, 5% CO₂)-saturated artificial cerebrospinal fluid that contained: 129 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1.8 mM MgSO₄, 1.6 mM CaCl₂, 26 mM NaHCO₃ and 10 mM glucose (pH 7.3). For induction of seizure-like events, MgSO₄ was omitted and KCl was elevated to 5 mM (0 Mg²⁺ artificial cerebrospinal fluid; Kovács et al., 2005). Salts, acetylcholine, rotenone and rhodamine-123 were from Sigma-Aldrich (Taufkirchen, Germany); physostigmine and tetrodotoxin were from Tocris (Biotrend, Köln, Germany).

### Electrophysiology

Direct current-coupled recordings of local field potentials (LFPs) and changes in extracellular potassium concentration ([K⁺]ₑ) were performed with double-barrelled microelectrodes (Heinemann et al., 1990; Kann et al., 2003). Changes in voltage were digitized at 10 kHz (low-pass filters: 3 kHz for LFPs, 0.3 kHz for [K⁺]ₑ; CED 1401 interface, Cambridge Electronic Design, Cambridge, UK). Recording electrodes (LFP/K⁺-sensitive, O₂-sensor) were placed in stratum pyramidale (CA3, CA1) as well as in the granular cell layer (dentate gyrus). For electrical stimulation, a bipolar tungsten electrode was positioned close to the dentate gyrus to activate fibre tracts to CA3.

### Oxygen sensor microelectrode

The O₂ sensor microelectrode (tip diameter of 10 μm, Unisense, Aarhus, Denmark) was connected to a polarographic amplifier (Chemical Microsensor II; Diamond General Development, Ann Arbor, MI, USA) and polarized overnight. Before and after each experiment, O₂ microsensors were individually calibrated (Huchzermeyer et al., 2008). Changes in voltage were low-pass filtered and digitized at 1 kHz (CED 1401).

### Fluorescence recordings of nicotinamide adenine dinucleotide (phosphate), flavin adenine dinucleotide and rhodamine-123

Nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] (excitation 360 ± 15 nm or 760 nm, emission 460 ± 10 nm), flavin adenine dinucleotide (FAD) and rhodamine-123 fluorescence imaging (excitation 490 ± 10 nm, emission 530 ± 10 nm) was previously described in detail (Kann et al., 2005; Huchzermeyer et al., 2008). NAD(P)H and FAD image pairs were recorded at 0.5 Hz using CellR imaging system (Olympus, Hamburg, Germany). Changes in NAD(P)H and FAD fluorescence are presented as changes in ∆F/F₀ (in percentage).
For technical reasons, NAD(P)H and FAD fluorescence imaging was performed under submerged recording conditions (Schuchmann et al., 2001; Kann and Kovács, 2007). For high-spatial resolution images of mitochondria, slice cultures were stained with 5 μM rhodamine-123 and a 2-photon fluorescence microscope (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) was used. To remove haze and pixel shot noise, NAD(P)H images were band-pass filtered offline (Kovács et al., 2005).

**Preparation of Digoxigenin-labelled riboprobes**

Complementary DNA from mouse muscle was used to generate the ~300 bp templates for in situ hybridization probes via polymerase chain reaction using proof-reading Phusion™ polymerase (Finzymes, Espoo, Finland) and gene-specific primers. The products were cloned into pGEM T-easy (Promega, Mannheim, Germany) and verified by automatic sequencing. Contaminating RNases were removed by phenol–chloroform extraction. To generate anti-sense and sense (negative control) Digoxigenin-labelled riboprobes (DIG RNA Labelling Kit, Roche, Grenzach-Wyhlen, Germany), 1 μg of template DNA was incubated with RNA polymerase. After DNase treatment the labelled product was ethanol precipitated and resuspended in diethylpyrocarbonate water.

