Original Article

Temperature- and concentration-dependence of kainate-induced γ oscillation in rat hippocampal slices under submerged condition

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Aim: Fast neuronal network oscillation at the γ frequency band (γ oscillation: 30–80 Hz) has been studied extensively in hippocampal slices under interface recording condition. The aim of this study is to establish a method for recording γ oscillation in submerged hippocampal slices that allows simultaneously monitoring γ oscillation and the oscillation-related intracellular events, such as intracellular Ca^{2+} concentration or mitochondrial membrane potentials.

Methods: Horizontal hippocampal slices (thickness: 300 μm) of adult rats were prepared and placed in a submerged or an interface chamber. Extracellular field recordings were made in the CA3c pyramidal layer of the slices. Kainate, an AMPA/kainate receptor agonist, was applied via perfusion. Data analysis was performed off-line.

Results: Addition of kainate (25–1000 nmol/L) induced γ oscillation in both the submerged and interface slices. Kainate increased the γ power in a concentration-dependent manner, but the duration of steady state oscillation was reduced at higher concentrations of kainate. Long-lasting γ oscillation was maintained at the concentrations of 100–300 nmol/L. Under submerged condition, γ oscillation was temperature-dependent, with the maximum power achieved at 29°C. The induction of γ oscillation under submerged condition also required a fast rate of perfusion (5–7 mL/min) and showed a fast dynamic during development and after the washout.

Conclusion: The kainate-induced γ oscillation recorded in submerged rat hippocampal slices is useful for studying the intracellular events related to neuronal network activities and may represent a model to reveal the mechanisms underlying the normal neuronal synchronizations and diseased conditions.

Keywords: γ oscillation; neuronal network; hippocampus; kainate; submerged slice

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Introduction

Neuronal network oscillations at the gamma frequency band (γ oscillations, 30–80 Hz) generated in the cortex play an important role in learning and memory^[1,2]. These oscillations provide a timing mechanism for controlling information processing^[1,3]. Impairments of γ oscillations have been associated with Alzheimer's disease^[4] and normal aging. Both *in vivo*^[5] and *in vitro*^[6,7] studies have suggested that intrahippocampally generated γ activity emerges from the rhythmic activity of interneurons that receive converging inputs from pyramidal neurons and control their firing through divergent outputs. The similarity in the timing of the unit discharges to the field potentials observed both *in vivo* and *in vitro* have validated the

in vitro γ models.

In vitro models of hippocampal γ oscillations rely on a depolarizing drive provided by specific agonists for the metabotropic glutamate, muscarinic acetylcholine or kainate receptors. Most *in vitro* oscillations have been shown to be accurate experimental models for the study of *in vivo* γ oscillations^[5,8,9]. Although *in vitro* γ oscillations are primarily studied under interface conditions and have provided valuable information for an understanding of the mechanisms of oscillatory activities, the limitation of this model is its inability to detect intracellular events, such as intracellular calcium concentrations or mitochondrial functions in individual neurons, which are critical for neuronal network oscillations^[10]. To study intracellular events related to γ oscillations, an *in vitro* γ recording under submerged conditions is essential. Previous studies reported the difficulty of inducing and maintaining γ oscillations under submerged conditions^[7,11,12]. Here, we aim to develop

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a method for recording persistent γ oscillations under submerged conditions that is capable of simultaneously monitoring γ oscillations and oscillation-related intracellular events.

By optimizing the experimental conditions, we established a method in which persistent γ oscillations can be reliably induced in the hippocampal CA3 area and that provides a superior model for the study of cellular mechanisms underlying γ oscillations.

Materials and methods

Animal model

All procedures were carried out under UK home office license and in accordance with the regulation of the UK Animals Act, 1998 and associated guidance. All efforts were made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to *in vivo* techniques, if available.

Young adult rats (3–4 months) were anesthetized by intraperitoneal injection of a ketamine (76 mg/kg)/medetomidine (1 mg/kg) mixture and then sacrificed by cervical dislocation. The brain was quickly removed and immersed in an ice-cold sucrose-ACSF solution saturated with 95% O₂/5% CO₂ and contained the following (in mmol/L): 189 sucrose, 2.5 KCl, 0.5 CaCl₂, 10 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose. Horizontal hippocampal slices were cut into 300- μ m sections using an Integraslice (Campden Instruments, UK). The slices were then stored in an interface chamber at room temperature (22–23°C) bubbled with a mixture of 95% O₂/5% CO₂.

