Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 982 (2003) 79–91
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research report

Inhibitory control of intrinsic hippocampal oscillations?

Yacov Fischer*, Roland Dürr

Brain Research Institute, University of Zurich, CH-8057 Zurich, Switzerland

Accepted 2 May 2003

Abstract

An oscillatory mode of activity is a basic operational mode of the hippocampus. Such activity involves the concurrent expression of several rhythmic processes, of which theta (4–15 Hz) and gamma (20–80 Hz) oscillations are prominent and considered to be important for cognitive processing. In an experimental model that preserves the intrinsic network oscillator, exhibiting the dependency on cholinergic inputs and consequent expression of concurrent theta and gamma oscillations, we investigate the intrinsic mechanisms underlying such integrated hippocampal network responses. This experimental framework is used here to examine the currently prevailing dogma, that interneurons control hippocampal oscillations. The spontaneous response of individual pyramidal cells (in areas CA3 and CA1) and interneurons (area CA3), during oscillatory activity, was monitored intracellularly. Particular attention was given to the initiation of interneuron discharge during oscillations, to the impact of the synaptic output of discharging interneurons on the oscillatory activity, and to the time at which interneurons discharge in relation to the oscillatory cycles. Analysis of the spontaneous patterns of activity in individual interneurons and their outcome, during the oscillatory activity, revealed that interneuron activity is incompatible with initiating, pacing or determining the oscillatory frequencies, although contributing to the apparent rhythmic patterns. Moreover, our results show that non-interneuronal members of the network control interneuron activity. We therefore suggest that the activity of the excitatory cells, i.e., principle cells, is critical toward the initiation, pacing and synchronization of intrinsic hippocampal network oscillations.

Theme: Other systems of the CNS

Topic: Limbic system and hypothalamus

Keywords: Theta; Gamma; Synchronization; Intrinsic network oscillator; Acetylcholine; Integrated network activity

1. Introduction

The hippocampal formation is a source of the brain's rhythmic activities [2,20,23,33]. Hippocampal adaptation to an oscillatory mode of activity depends on inputs from the medial septum or enthorinal cortex [1,4,19,25] and on the intrinsic properties of its network. In response to septal cholinergic inputs the intrinsic oscillator [13,24] is activated, and concurrent theta (4–15 Hz) and gamma (20–80 Hz) oscillations, with underlying sustained synaptic activity, emerge [13,14]. Interneurons, an integral part of the hippocampal network, are the major cell-class to spontaneously discharge during oscillatory activity [8,14], and are suggested to control hippocampal oscillations [3,5,7,12,15,22,30,31,35].

The experimental model of hippocampal slice cultures, which uniquely preserve the local network interaction, offers to date the singular in vitro system where the hippocampus spontaneously adapts the oscillatory mode of activity in response to inputs from the medial septum [13]. During this cholinergically mediated oscillatory activity, theta, common in pyramidal cells, and gamma, prevalent among interneurons, are simultaneously expressed [14], through mechanisms intrinsic to the hippocampal network [14]. Moreover, in the course of oscillatory activity, about half of the interneuron population, including basket, O-LM, bistratified, str. lucidum-specific and multi-subfield cells, spontaneously discharge [14]. Furthermore, prevention of action potential discharge, by TTX, abolishes the oscillatory activity [14]. We therefore set out to examine to what extent this spontaneous discharge determines the oscillatory activities, i.e., theta in CA1 and CA3 pyramidal cells (PCs) and gamma in CA3 interneurons.

*Corresponding author. Tel.: +41-1-635-3364; fax: +41-1-635-3303.
E-mail address: kfischer@hifo.unizh.ch (Y. Fischer).

0006-8993/03/$ – see front matter
doi:10.1016/S0006-8993(03)02974-3

Experiments and analytical procedures were designed to answer the following questions: (1) is the spontaneous discharge of interneurons a direct result of the activation of their own cholinergic receptors, (2) can the autonomous inhibitory network support theta and gamma oscillations, (3) does the synaptic output of spontaneously discharging interneurons, i.e., the IPSP, determines the oscillatory frequency, (4) is interneuron discharge synchronized, and (5) does the phase of interneuron discharge, i.e., the relative time at which an action potential in an interneuron occurs in relation to the oscillatory cycle, supports a pacing function?

2. Materials and methods

2.1. Slices cultures

All experiments were done according to the guidelines set forward by the Department for Veterinary Affairs of the Kanton of Zurich. Hippocampal slice cultures were prepared from 6-day-old rat, and maintained for 3–6 weeks in vitro [16].

2.2. Electrophysiological recordings

For electrophysiological recordings, cultures were transferred to a recording chamber and continuously superfused with warmed (32–33 °C) saline containing (in mM): Na^+ 145, Cl^- 149, K^+ 2.7, Ca^{2+} 2.8, Mg^{2+} 2, HCO_3^- 7.7, $H_2PO_4^-$ 0.4, glucose 5.6, and Phenol Red (10 mg l^{-1}) at pH 7.4.

Simultaneous electrophysiological recordings were obtained, either from individual PCs of areas CA1 and CA3 or from individual interneurons and PCs of area CA3, using sharp microelectrodes containing 1 M potassium methylsulfate either alone or with 2% biocytin (electrode resistance: 30–60 MΩ), as described previously [13,14]. Only a fraction of these cell-pairs were monsynaptically connected.

2.3. Drugs

Methacholine (MCh) and midazolam were purchased from Sigma–Aldrich (Buchs, Switzerland), NBQX from Tocris-Cookson (Bristol, UK), CPP was a gift from Novartis (Basel, Switzerland), and flumazenil was a gift from Hoffmann-La Roche (Basel, Switzerland). Biocytin was purchased from Molecular Probes (Eugene, OR, USA).