**In situ hybridization**

Slides were fixed in paraformaldehyde and incubated in acetylation buffer (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0). After prehybridization in hybridization buffer (50% formamide, 5 × saline sodium citrate buffer, 5 × Denhardt’s solution, 150 μg/ml transfer RNA, 50 μg/ml sperm DNA) in situ hybridization was carried out overnight at 60°C using 1 μg/μl specific DIG-labelled riboprobe in hybridization buffer. Slides were washed with increasing stringency, incubated in blocking buffer (5% normal goat serum in buffer 0.1 M Tris pH 8.0; 0.15 M NaCl) and with 150 μl (1:2500) anti-digoxigenin, alkaline phosphatase-conjugated antibody from sheep. Subsequently, the enzymatic colorimetric reaction proceeded over two days with BM DMLB microscope at 63 magnification, one with bright-field illumination (DAPI) for estimation of the nuclear packing density. All images were processed using the open source ImageJ v1.14 software (http://rsb.info.nih.gov/ij/). In situ hybridization images were inverted, regions of interest were demarcated and their integrated density was determined (Supplementary Fig. 2A). For estimation of cellular packing density, we counted DAPI-stained nuclei with the ImageJ ‘watershed’ algorithm (Supplementary Fig. 2B–E). As in situ hybridization signals are neither normally distributed nor linear and can only be evaluated semi-quantitatively on an ordinal scale, we used the non-parametric Mann–Whitney U-test to calculate significance levels. Additionally, the relative signal intensities (in situ hybridization signal density per nucleus) were z-transformed and the z-values were visualized as heat-scale images (Supplementary Figs 2F and 2K).

**Relative quantification of mitochondrial DNA copy numbers**

CA1, CA3 and dentate gyrus were quickly excised from acute hippocampal slices and immediately frozen at ~80°C. Mitochondrial DNA quantification was performed as described (Amthor et al., 2007). The results were depicted as the ratio between mitochondrial DNA and nuclear gene copy numbers.

**Calculations and statistics**

To translate the recorded potential values (mV) in [K+]o, a modified Nernst equation was used (Kann et al., 2005). Power spectra (Fast Fourier transform algorithm), auto- and cross-correlograms were calculated from data segments of 120 s according to standard procedures (Fisahn et al., 1998; Huchzermeyer et al., 2008). Data are reported as mean ± standard error (SEM) and are derived from at least three animal preparations per experimental group. Statistical significance (P < 0.05) was determined as indicated by applying paired Student’s t-test (comparison of two groups), one-way or two-way repeated-measure ANOVAs with Bonferroni’s post hoc test (comparison of multiple groups), and non-parametric Mann–Whitney U-test.

**Results**

**Gamma oscillations in hippocampal slice preparations of mouse and rat**

LFPs were recorded in subfields CA3, CA1 and dentate gyrus in mouse acute slices being stored at the interface between recording solution and gaseous environment (95% O2, 5% CO2, interface condition). Permanent bath application of acetylcholine resulted in robust gamma oscillations in both CA3 and CA1 (Fig. 1A); these were blocked by the competitive muscarinic receptor antagonist, atropine (Fig. 1C). By contrast, gamma oscillations were absent in the dentate gyrus. Highest amplitudes of oscillations were observed in the distal part of CA3 as compared with the proximal part (not shown) and CA1. In auto-correlograms, the mean peak frequency of gamma oscillations was ~39 Hz (n = 9) and did not differ in CA3 and CA1 (Supplementary Table 1). Cross-correlograms showed high synchrony between the oscillations (r = 0.62 ± 0.06; n = 4) with a phase lag of 1.0 ± 0.05 ms (n = 4), indicating propagation of the activity from CA3 to CA1. Fast Fourier transform algorithms revealed high power of oscillations in bins from 30 to 60 Hz in both subfields (Fig. 1B). Each power spectrum in this range was precisely approximated with a single Gaussian fit. Area and height of the fits were significantly greater in CA3 (Supplementary Table 1), clearly demonstrating a higher power of gamma oscillations. Full width at half maximum did not differ in both subfields. We observed a similar pattern of gamma oscillations in acute slices from adult mice (Supplementary Table 2), despite the fact that the amplitudes were somewhat
lower overall. This showed that our electrophysiological data were widely independent of age (p20–30 versus adult). We also obtained similar results from CA3 and CA1 in rat slice cultures for oscillation frequency ($\frac{C41}{C24}$ Hz; $n = 7$), cross-correlation ($r = 0.58$; $n = 5$) and phase lag ($1.19$ ms; $n = 5$).

Also in this preparation, area and height were significantly greater in CA3 while full width at half maximum did not differ (Fig. 2 and Supplementary Table 3). These data consistently show that cholinergically induced gamma oscillations are most prominent in CA3 and propagate to CA1 in slice preparations from rodents (Fisahn et al., 1998). Highest oscillation power in CA3 was also found during kainate-induced gamma oscillations in both mouse (unpublished observation) and rat acute slices (Wójtowicz et al., 2009).
We then investigated whether gamma oscillations were associated with an increase in O₂ consumption.