Test procedure

A slice was placed in an interface or submerged chamber and allowed to equilibrate for an hour in artificial ACSF before recording. The bath temperature was measured using a digital temperature meter (Hanna Instruments, Ann Arbor, Michigan, USA). Temperature alterations in the recording chambers were achieved by either increasing or decreasing the water temperature in the water bath. For interface slices, the temperature and perfusion rate were maintained at 31–32°C and 2–3 mL/min, respectively. The optimal conditions for γ activity in the submerged slices were 28–29°C and 5–7 mL/min for temperature and perfusion rate, respectively. The composition of ACSF was (in mmol/L) 125 NaCl, 26 NaHCO₃, 3 KCl, 2 CaCl₂, 1.25 NaH₂PO₄, 1 MgCl₂, and 10 D-glucose, and its pH was equilibrated with a mixture of 95% O₂/5% CO₂ at pH 7.4. The slices were visualized with a stereo microscope (Leica MZ8, Micro Instruments, Oxford, UK). Electrodes (2 to 4 M Ω) were pulled from borosilicate glass capillaries of 1.2 mm OD $\times$ 0.69 mm ID (Harvard Apparatus, Edenbridge, Kent, UK) using a P-97 horizontal micropipette puller (Sutter Instrument Company, Novato, CA, USA) and filled with ACSF.

The electrodes were placed in the stratum radiatum (SR) of area CA3 in the hippocampus for the interface chamber, as the SR appears to be most reliable location to induce γ oscillation. For the same reason, the stratum pyramidale (SP) of the CA3 is used for the submerged recording of KA-induced γ oscillation. The coherence of the γ oscillations was studied by placing one electrode in CA3c and moving a second electrode in $\sim$ 200- μ m

steps along the cell layer of the Ammon horn. After recording a baseline for approximately 5–10 min, γ oscillations were induced by adding various concentrations of KA.

The extracellular microelectrodes were connected to an AC-DC amplifier (Neurolog System, Digitimer, Welwyn Garden City, UK). The signals were sampled at 5 kHz using a CED 1401 interface and low-pass filtered at 2.5 kHz using a Neurolog NL-125 filter unit (Digitimer). For the continuous recording in submerged slices, signals were sampled at 1 kHz and filtered at 0.5 kHz. A Humbug noise-eliminator (Digitimer) was used to remove line noise. Signal 2 and Spike 2 were used for data acquisition. Data analysis was performed off-line using Spike 2 and Signal 2 (Cambridge electronic design [CED], Cambridge, UK), SigmaStat (SPSS Inc, USA) and Microsoft Excel software.

The power of network oscillations was measured using fast Fourier transformation (FFT) over 20–100 s of data, which quantifies the proportional power of each wavelength within the data section. The summated power value of the γ oscillation is a summation of powers ranging between 20–60 Hz *in vitro*, first adopted by Fisahn *et al*^[6].

Statistical analysis

The data are expressed as the mean and standard error of the mean, with *n* indicating the number of slices.

Results

KA-induced γ oscillations

Extracellular field recordings were made in the hippocampal CA3c pyramidal layer in both submerged and interface slices. KA-induced γ oscillations developed in a time-dependent manner. At 100 nmol/L, KA-induced oscillations required 2–10 min to reach the peak (*n*=8) for submerged slices and 30–70 min (*n*=7) for interface recordings. The oscillations lasted longer than 30 min and for a few hours for the submerged and interface slice recordings, respectively. After washout of KA, the oscillations generally required 20–30 min to recover in submerged recordings but could take hours to recover in the interface recordings.

Power spectra analysis indicated that the dominant oscillation frequency in the submerged and interface recordings were approximately 25 and 40 Hz, respectively (Figure 1). A frequency of 25 Hz was at the lower range of γ oscillations (20–59 Hz). This frequency was associated with the lower temperature in the recording chamber in the submerged slices (29°C).

Temperature dependence

The optimum temperature for KA-induced γ oscillations under submerged conditions was determined, and the temperature affected γ oscillations in the submerged slices. Increasing the temperature from 23–36°C altered the frequency and power of the oscillation. Representative traces of oscillatory activity at 25 and 29°C are shown in Figure 2A. The frequency of oscillations increased from 18 to 25 Hz as the temperature increased from 25 to 29°C (Figure 2B). The temperature-dependence

Figure 1. Kainate-induced γ oscillations. A) Example traces of oscillatory activities induced by various concentrations of KA in submerged slices. B) Power spectra from oscillations induced by KA (250 nmol/L) show that the peak frequency for submerged slice is 25 Hz. C) Bar graphs show the average peak frequency of oscillatory activity induced by various concentration of KA in submerged slices. $^{\circ}P < 0.01$, compared with 25 nmol/L KA group, ANOVA.

of oscillation frequency over a range of temperatures from 23–33°C is depicted in Figure 2C. Based on the linear fit, the calculated change in response to a 1°C increase in temperature corresponds to a 2.3 ± 0.4 Hz increase in the oscillation frequency ($n=7$). Although the oscillation frequency was positively correlated with temperature over a range of 23–33°C, γ power was only correlated with temperature within a limited temperature range. Increasing the temperature increased the γ power (Figure 2C). The highest γ power was achieved at 27–29°C. As the temperature was further increased, the γ power was reduced (Figure 2C).