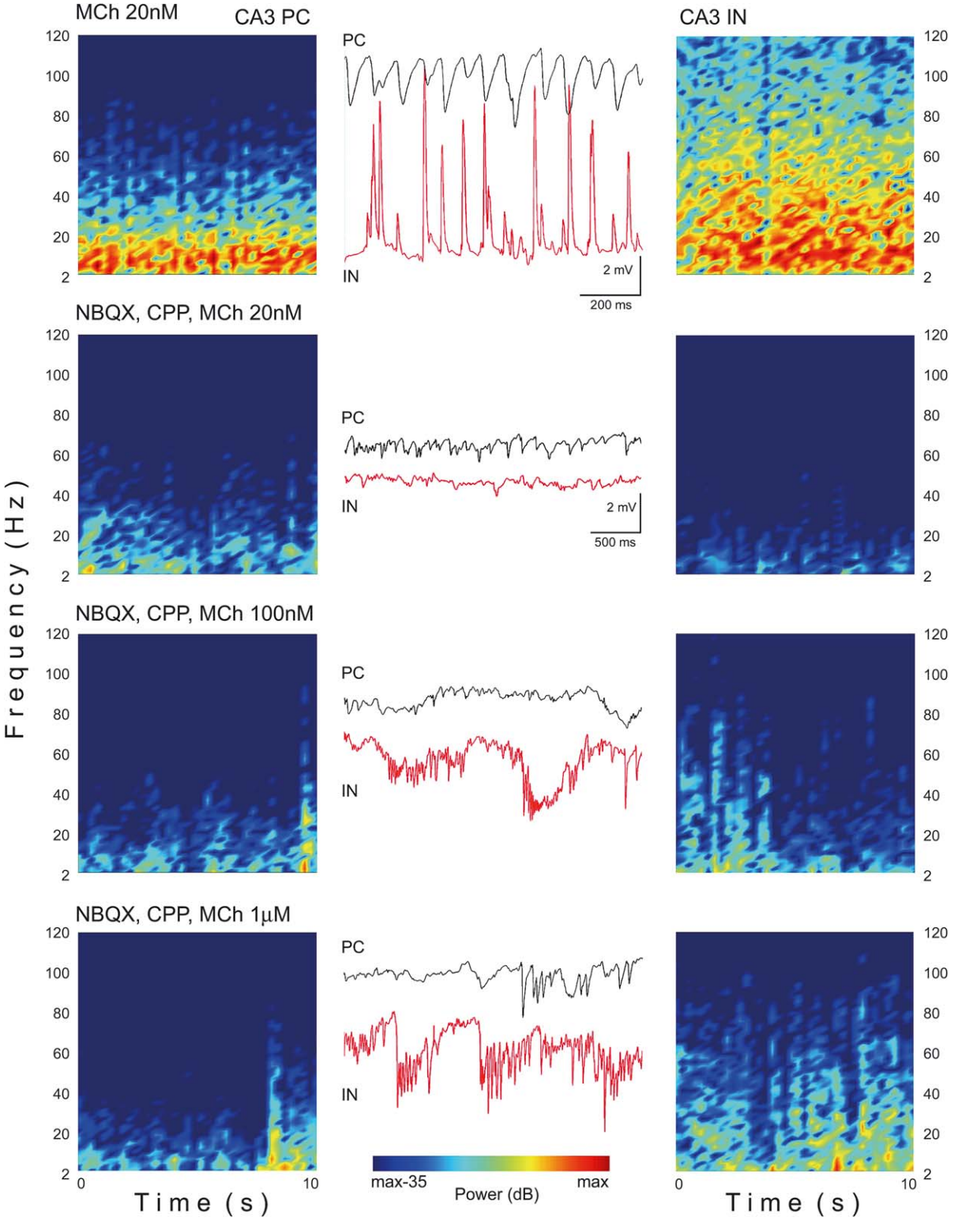
2.4. Data analysis

The analog signal was recorded on videotape at 22 kHz and digitized at 1 kHz. Analysis of oscillatory activity was based on windowed Fourier analysis, using 500 ms windows with 50% overlap, as previously described [14]. Results of the windowed FFT analysis are presented in the form of spectrograms, which illustrate the Fourier analysis power spectrum (frequencies: 2–500 Hz) as a function of time, using a false color scale to indicate the power in dB (warm colors represent high power and thus the dominant frequencies). Rhythmic activity in the cells was evaluated by calculating the mean of the dominant frequencies obtained from the FFT of each window (during 1 min or 10 s period of activity). For assessing gamma activity in the cells, our previously described subtraction method was used, where the slow activity content of the PCs and interneurons signals was calculated and subtracted and windowed FFT was recalculated [14]. Due to transient interruptions of the oscillatory activity during application of midazolam (see Section 3), only data from segments expressing theta oscillations were used for the analysis.