**Oxygen consumption during gamma oscillations in mouse acute slices**

The Clark-type microelectrode has been commonly used to monitor interstitial pO₂ levels in hippocampal slice preparations (Huchzermeyer et al., 2008) and in the brain in vivo (Offenhauser et al., 2005; Takano et al., 2007). We measured pO₂ at various depths (40–160 μm below the slice surface) in mouse acute slices during spontaneous neuronal network activity and, subsequently, during acetylcholine-induced gamma oscillations that were electrophysiologically verified in each subfield.

Depth profiles showed that pO₂ strongly decreased in spontaneously active slices (Fig. 3A). Blockade of action potentials with tetrodotoxin and pharmacological interference with cellular energy metabolism (NaN₃, 2-Deoxy-D-glucose) demonstrated that this strong pO₂ decrease was attributable to substantial O₂ consumption of active neurons and glial cells (Attwell and Iadecola, 2002) rather than reflecting a general lack of gas penetration in consequence of slice thickness (Supplementary Material). Overall, pO₂ was lower in CA3 (distal part) compared with CA1 (Fig. 3B, lower left histogram), presumably reflecting differences in spontaneous activity and concomitant energy demands.

Importantly, during persistent gamma oscillations (>30 min of induction), pO₂ further decreased significantly in both CA3 and CA1 (Fig. 3B, upper histograms), indicating an additional, sustained increase in O₂ consumption. Calculating the decrease in pO₂ (in %) during gamma oscillations relative to pO₂ during spontaneous activity revealed that O₂ consumption was highest in the distal CA3 (Fig. 3B, lower right histogram), which was consistent with the electrophysiological data (Fig. 1). The fact that higher O₂ consumption in CA3 was only evident at a depth of 160 μm might be explained by the more intact network structure and thus network function at a longer distance from the slice cut surface. This was partially supported by electrophysiological recordings that revealed higher amplitudes of gamma oscillations in the deeper slice layers (Supplementary Material).

These data suggest that persistent gamma oscillations are associated with considerable sustained O₂ consumption and thus require proper mitochondrial oxidative metabolism. We next explored mitochondrial gene expression in the hippocampus.

**Expression profiles of mitochondrial complex I subunits in the mouse hippocampus**

The electron transport chain of mitochondria consists of four enzyme complexes (I, II, III and IV) that are encoded by nuclear and mitochondrial DNA (Wallace, 2005). We focused on complex I (NADH:ubiquinone oxidoreductase) that has a large and complicated enzyme structure, with up to 46 individual subunits (Janssen et al., 2006; Zickermann et al., 2008; Distelmaier et al., 2009) and appears to exert major control over oxidative phosphorylation (Pathak and Davey, 2008). We determined the expression pattern of 33 nuclear-encoded subunits in the hippocampus of wild-type mice by in situ hybridization of messenger RNA and semi-quantitative analysis. In situ hybridization signal intensity was normalized to the nuclear packing density that was estimated by DAPI staining on exactly the same slide, automatic counting and densitometric analysis (Supplementary Fig. 2). For most of the 33 subunits, cluster analysis of relative expression intensities revealed the following spatial pattern (from high to low intensity): distal CA3 > proximal CA3 > CA1 > dentate gyrus (Fig. 4A; the

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**Figure 3** Oxygen consumption during gamma oscillations in mouse acute slices. (A) Depth profiles of interstitial pO₂ ranging from 40 to 160 μm below the slice surface were determined in the distal CA3 (trace) and in CA1 using O₂-sensor microelectrodes. (B) Absolute values of pO₂ in a given slice were obtained during spontaneous network activity (spon) and, subsequently, during gamma oscillations (gam) that were induced by bath application of acetylcholine (2 μM) and physostigmine (400 nM). Note that persistent gamma oscillations had been present for >30 min when the second depth profile was made. The histograms summarize pO₂ values from multiple depth profiles (n = 16) in eight slices. Note that the interstitial pO₂ decreases significantly during gamma oscillations in both CA3 and CA1 (upper histograms) while O₂ consumption during gamma oscillations is highest in CA3 as revealed by normalization (lower right). Recordings were made under interface conditions. *P < 0.05 (two-way repeated-measure ANOVA).
colours depict the average densities of riboprobe signals in each region of interest normalized to nuclear packing density as determined by automatic counting. Examples of highly expressed nuclear-encoded subunits were the flavoproteins Ndufv1 (NADH-binding subunit) and Ndufv2, the iron–sulphur proteins Ndufs1 to Ndufs8 in addition to Ndufa1, Ndufa2, Ndufa10 and Ndufb7 from the hydrophobic fraction. We found similar expression patterns in the hippocampus of mice at the age of p11 and adult mice (Supplementary Figs 3 and 4).