Concentration dependence

KA increased the γ power (Figure 3) in both interface and submerged slices in a concentration-dependent manner. At 25 nmol/L KA, the average γ power was $303 \pm 154 \mu V^2$ ($n=7$) and $26 \pm 13 \mu V^2$ ($n=8$) for the interface and submerged slices, respectively. At 100 nmol/L KA, the γ power increased to $2308 \pm 691 \mu V^2$ ($n=7$) and $195 \pm 91 \mu V^2$ ($n=8$) for the interface and submerged slices, respectively. At 250 nmol/L KA, the

Figure 2. Oscillatory activity is temperature-dependent. A) Examples of KA-induced field potentials in a submerged slice at 25°C and 29°C. Recordings were made in SP of CA3. B) Power spectra at different temperatures, corresponding to the field potentials in A. C) Plots of oscillation frequency (diamond) and the peak power of oscillations (square) as a function of temperature for the submerged slices.

γ power further increased for both interface and submerged conditions (Figure 3C, 3D). Increasing the KA concentration also accelerated the development of γ oscillations, as represented by a reduction in the time to reach peak power. For the interface slices, the time to peak γ power was 110 ± 16.4 min, 103 ± 4.6 min, and 27 ± 4.9 min for 25 nmol/L, 100 nmol/L, and 250 nmol/L KA, respectively. For the submerged slices, the time to peak γ power was 68 ± 7.9 min, 12.2 ± 2.2 min, and 4.7 ± 1.97 min for 25 nmol/L, 100 nmol/L, and 250 nmol/L KA, respectively.

Although the maximum γ powers under both conditions increased by KA in a concentration-dependent manner, γ maintenance appeared to be reduced at higher KA concentrations. At 25 nmol/L KA, the steady state of γ oscillations lasted for a few hours in the interface slices ($n=7$) and longer than 50 min in the submerged slices ($n=8$), but at 250 nmol/L, the steady state of γ oscillations was maintained for less than an hour for the interface slices ($P < 0.01$, compared with that of

the 25 nmol/L KA interface slice group) and less than 30 min for submerged slices ($P<0.01$, compared with that of the 25 nmol/L KA submerged slice group). In 5 submerged slices tested, high concentrations of KA (1.5 $\mu\text{mol/L}$) induced rapidly developing (2–3 min), strong γ oscillations, which lasted for only a couple of minutes (Figure 3F).

Area and layer specificity

In interface chamber recordings, the strongest γ oscillations were usually recorded in the CA3c area (closest to the hilus), followed by CA3b and CA3a^[13]. Representative results of γ oscillations recorded from these three areas (CA3a, CA3b, and CA3c) from an interface slice are shown in Figure 4A–4D. These recordings were repeated in the submerged slices, and similar results were obtained (data not shown). Therefore, γ oscillations were routinely recorded in the submerged slices in the CA3c area. Oscillatory activity can be recorded in the somatic (stratum pyramidale, SP) and apical dendritic layer (stratum radiatum, SR), with positive population synaptic potentials in the somatic region (SP) and negative potentials in the SR (Figure 4E). In the interface slices, the strongest γ

oscillations were recorded in the SR, followed by the SP and stratum oriens (SO). In the submerged slices, the γ power was highest in the SP, followed by the SR and SO (Figure 4F).

Oscillations are mediated by both excitatory and inhibitory synapses

KA-induced γ oscillations require KA receptor activation as well as excitatory glutamatergic and inhibitory GABAergic neurotransmission. The effects of the AMPA/KA receptor blocker, NBQX, and the GABA_A receptor blocker, GABAzine, were tested on KA-induced (100 nmol/L) γ oscillations. On average, the γ power was reduced to $0.9\pm0.3\%$ that of the control (100%) by NBQX ($n=4$, $P<0.01$) and to $3.1\pm2.1\%$ of the control by GABAzine ($n=4$, $P<0.05$). After 20 min washout of GABAzine, the γ oscillations partially recovered ($47\pm20\%$ of control, $n=4$), but no significant recovery was observed after NBQX washout ($1.2\pm0.2\%$ of control). TTX (1 $\mu\text{mol/L}$) reduced the γ powers to $24.2\pm3.8\%$ ($P<0.05$, $n=4$) that of the control at 20 min of application, which did not recover at washout ($0.54\pm0.26\%$ of the control, data not shown).