For phase-analysis we used a program that was designed to determine the firing phase of each action potential in the interneuron in relation to the relevant local (theta) cycle in the co-recorded PC. Action potentials in interneurons were detected by a threshold method. For each action potential we determined the corresponding oscillatory cycle and its duration. An action potential can be assigned to either the cycle it coincides with or the next closest one. This was determined by fuzzy-logic criteria that were based on sigmoid functions that search forward and backward. Next,

Fig. 1. Cholinergically activated network responses during blockade of fast glutamatergic transmission. MCh-mediated network responses were simultaneously recorded from a CA3 PC (black) and a CA3 interneuron (IN, red) at four experimental conditions, i.e., the control oscillatory response (MCh 20 nM) and the network responses during blockade of fast glutamatergic transmission (MCh: 20 nM, 100 nM and 1 μM). Traces depict changes in the membrane potential and are accompanied by corresponding spectrograms of 10 s of activity in the PC (left) and interneuron (right). Color bar: windowed FFT power relative to the maximum in a 35-dB scale. MCh (20 nM) mediated the emergence of oscillatory activity that was confined in the PC to the theta range, and that contained major contributions at the gamma range in the interneuron (top panels) (traces depict intracellular activity 2-min form MCh application). While oscillatory activity in control was confined in the PC to the theta band and continuous, only residual activity was seen in the presence of NBQX (20 μM) and CPP (10 μM), irrespective of MCh concentration (lower sets of panels) (traces for MCh 20 nM, 100 nM and 1 μM, depict the intracellular activity at 5, 7, and 4 min after MCh application, respectively). Moreover, during blockade of fast glutamatergic transmission (NBQX and CPP) the frequency response of the PC (see spectrogram) was found to be asynchronous. Gamma oscillations at the interneuron were abolished during blockade of fast glutamatergic transmission. Even at higher concentrations of MCh, gamma oscillations were not observed. Instead small, asynchronous and burst-like IPSP-patterns were detected, reflecting bursting activity in the interneuron population (see also Fig. 4). Note the different time base for trace obtained from the intact network and those obtained during application of NBQX and CPP. Resting potentials were −58 mV (IN) and −58 mV (PC). The interneuron was identified, in a previous study [14], as a basket cell.

the relevant local cycle length was approximated from the preprocessed PC signal, which included removal of slow DC effects and amplitude normalization. This approximation was then used in a search mode to determine the cycle length in the original signal to avoid aliasing. The beginning of each cycle was assigned to the start of the population IPSP, i.e., zero-phase by definition. The location of the cycle onset, i.e., IPSP onset, was found by identifying all possible local maximas at the corresponding PC cycle. Each position of local maxima was scored with



two evaluators: one for the amplitude and the other for the delay from the action potential (using two sigmoid likelihood functions). From all possible candidates the one showing the highest score was selected. The end of a cycle was taken as the beginning of the following cycle, and the difference of the two was the local cycle length (≡360°). For each action potential we determined its spike-delay: the time difference between the action potential and the cycle start, which could have either a positive or a negative value. The local firing phase was calculated as the ratio between the spike-delay and the local cycle length in degrees. For the construction of the firing-phase histograms, 360° were subtracted from the calculated phase if larger than 180°. For practical reasons the signal was digitized at 1 kHz. This creates a binning problem for the zero-bin, which equals 3.6° for a 100-ms cycle, and was solved by using a Gaussian distribution for this bin. The need for the randomization step of the zero-bin was apparent when we used the program to analyze the same signal after digitization at 10 kHz (the zero-bin for a 100-ms cycle is 0.36°). The same phase histograms were obtained for the analyzed signal, whether it was digitized at 10 kHz, or at 1 kHz with Gaussing of the zero-bin.

Analysis was performed using Matlab (The MathWorks, Natick, MA, USA). Variability of the mean was expressed in S.D.

3. Results

Antagonists for fast glutamatergic transmission (NBQX, CPP) were used in order to test (a) whether interneuronal discharge results from direct cholinergic activation, or from synaptic interactions within the hippocampal network, and (b) whether the autonomous interneuron network is sufficient for the expression of theta and gamma oscillations.

In the presence of NBQX (20 μM) and CPP (10 μM), none of the intracellularly recorded interneurons, including the previously identified basket, bistratified and multi-subfield cells [14], exhibited action potential firing or membrane potential depolarization in response to the cholinergic agonist MCh (20 nM) ($n=6$). Membrane potentials of the interneurons were (in mV) -58 ± 2.4 (MCh), -60 ± 3.6 (NBQX, CPP), and -60 ± 2.4 (MCh, NBQX, CPP) ($n=6$) ($P>0.05$, t-test). Moreover, also when the networks fast glutamatergic transmission is intact, interneuron discharge, during oscillatory activity, was not associated with depolarization of their membrane potential [14]. We therefore conclude that spontaneous interneuron discharge during oscillations does not result from the activation of their muscarinic receptors [21], but depends on excitatory, i.e., glutamatergic, synaptic network interactions.

Furthermore, application of NBQX and CPP also abolished the expression of cholinergically mediated (MCh, 10 or 20 nM) theta oscillations in CA3 PCs (i.e., population EPSP/IPSP sequences) ($n=10$) and gamma oscillations in interneurons (i.e., fast EPSPs) ($n=6$) (Fig. 1). The small inhibitory events seen in PCs after 4–10 min (Fig. 2), contrasted with the full-blown oscillations observed within 2 min of exposure to MCh at control, as they failed to reach theta frequencies (mean frequency: 0.43 ± 0.69 Hz, $n=10$), or to exhibit comparable amplitude with the population IPSPs in the PCs oscillatory cycles. The specificity of this manipulation was tested (Fig. 3A). The lack of comparable inhibitory events was due to the failure of interneurons to discharge, and not to the depres-