By contrast, mitochondrial DNA copy numbers per cell did not significantly differ in CA3, CA1 and dentate gyrus (Supplementary Fig. 5), which might indicate that the expression patterns reflect variations in complex I composition rather than differences in mitochondrial numbers.

These data suggest subfield-specific compositions of mitochondrial complex I in the hippocampus.

**Oxygen consumption during gamma oscillations in rat slice cultures**

Application of acetylcholine and physostigmine also resulted in robust and persistent gamma oscillations in slice cultures that
were entirely stored in recording solution saturated with a gas mixture of 95% O₂ and 5% CO₂ (submerged condition, solution flow rate at 5 ml/min). Gamma oscillations were associated with a rapid decrease in pO₂ of 93 mmHg in CA3 (Fig. 5A and B), which confirmed our data from mouse acute slices suggesting a considerable increase in O₂ consumption (Fig. 3). The oscillations were accompanied by a transient increase in [K⁺]₀ of 0.56 ± 0.08 mM (n = 12) and a fluctuating, very low steady-state level of <0.1 mM after ~10 min (Fig. 7A), which presumably reflects an optimal distribution of physiological synchronized

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**Figure 5** Oxygen consumption and mitochondrial complex I inhibition in rat slice cultures. (A) The interstitial pO₂ (trace) was monitored in the slice core (100-μm depth) of CA3. Acetylcholine (5 μM) and physostigmine (1 μM) were applied with the bath solution (upper light grey bar). (B) Histogram summarizing absolute values of pO₂ during the six different conditions of the experiment (n = 7). Note the significant increase in O₂ consumption during gamma oscillations (Conditions 2 and 3) and its inhibition by bath application of selective mitochondrial complex I inhibitor, rotenone (1 μM) (Conditions 4–6; lower dark grey bar). Values of pO₂ were taken at the time points as indicated by numbers. (C) Representative LFP recordings in CA3 during the time course of the experiment as shown in (A). Note that robust and persistent gamma oscillations (Conditions 2 and 3) are completely blocked after 4–6 min of rotenone application (Condition 6). (D) Power spectra were calculated for data segments at a given time point (±60 s; as shown in (A)) and precisely approximated with a single Gaussian fit. (E and F) Area and height of the Gaussians fits are significantly smaller when normalizing Condition 3 to Condition 2 as well as Conditions 4 and 5 to Condition 3 (n = 7). Recordings were made under submerged conditions. *P < 0.05 (one-way repeated-measure ANOVA). n.s. = not significant.
neuronal activity in the network and effective K⁺-uptake mechanisms. The initial [K⁺]₀ transient that promotes the induction of gamma oscillations per se (LeBeau et al., 2002) probably explains the slightly higher values in both power of gamma oscillations and O₂ consumption during the first 10–12 min of acetylcholine application (Fig. 5A–F).

To be able to give a ranking, we also determined O₂ consumption in CA3 during seizure-like events that represent strong pathological neuronal activity (Kovács et al., 2005) as well as during electrical stimulation (10 s, 20 Hz) that was adjusted to evoke [K⁺]₀ transients of <2 mM, which was still in the physiological range (Heinemann et al., 1990; Kann et al., 2003). Intriguingly, seizure-like events resulted in transient decreases in pO₂ of ~114 mmHg similar to acetylcholine-induced gamma oscillations while O₂ consumption during electrical stimulation was considerably lower (Fig. 6A and B).

These data suggest that gamma oscillations represent neuronal network activity of high degree and are associated with high O₂ consumption due to enhanced oxidative energy metabolism. We further addressed this aspect by pharmacological interference with complex I activity, which also provided an experimental tool to mimic mitochondrial dysfunction during pathological processes.

### Mitochondrial redox state during gamma oscillations in rat slice cultures

These pharmacological data strongly support our genetic findings and suggest that gamma oscillations require high complex I expression and enhanced mitochondrial performance. We then explored the mitochondrial redox state during gamma oscillations.