Figure 3. Time-effect curves of summed power at various KA concentrations. Left panel (A, C, E) and right panel (B, D, F) show the summed powers of γ oscillations recorded under interface and submerged conditions, respectively. Larger concentrations of KA (250 nmol/L and 1.5 $\mu\text{mol/L}$) induced larger amplitude of γ oscillations but reduced temporal stability in both interface (C, E) and submerged slices (D, F). Each data point represents the mean $\pm$ SEM.

Figure 4. Area and layer specificity of γ oscillations. A) Representative recordings of γ oscillations from CA3c (red) and CA3b (blue). B) Representative recordings of γ oscillations from CA3c (red) and CA3a (black). C) Power spectra from the recordings of γ oscillations shown in panel A. D) Power spectra from the recordings of γ oscillations shown in panel B. E) γ oscillations show positivity recorded in SP and negativity recorded in SR. The recording electrode was moved from SO to SP and then to SR and recordings were made at each position. F) Normalized γ powers at different layers of hippocampus in CA3c area. For the comparison, summated γ power from each location was normalised to the power in SP. Data are given as mean $\pm$ SEM. For interface slices, $n=17$ for the recordings from all the three layers; for submerged slices, $n=13$ for the recordings at SP and SR and $n=5$ for the recordings at SO.

Effect of perfusion rate on γ oscillations

The effect of perfusion rate on γ oscillations over the range of 1.35–10.5 mL/min in both interface and submerged recordings is shown in Figure 5. In the interface recordings, there was no obvious change in the power of γ oscillations with increased perfusion rate (line with open circles). In the submerged recordings, increasing the perfusion rate from 1.35–7 mL/min increased the power of the γ oscillations, whereas the higher perfusion rates (10.5 mL/min) reduced the power (line with filled diamonds). The optimal perfusion rate was determined to be 7 mL/min.

Figure 5. Effect of perfusion rate on γ oscillations in interface and submerged slices. Varying the perfusion rate did not alter γ oscillation strength in interface slices at 1.35–10.5 mL/min, but affect γ oscillation in submerged slices. The optimum perfusion rate for the submerged slices is 5–7 mL/min. Data are presented as mean $\pm$ SEM. $n=5$ slices

Discussion

We determined the optimal conditions for the induction of

persistent γ oscillations in submerged slices. Previous *in vitro* studies showed that γ frequency network oscillations lasting for tens of minutes could be routinely induced in hippocampal slices using an interface-type recording chamber^[6, 14–16]. In contrast, oscillations induced using submerged-type slice chambers, designed for visually guided patch-clamp recordings using infrared DIC optics, lasted only a few seconds and were qualitatively different from those recorded under interface conditions^[11, 13].

The optimal temperature for γ oscillations recorded under submerged conditions is 28–29°C, a few degrees less than that under the interface conditions (32–33°C). The reason that a relatively low temperature is required for γ oscillations under submerged conditions remains to be determined, but the possibilities include the following situations: 1) because tissues consume less oxygen at lower temperatures, the relatively low temperature may help maintain the γ oscillations and 2) the relatively low temperature may help maintain the mitochondrial membrane potential ($\Delta\Psi_m$)^[17], which is critical for the maintenance of the proton gradient necessary for ATP synthesis in mitochondria. Warming the chamber from 22 to 32°C was reported to depolarize the mitochondrial membrane potential and reduce ATP synthesis in cells of the carotid body^[17].

The oscillation frequency induced by KA in the submerged slices was at the lower range of the γ band. Previous studies have shown that γ oscillations typically have a peak frequency of ~35 Hz^[6] under interface conditions. The difference in the peak frequency in this study and those of previous reports may be explained by the temperature at which the experiments were conducted. Our recordings indicate that a 1°C

change in temperature correlates with an alteration of oscillation frequency of 2.3 Hz (a 9.2% change per degree). Our results are in line with a previous report in interface slices^[18], which showed an 8.3% reduction in frequency per degree of change in CCh-induced oscillations, and another report in hippocampal slices^[19], which showed an 8.8% reduction per degree in tetanically evoked γ oscillations. Temperature-related changes in the oscillation frequency are likely associated with changes in inhibitory postsynaptic potential (IPSP) duration. GABAergic IPSP duration is also known to be a key determinant of oscillation frequency^[20], and GABAergic IPSPs are prolonged at lower temperatures^[18, 21]. Temperature may also affect excitatory synaptic transmission^[22] and contribute to the temperature dependence of oscillation frequency; indeed, gamma oscillation is dependent on both excitatory and inhibitory neurotransmissions.