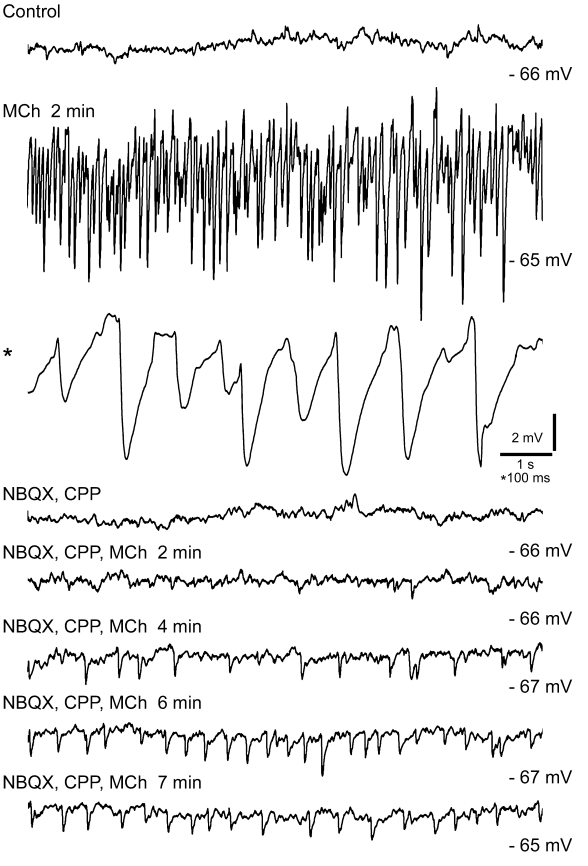


Fig. 2. The time course of cholinergically activated network responses during blockade of fast glutamatergic transmission. In a CA3 PC, in response to blockade of fast glutamatergic transmission (NBQX (20 μM) and CPP (10 μM)), resting membrane potential was unaltered, basal activity was reduced, and MCh mediated (10 nM) theta oscillations were abolished. At the time point (2-min) where MCh evoked full-blown oscillations under control conditions, PCs were silent. At later time points, inhibitory events started to appear. However, these IPSPs were smaller in amplitude, and the frequencies never reached the theta range (mean frequency: 1.53 ± 0.54 Hz). Note in Fig. 1 that the pattern of emerging IPSPs is asynchronous. Membrane potential is indicated to the right of the 10-s traces. All traces are on the same time/voltage scale. Asterisk: a 1-s zoom of the MCh-mediated activity, in control.

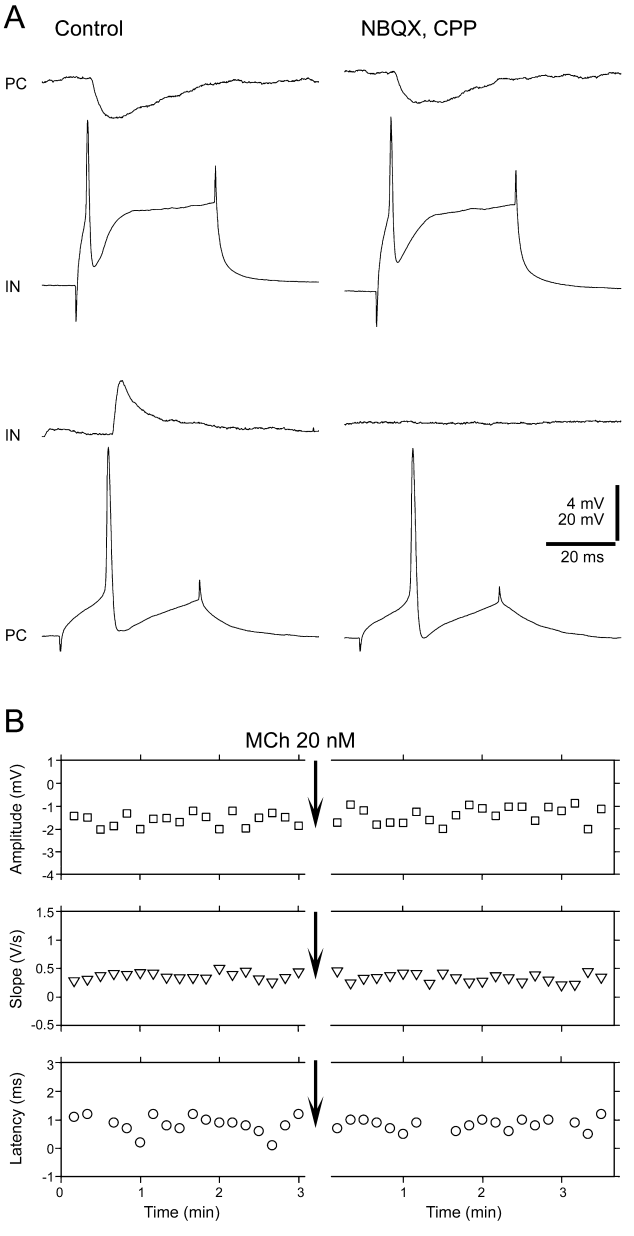


Fig. 3. Inhibitory transmission between CA3 interneuron–PC pairs is unaltered by MCh (20 nM). (A) The specificity of NBQX and CPP action to the networks synaptic interactions was tested in all CA3 interneuron–PC pairs ($n=6$) used for the study of network responses during the blockade of fast glutamatergic transmission. In mutually connected CA3 interneuron–PC pairs, the unitary EPSPs in the interneuron were abolished in the presence of NBQX and CPP ($n=2$), while unitary IPSPs in the co-recorded PC persisted ($n=5$). Note the fast kinetics of the unitary EPSP in the interneuron (the unitary gamma event, see Ref. [14]), as compared to the unitary IPSP in the PC. Resting potentials were −60 mV (interneuron) and −60 mV (PC). (B) The properties of inhibitory synaptic transmission from a CA3 interneuron to a CA3 PC, i.e., the unitary IPSP amplitude, initial slope and latency, at control and during the application of 20 nM MCh are depicted. The strength of this synapse was unaltered by application of MCh. At control the unitary IPSP had an amplitude of −0.53±0.08 mV, slope of 0.128±0.04 V/s and latency of 1.88±0.55 ms. During MCh application (20 nM) the unitary IPSP amplitude was of −0.59±0.11 mV, slope of 0.133±0.03 V/s and latency of 1.58±0.69 ms. For all $P>0.05$ (t-test). Each point represent the raw data obtained at 0.1 Hz stimulation. Arrow: MCh application.

sion of inhibitory transmission to PCs, which remained unaltered (paired recordings between CA3 interneurons and PCs, $n=3$, MCh 20 nM, Fig. 3B).