#### Inhibition of mitochondrial complex I in rat slice cultures

Rotenone is a potent and selective complex I inhibitor that, because of its lipophilic nature, rapidly interferes with mitochondrial function in slice preparations (Schuchmann et al., 2001; Kann et al., 2003). Fast bath application of rotenone during acetylcholine-induced gamma oscillations promptly reduced O₂ consumption as reflected by a sustained pO₂ increase in CA3 (Fig. 5A and B, Supplementary Fig. 6 for kinetics of recording solution exchange). In some experiments, we applied rotenone for up to 20 min, which resulted in a new steady-state of 83 ± 9 mmHg (n = 3) above baseline pO₂ prior to induction of gamma oscillations (not shown). Intriguingly, rotenone application was also associated with a rapid and complete loss in the power of gamma oscillations within 4–6 min (Fig. 5C–F). Thus, gamma oscillations were exquisitely sensitive to rotenone because its effects on both neuronal activity as evoked by electrical stimulation (10 s, 20 Hz) and seizure-like events were much weaker (Fig. 6C and D). These data are consistent with other reports showing that rotenone had only minor effects on neuronal activity evoked by electrical stimulation (Schuchmann et al., 2001) and on hypoxia-induced spreading depression (Gerich et al., 2006).

Rotenone application also rapidly lowered mitochondrial oxidation rates as indicated by an immediate and sustained increase of NAD(P)H fluorescence and marked alterations of electrically evoked biphasic NAD(P)H fluorescence transients (Fig. 6C), which is discussed below and in detail by Schuchmann et al. (2001). We note that the baseline in [K⁺]₀ was only slightly increased by 0.1 ± 0.05 mM (n = 6) after 15 min of rotenone application and that even longer application duration did not result in spreading depression because of generalized energy failure.

Changes in the intensity of NAD(P)H and FAD fluorescence primarily reflect changes in mitochondrial redox state, in particular in brain slice preparations in which artefacts due to adaptations in blood flow are absent (Lipton, 1973; Kann et al., 2003). Acetylcholine application resulted initially in a biphasic NAD(P)H transient that was followed by a long-lasting elevation of NAD(P)H fluorescence when persistent gamma oscillations were present (Fig. 7A). Thus gamma oscillations are associated with a significant shift in mitochondrial redox state towards reduction that might reflect stimulation of reducing processes (Krebs–Szent–Györgyi cycle, glycolysis) and/or limitation of oxidizing processes (electron transport chain).

To gain further insight into the underlying mechanisms, we also used electrical stimulation (10 s, 20 Hz) to evoke biphasic NAD(P)H and FAD fluorescence transients (Brennan et al., 2006; Huchzermeyer et al., 2008) during different activity states of slice cultures. In the absence of gamma oscillations, biphasic NAD(P)H transients in CA3 were characterized by an initial ‘dip’ component reflecting enhanced oxidation and a sustained ‘overshoot’ component (Fig. 7C). Evoked biphasic FAD transients showed similar characteristics with an inverse shape (‘peak’ and ‘undershoot’) due to different fluorescence properties of the di-nucleotides (Kann and Kovács, 2007). Thus, biphasic NAD(P)H and FAD transients clearly indicate that mitochondrial oxidation can be further increased by electrical stimulation in CA3. In the presence of gamma oscillations (>15 min of induction), components of electrically evoked NAD(P)H and FAD transients differed markedly. Most importantly, the peak component of FAD transients was absent and the dip component of NAD(P)H transients was significantly smaller in amplitude and showed faster kinetics (Fig. 7C). This suggests that the mitochondrial oxidative capacity was already operating near limit during gamma oscillations.

Our observation that electrically evoked NAD(P)H transients showed weaker alterations than FAD transients might be partially explained by the fact that NAD(P)H fluorescence is less specific for mitochondria and that NAD(P)H is cofactor for a variety of extra-mitochondrial reactions (Huang et al., 2002; Kann and Kovács, 2007). Indeed, applying confocal laser scanning microscopy in CA3 clearly demonstrated that NAD(P)H fluorescence originated from both mitochondria and cytosol as compared with specific mitochondrial markers such as rhodamine-123 (Fig. 7B; Kovács et al., 2005).