The oscillation polarity is positive in the SP, where the neuronal somata are located, but negative in the apical dendrites in both interface and submerged slices. The polarity at the soma is positive because GABA, released from presynaptic interneurons, activates GABAA receptors at the postsynaptic membrane of the principle cell, causing Cl⁻ influx and thereby leaving a positive charge outside the soma. The positive potential in the extracellular recordings is caused by a positive charge flowing out of the cell, a process called the “source” in current source density analysis^[23, 24]. Whether the source is an active or passive process depends on the intracellular recording of the soma; if this recording shows a hyperpolarized current (IPSC) coinciding with the source, then the source is likely to be active^[25] because hyperpolarization is caused by Cl⁻ influx. In contrast, the negative field potential in the stratum radium (neuronal dendrite location) is due to the positive charge flowing into the dendrite. This process is called a “sink” in current source density analysis. A passive sink is caused by an active source of GABA in the soma, whereas an active sink is caused by the release of glutamate from recurrently excited synapses activating synaptic receptors and causing a positive charge (Na⁺) influx. The positive polarity of the KA-induced γ oscillations recorded in the SP in the slices in this study is likely due to active sources^[25].

KA-induced γ oscillations in the submerged slices in the hippocampal CA3 can be blocked by either NBQX or GABAzine, suggesting that both pyramidal neurons (excitatory) and interneurons (inhibitory) are involved in γ oscillations. These oscillations are similar to those recorded *in vitro* in the entorhinal cortex caused by kainate receptor activation^[26], *in vitro* in the hippocampus caused by cholinergic activation^[6, 16, 27] and *in vivo* in the hippocampus^[5] but are different from the γ oscillations recorded in the CA1^[14, 15, 28, 29], which showed that both chemically and electrically interconnected interneuronal networks were involved without any contribution from pyramidal cells. The sodium channel blocker, TTX, also largely reduced γ oscillations, suggesting that neuronal action potentials (spikes) contribute to the network oscillations^[30].

The recovery after a 20-min drug washout showed different dynamics for different blockers: partial recovery for GABA-

zine, limited recovery for NBQX and no recovery for TTX. The difference in washout dynamics may be related to the chemical and physical properties of the different drugs. For example, the antagonist NBQX typically cannot be washed out easily due to its lipophilic nature^[31]. The limited washout time (20 min) in this study may have contributed to the limited recovery of these drugs. Under prolonged washout times (up to 1 h), there was a dramatic recovery of the γ oscillation for the NBQX and TTX treatment groups in some cases.

The development dynamics of KA-induced γ oscillation are largely dependent on the concentration of KA. High concentrations of KA (1–2 μ mol/L) induced a rapid collapse in γ oscillations, suggesting that strong membrane depolarization blocks neurotransmission^[32]. The micromolar KA-induced rapid collapse of γ oscillations may be related to the high demand for ATP in fast network oscillations, causing the neurons to rapidly run out of energy. Therefore, a concentration of KA in the nanomolar range is suitable for the induction of persistent γ oscillations.

Under submerged conditions, γ oscillation requires a fast perfusion rate (5–7 mL/min), indicating the nature of high oxygen demand for fast network oscillations. A high perfusion rate, although providing a rapid exchange of fresh solution, may cause mechanical damage to the brain slice, which likely explains the reduced power of the γ oscillations under a high perfusion rate of 10.5 mL/min in this study.

In summary, the KA-induced submerged oscillatory activity in the γ frequency band in this study is similar to that recorded in the interface recordings with respect to the neurotransmitters and synapses involved but with faster dynamics during development and after the washout. Under submerged conditions, γ oscillations can be induced by KA at nanomolar concentrations and at a relatively low temperature. This model will provide neuroscientists, pharmacologists and clinical researchers with a useful tool to study the network mechanisms underlying normal neuronal synchronization and neuronal disorders, such as Alzheimer's disease and schizophrenia, which are known to involve cognitive dysfunction and the impairment of oscillatory activity.

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Author contribution

Lu CB: designed, performed experiments and wrote paper; Wang ZH: performed experiments; Zhou YH: analyzed data; Vreugdenhil M: designed research and wrote paper.

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