To determine whether the lack of oscillatory response at theta or gamma frequencies, during the blockade of fast glutamatergic transmission, was due to insufficient cholinergic activation we increased the concentration of MCh to 100 nM and 1 μM. In the presence NBQX and CPP, elevation in MCh concentration resulted in increased asynchronous inhibitory events, but failed to produce theta or gamma oscillations ($n=6$) (Fig. 1). No change in the resting membrane potential of the interneurons was observed with 1 μM MCh, which remained at −61±2.7 mV ($n=6$).

Nevertheless, action potential could be detected in the intracellularly recorded interneurons with elevated concentrations of MCh. In particular, during application of 1 μM MCh (with NBQX and CPP), where four of the interneurons exhibited slow episodic depolarizations (about every 15 s), which were accompanied by bursting activity in two of the cells (Fig. 4). We attribute such discharge to the appearance of slow quasi-rhythmic synaptic modulation of the co-recorded PCs membrane potential (Fig. 4) (see also Fig. 1).

Thus, even micromolar concentrations of MCh, which in the absence of NBQX and CPP induces epileptiform activity [13], failed to elicit theta or gamma oscillations.

These data show that the autonomous interneuron network is incapable of expressing the patterns and rhythmicities observed during hippocampal oscillations. Similarly, antagonists for fast glutamatergic transmission are reported to abolish rhythmic activity in a number of different cholinergic in vitro models [11,12,26,37].

We thereafter tested the hypothesis that the output of discharging interneurons (the IPSP), in the intact network, determines the frequency of the oscillatory activities. One way of examining the influence of inhibitory elements would be to reduce their output. Unfortunately, hippocampal slice cultures exhibit epileptiform activity in response to application of GABA antagonists. We therefore used an opposite approach of enhancing inhibition through application of the benzodiazepine agonist midazolam, known to prolong IPSPs by 50% in this preparation [27].

Control MCh-mediated (10–20 nM) oscillatory response was observed in all CA3–CA1 PC pairs ($n=13$) (Fig. 5) and CA3 interneuron–PC pairs ($n=8$) (Fig. 6). Sustained theta oscillations of rhythmic excitatory and inhibitory events were observed in CA3 PCs and of rhythmic excitatory events (i.e., population EPSPs) in CA1 PCs [13], while sustained gamma oscillations of fast depolarizing rhythmic events were observed in CA3 interneurons.

Increase in inhibitory tone, by applying midazolam, revealed interneurons involvement in the dynamic regulation of the oscillatory activity of the network, as described here.