Amplitudes of electrically evoked [K⁺]₀ transients did not differ in the absence and presence of gamma oscillations (Fig. 7C), indicating the same degree of neuronal activation. Nevertheless, the decay time of [K⁺]₀ transients as electrically evoked during gamma oscillations was significantly prolonged, which might
reflect limitations of Na⁺/K⁺-ATPase activity and/or glial K⁺-buffering (D’Ambrosio et al., 2002).

These data strongly suggest that gamma oscillations are associated with the operation of mitochondrial oxidative capacity near limit.

Discussion

In this study, we addressed the fundamental relationship between gamma oscillations, mitochondrial gene expression and oxidative metabolism in hippocampal slice preparations. Our main findings are: (i) power of gamma oscillations, oxygen consumption and expression of complex I subunits are highest in hippocampal subfield CA3; (ii) oxygen consumption during gamma oscillations is high; (iii) gamma oscillations are exquisitely sensitive to rotenone; and (iv) gamma oscillations make use of mitochondrial oxidative capacity near limit.

Gamma oscillations in hippocampal slice preparations

We report that cholinergically induced gamma oscillations are consistently more prominent in CA3 than in CA1, which is independent from type of preparation, species and developmental stage and in line with in vivo reports (Csicsvari et al., 2003; Montgomery and Buzsáki, 2007). This probably reflects a general feature of the rodent hippocampus because highest oscillation power in CA3 has been also reported for kainate-induced stimulation (ES; 10 s, 20 Hz; black bar) (left) and during seizure-like events (SLE) that occurred spontaneously in the presence of 0 Mg²⁺ artificial cerebrospinal fluid (right, light grey bar). (B) Histogram summarizing decreases in pO₂ (left) and increases in [K⁺]₀ (right) during electrical stimulation (ES; n = 12), gamma oscillations (gam; Condition 3 in Fig. 6; n = 7) and seizure-like events (SLE; n = 6). (C) Changes in NAD(P)H fluorescence and [K⁺]₀ (see also Fig. 7) were recorded in CA3 during spontaneous neuronal network activity (normal artificial cerebrospinal fluid) and additional electrical stimulation (ES; 10 s, 20 Hz; black bars). Application of rotenone (1 μM, grey bar) was associated with a rapid cumulative elevation of NAD(P)H fluorescence (in %F/F₀), indicating less oxidation due to inhibition of mitochondrial complex I. Electrical stimulation after 5 min (15 min) in the presence of rotenone revealed that the dip component of biphasic NAD(P)H transients was reduced by 28 ± 4% (48 ± 4%; n = 6, P < 0.01, respectively), the overshoot component by 43 ± 3% (86 ± 2%; n = 6, P < 0.01, respectively). Electrical stimulation after 5 min (15 min) in the presence of rotenone revealed that transient increases in [K⁺]₀ were reduced by 33 ± 5% (52 ± 5%; n = 9, P < 0.01, respectively). (D) In the presence of rotenone (1 μM, grey bar), the transient increase in [K⁺]₀, which was associated with spontaneous (n = 3) and triggered (n = 2) seizure-like events in 0 Mg²⁺ artificial cerebrospinal fluid (light grey bar), was reduced by 20 ± 7% after 5–7 min (P = 0.05) and by 33 ± 7% after 10–12 min (P < 0.01). Recordings were made under submerged conditions. *P < 0.05 (one-way ANOVA).
Figure 7  Mitochondrial redox state during gamma oscillations in rat slice cultures. (A) Simultaneous recordings of NAD(P)H fluorescence, $[\text{K}^+]_o$, and LFPs (not shown) were made in CA3. Acetylcholine (Ach; 5 μM) and physostigmine (1 μM) were applied with the bath solution (upper grey bar). Note that the increase in $[\text{K}^+]_o$ was initially associated with a biphasic NAD(P)H fluorescence transient (small dip and overshoot component) that transformed into a persistent NAD(P)H elevation (red arrow) when gamma oscillations were fully established (lower grey bar). (B) Images as obtained by confocal laser scanning microscopy in stratum pyramidale of CA3. Note that NAD(P)H fluorescence originated from both mitochondria and cytosol (left) compared with specific mitochondrial marker, rhodamine-123 (right). Neuronal nuclei showed the weakest fluorescence intensity. Scale bars denote 10 μm. (C) Simultaneous recordings of biphasic NAD(P)H and FAD fluorescence transients as well as of $[\text{K}^+]_o$ transients as evoked by electrical stimulation (10 s, 20 Hz; black spots) during spontaneous activity (spon) and in the presence of persistent gamma oscillations (gam) in CA3 (A). Note that during gamma oscillations, amplitudes and kinetics of both NAD(P)H dip and FAD peak were clearly altered (red arrows, histograms). Note that the decay time of $[\text{K}^+]_o$ transients was significantly prolonged (red arrow) during gamma oscillations while the amplitude was unaffected (histograms). Rise and decay times are given for 10–90% intervals. Histograms summarize data from different slice cultures ($n = 7$). Recordings were made under submerged conditions. *$P < 0.05$ (paired Student’s t-tests).
gamma oscillations in both mouse (Kann, unpublished results) and rat acute slices (Wójtowicz et al., 2009). Nevertheless, we note that both power and duration of gamma oscillations might be different in living animals and humans because gamma oscillations in vivo occur in more complex scenarios that are governed by a variety of factors, including intrinsic network properties, transient activation of afferent inputs as well as glutamatergic and cholinergic modulation (Rodríguez et al., 2004; Hentschke et al., 2007; Cardin et al., 2009; Mann and Mody, 2010). Moreover, alterations such as synaptic reorganization occur in long-term hippocampal slice cultures (Zafirov et al., 1994; Gutíerrez and Heinemann, 1999; De Simoni et al., 2003), while other neuronal features are similar to the hippocampus in vivo (Bahr et al., 1995; De Simoni et al., 2003).