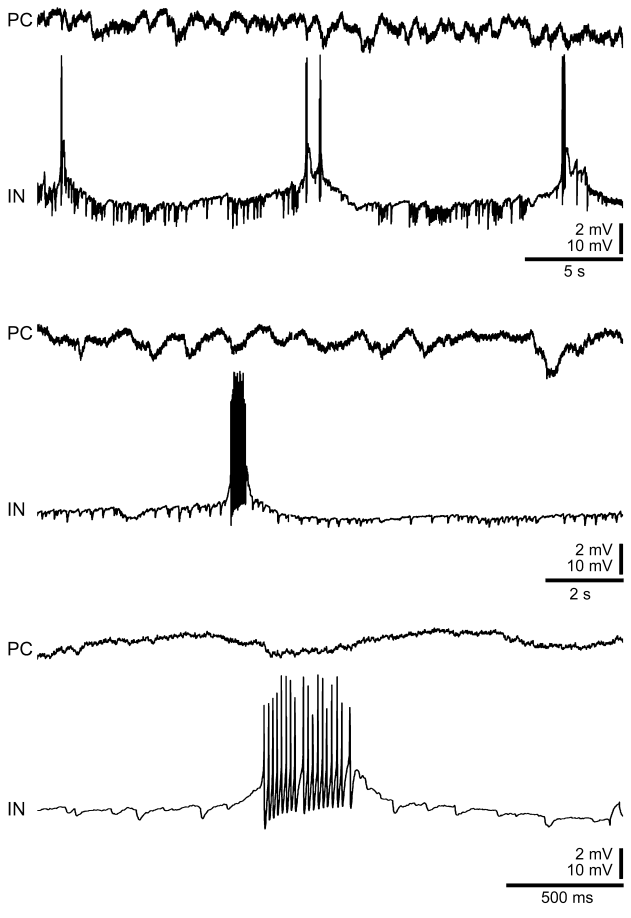


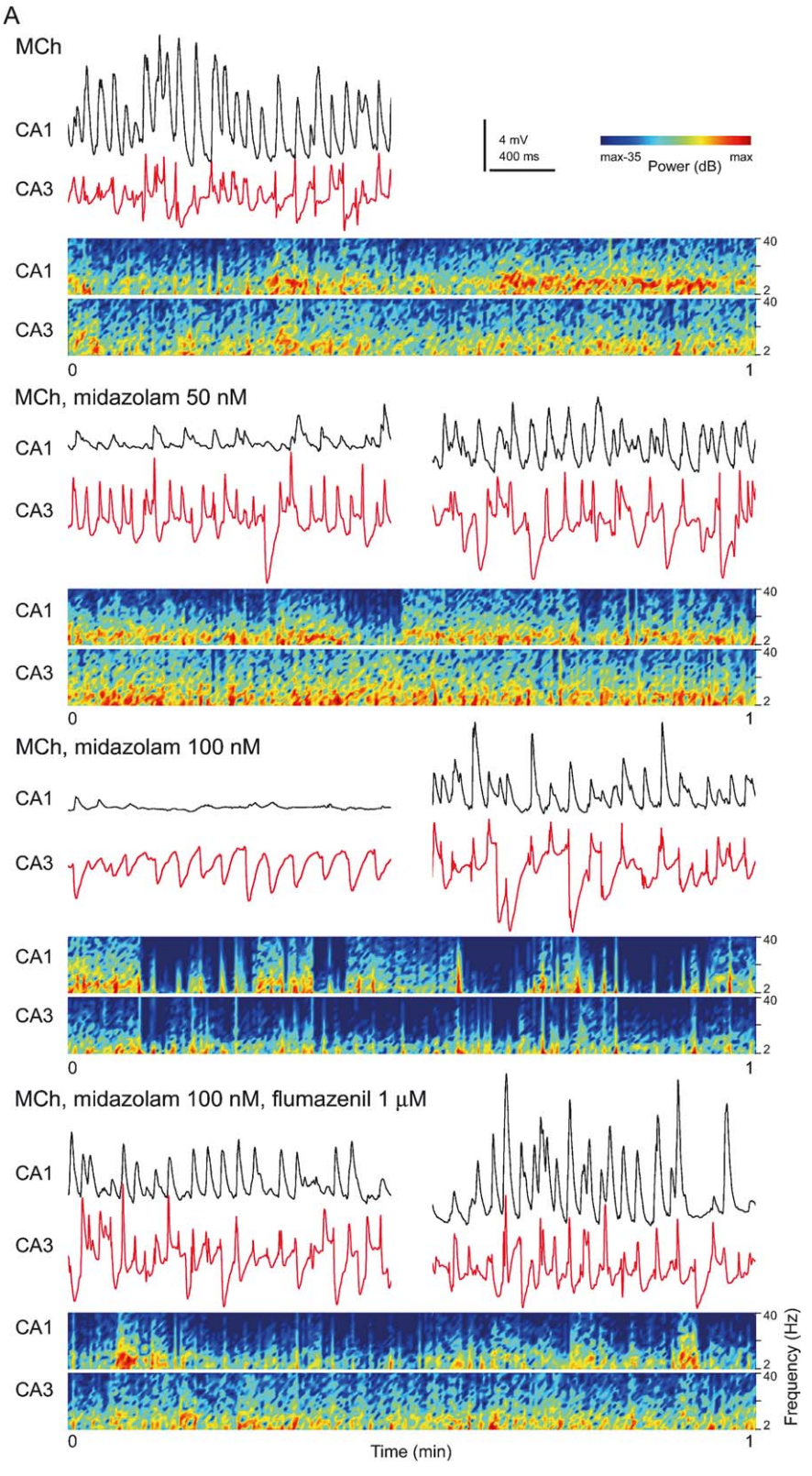
Fig. 4. Interneurons discharge at elevated concentration of MCh. Fluctuation in the membrane potential of an intracelluarly recorded CA3 interneuron (IN) and a CA3 PC, in the presences of NBQX (20 μM), CPP (10 μM) and MCh (1 μM). Three sets of traces (at three different time bases) are provided. They depict the global and local details of the spontaneous activity. A slow quasi-rhythmic pattern is observed in the PC. These events coincide and correspond to bursts of action potentials in individual interneurons (at different time points). This discharge was driven by an episodic depolarization of the interneuron membrane potential.

Application of 50 nM midazolam increased the weight of IPSPs, i.e., amplitude, duration and relative portion in the signal, in the mixed oscillatory sequence of CA3 PCs. At the same time, the rhythmic synaptic sequence in the co-recorded CA1 PCs underwent a 2–5-fold time-variable reduction in amplitude (Fig. 5). Further increase in the inhibitory tone, by elevating the concentration of midazolam to 100 nM, resulted in CA3 PCs in an oscillatory pattern that fluctuated between the mixed control sequence (with enhanced IPSPs) and an IPSP-predominated sequence. Appearance of an IPSP-predominated sequence in CA3 PCs coincided with an abrupt blockade of theta oscillations in 11 out of 13 co-recorded CA1 PCs (Fig. 5). CA1 PCs regained their population EPSP theta sequence, of reduced amplitude, when the oscillatory sequence of CA3 PCs spontaneously switched to exhibit the mixed control sequence. All these effects of midazolam were reversed by the benzodiazepine antagonist flumazenil (1 μM) ($n=5$).

The time-variable reduction in amplitude of the oscillatory sequence in CA1 PCs is likely to reflect the depression of the Schaffer-collateral pathway output, necessary for the expression of rhythmic activity in area CA1 [13]. The dependency and sensitivity of the oscillatory activity of CA1 PCs to external inputs corresponds with in vivo data demonstrating that area CA1 cannot generate rhythmic activity on its own, during medial septum-mediated hippocampal oscillations [6,38].

At the level of CA3 interneurons, IPSPs that were rare and in many cases absent from the oscillatory sequence (Fig. 6, and Ref. [14]), became visible (Fig. 6) upon midazolam application (50, 100 nM). This coincided with a reduction in amplitude of excitatory gamma activity. All these effects were reversed by flumazenil (1 μM) ($n=8$). Spectrograms, generated according to our previously reported method [14], depict gamma activity in interneurons

Fig. 5. The effect of benzodiazepines on the oscillatory activity of CA1 and CA3 PCs. (A) Simultaneous intracellular recordings from a CA1 (black) and CA3 (red) PC during MCh-mediated (10 nM) oscillations. Two sets of 2-s traces are provided for the different manipulations, in order to illustrate the range of effects. Traces are accompanied by corresponding spectrograms of 1 min of activity in the PCs. Color bar: windowed FFT power is relative to the maximum in each segment, in a 35-dB scale. Oscillatory activity was confined to the theta band in both cells and persisted during addition of 50-nM midazolam. Inhibitory responses were facilitated in the CA3 PC, while CA1 PC activity was variably reduced in amplitude. Nevertheless, oscillatory activity remained continuous and was not altered in frequency. With 100 nM midazolam the CA3 PC sequence was transiently predominated by inhibitory responses, which coincided with blockade of oscillatory activity in the CA1 PC. When rhythmic activity reappeared in the CA1 PC the sequence consisted of population EPSPs, of reduced amplitude, whereas the CA3 PC expressed the mixed control sequence, with population IPSPs of increased amplitude and duration. These dynamic and transitory effects are seen as flickering in the CA1 PC spectrogram. Midazolam effects were blocked by flumazenil (1 μM). Resting potentials were −67 mV (CA1) and −64 mV (CA3). Dominant frequencies were (in Hz, 1 min) CA3: 7.2±4.2 (MCh), 7.5±3.2 (MCh, midazolam 50-nM), 6.7±3 (MCh, midazolam 100-nM) and 7.3±3 after additionally adding flumazenil, CA1: 7.8±3.7, 7.8±2.4, 7.4±3 and 7.4±2.8, respectively ($P>0.05$, t-test, for each manipulation). Similar results were observed in five such experiments ($P>0.05$, t-test, for each manipulation in each experiment). (B) The temporal variation in the dominant frequencies power (circles) and the dominant frequencies (lines) in the PCs (top, CA1; bottom, CA3) (for the data presented in A). Mean dominant frequencies as above. Dashed lines: theta band. Mean dominant frequencies power (in dB): CA1, MCh, 43.3±3.93; MCh and midazolam (50 nM), 42.12±3.55; MCh and midazolam (100 nM), 34.46±7.28; MCh, midazolam (100 nM) and flumazenil, 40.29±4.7; CA3, 39.85±3.07, 41.81±3.1, 42.45±4.95 and 42.46±3.35, respectively. Application of the benzodiazepine midazolam (50 and 100 nM) produced a significant decrease in the power of the CA1 PC, and an increase in the CA3 PC (for both: $P<0.05$, t-test). The increase in CA3 PC power reflects the increase in inhibitory tone and the enhancement of the population IPSPs. The decrease in CA1 PC power reflects the depression of population EPSPs amplitude and blockade of oscillatory activity in the cell. Application of the benzodiazepine antagonist flumazenil reversed the effects of midazolam, the power of the CA1 PC signal increased to 40.29 dB, and the overall power of the CA3 PC remained at 42.46 dB (in spite of the change in pattern).



(38.5±7 Hz, $n=6$) (Fig. 6, right-hand spectrograms); however, due to emerging IPSPs (Fig. 6), it is difficult to calculate the reduction in the power of gamma activity. Appearance of IPSPs in the rhythmic sequence of interneurons upon midazolam application was not due to a change in their resting membrane potential, which were (in mV) −60.8±4.1 (MCh), −61.2±4.5 (MCh, midazolam 50 nM), and −61.4±4.5 (MCh, midazolam 100 nM) ($n=7$).