The rhythmic synchrony of cholinergically induced hippocampal gamma oscillations is determined by AMPA receptor-mediated excitation and to a large extent by fast GABAA receptor-mediated inhibition (Bartos et al., 2007). In this process, pyramidal cells generate action potentials at 1–3 Hz in vitro and in vivo while fast-spiking interneurons generate action potentials coupled to each gamma cycle (Csicsvari et al., 2003; Hájos et al., 2004). We note that induction of gamma oscillations by receptor agonists or high [K+]o is associated with membrane depolarization and increases in firing rate of both excitatory and inhibitory neurons while the underlying synaptic mechanisms differ (Bartos et al., 2007). Because they were associated with high O2 consumption (see below), we propose that gamma oscillations fundamentally represent physiological neuronal activity of high degree, likely reflecting optimal recruitment of the vast majority of neurons in the network.

By contrast to electrically evoked neuronal activity and seizure-like events, gamma oscillations were especially sensitive to a complex I inhibitor, rotenone. Rotenone might, foremost, affect mitochondrial energy and/or neurotransmitter metabolism of fast-spiking interneurons that contain large numbers of mitochondria with high cytochrome c levels and that are essential for rhythmic synchronization of gamma oscillations (Gulyás et al., 2006; Fuchs et al., 2007; Hájos et al., 2009). Thus, mechanistically the effect of rotenone is most probably closely related to our observation that gamma oscillations were highly sensitive to decreases in interstitial pO2, while electrically evoked neuronal activities were resistant (Huchzermeyer et al., 2008).

High oxygen consumption during gamma oscillations

We demonstrate that gamma oscillations were associated with rapid and sustained decreases in interstitial pO2, reflecting enhanced mitochondrial O2 consumption. Highest O2 consumption was observed in the distal CA3, being consistent with the findings that gamma oscillations and mitochondrial gene expression were also most prominent in this subfield. We note that such decreases in pO2 have been reported in vivo only under exceptional conditions such as inhibition of vasodilatation (Offenhauser et al., 2005) and cortical spreading depression (Takano et al., 2007). Typically, enhanced neuronal activity in vivo is associated with a local rise in blood flow within a few seconds that markedly increases O2 availability (Masamoto et al., 2003; Offenhauser et al., 2005). We mimicked such blood flow responses by hyperoxic experimental conditions while accepting a putatively altered efficacy of O2 consumption in hyperoxic slice layers in consequence of respiratory uncoupling (Andrews et al., 2005). Nevertheless, the decrease in pO2 during sustained gamma oscillations in slice cultures was similar to that recorded during seizure-like events and >2-fold of that recorded during electrical stimulation. Thus, we propose that O2 consumption during gamma oscillations is high. This is supported by our data suggesting utilization of mitochondrial oxidative capacity near limit (see below) and a report on tight correlation between gamma oscillations and haemodynamic signals in vivo (Niessing et al., 2005).

Expression profiles of mitochondrial complex I subunits

For nuclear-encoded complex I subunits the relative expression intensity was highest in the distal CA3. This predominance was found in particular for iron–sulphur subunits carrying important redox centres of complex I (Zickermann et al., 2008), for all subunits related to human diseases (Janssen et al., 2006) and for nuclear-encoded subunits with high homology to Escherichia coli orthologues (Nuo operon), thus comprising the evolutionarily highly conserved core of complex I (Falk-Krzesinski and Wolfe, 1998). Because mitochondrial DNA copy numbers were similar in hippocampal subfields these data suggest high expression levels of complex I subunits in individual mitochondria in CA3. This provides further evidence for tissue- and cell-specific mitochondrial enzyme compositions (Mootha et al., 2003; Gulyás et al., 2006) and might explain the higher vulnerability of CA3 in certain mitochondria-related neurological disorders (Kunz et al., 2000; Kronenberg et al., 2008).

Importantly, the spatial pattern of complex I subunit expression exactly corresponded to gamma oscillation power and O2 consumption in mouse slices, indicating a close relationship. We note that the unique complex I gene expression in the hippocampus might also be relevant for other forms of fast network activities: sharp wave-ripple complexes (~140–200 Hz) that are thought to be crucial for memory consolidation are generated in CA3 and propagate to CA1 in mouse and rat (Maier et al., 2003; Behrens et al., 2005).

Mitochondrial redox state during gamma oscillations

Hyperoxic environments have been required for studying fast network oscillations in slice preparations (Fisahn et al., 1998; Huchzermeyer et al., 2008; Hájos et al., 2009). As a consequence, NAD and FAD pools might be overly oxidized to a certain degree (Mayevsky and Chance, 2007). Nevertheless, prominent dips of NAD(P)H transients (peaks of FAD transients) as evoked by electrical stimulation during spontaneous network activity indicated that mitochondrial oxidation could still be increased under these experimental conditions. Electrically evoked NAD(P)H transients
were similar to those observed in the cortex in vivo, where sustained neuronal activation was associated with large NAD(P)H dips, indicating rapid increases in mitochondrial oxidation and O2 availability (LaManna et al., 1984).

Strikingly, we demonstrate that gamma oscillations caused long-lasting elevations in NAD(P)H fluorescence although the interstitial pO2 was hyperoxic. Moreover, we observed a clear attenuation of electrically evoked NAD(P)H dips and the absence of electrically evoked FAD peaks during gamma oscillations, despite the fact that the stimuli resulted in an additional >15-fold transient increase in [K+]o. These observations most likely reflect that, during gamma oscillations, the electron transport chain and/or its control mechanisms already operate near limit, thus barely permitting a further increase in mitochondrial oxidation. By contrast, glycolysis and the Krebs–Szent–Györgyi cycle activity might be excessively stimulated by increased substrate availability and Ca2+ ions (Chih and Roberts, 2003; Kann and Kovács, 2007), which would result in stronger reduction of dinucleotide pools relative to oxidation. Alternatively, oxidative capacity of the electron transport chain might be limited by a critical decrease in pO2 relative to oxidation. Alternatively, oxidative capacity of the electron transport chain might be limited by a critical decrease in pO2 relative to oxidation.

Functional consequences and clinical relevance

Our genetic and functional data demonstrate high energy demand and requirement of strong functional performance of mitochondria during gamma oscillations. Therefore, our data might help to explain the strong influence of mitochondrial DNA genotype and nuclear-mitochondrial interaction on complex cognitive functions, which was elegantly shown in cross-breeding experiments of mice (Roubertoux et al., 2003).

Moreover, alterations of mitochondrial DNA have been reported to be critically involved in age-dependent memory loss and mood disorder-like phenotypes in rodents (Liu et al., 2002; Kasahara et al., 2006). Thus, our recent study (Huchzemeyer et al., 2008) and the present study might contribute to explain the exceptional vulnerability of complex brain functions in ageing, ischaemia, neurodegenerative and psychiatric diseases that are associated with mitochondrial dysfunction (Wallace, 2005; Verweij et al., 2007; DiMauro and Schon, 2008; Krishnan et al., 2008; Mattson et al., 2008; Uhlhaas et al., 2008).

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Supplementary material

Supplementary material is available at Brain online.

References