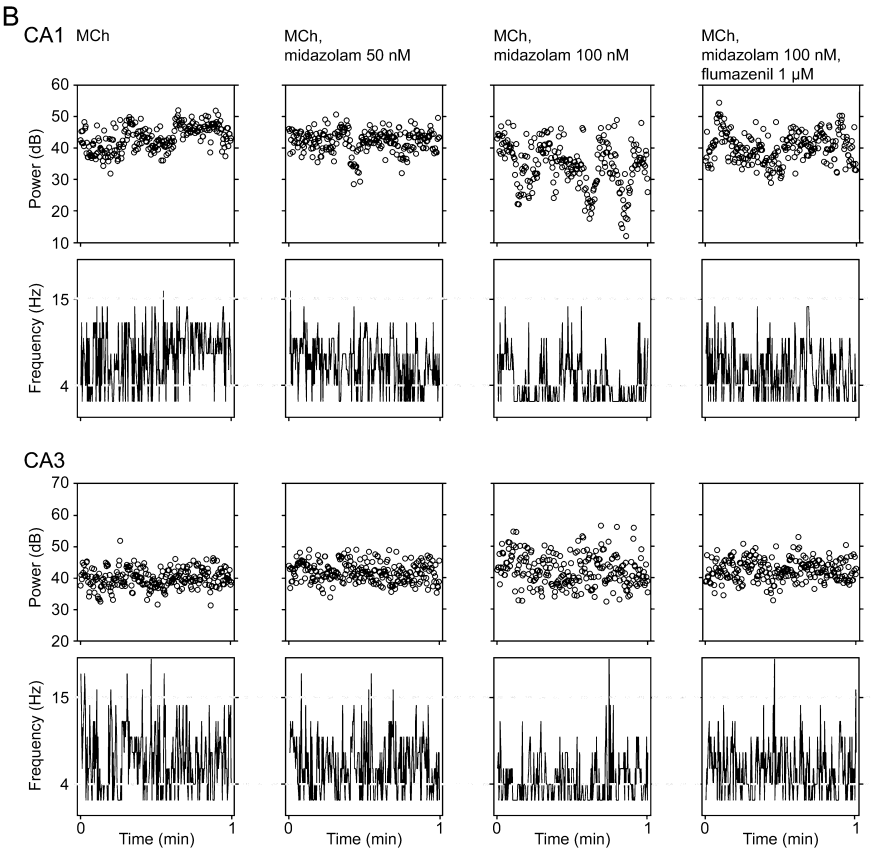


Fig. 5. (*continued*)

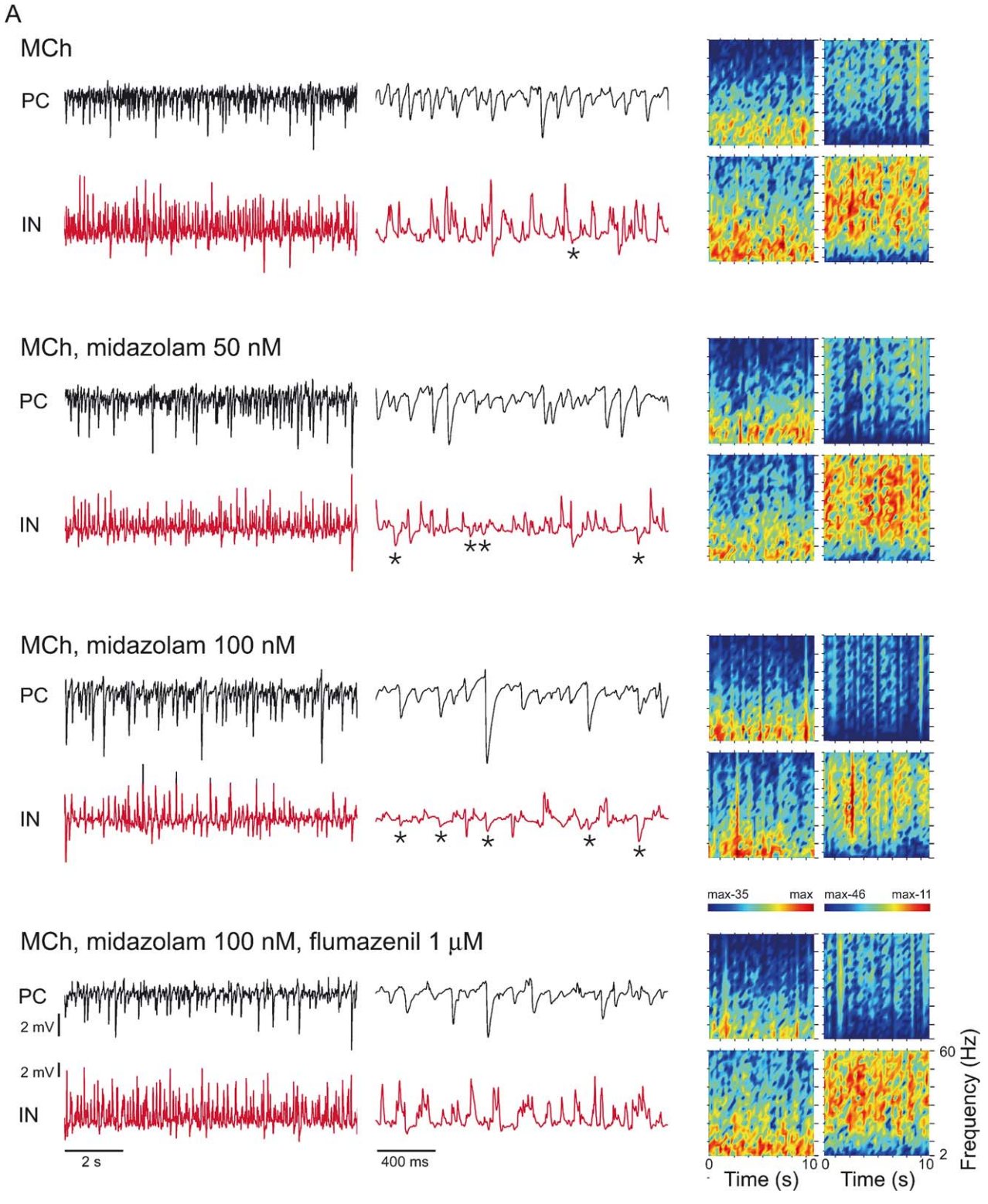
Importantly, modulation of the inhibitory weight and the duration of unitary IPSP by midazolam and flumazenil failed to change the frequency of oscillatory activity in CA1 and CA3 PCs ($n=5$) (Fig. 5), and in CA3 interneurons ($n=6$) (Fig. 6). Thus indicating that the duration of the synaptic output of individual interneurons does not directly determines the frequencies of oscillations.

In other cholinergic in vitro models, exclusively expressing hippocampal gamma oscillations, rhythmicity was found to be weakly sensitive [28] or insensitive [10] to benzodiazepines.

To determine whether the precise time, at which action potentials occur, in relation to the oscillatory cycles, serves as a pacing signal, we calculated their firing phase. Moreover, such analysis should resolve whether the interneuron-population discharges in synchrony, suggested to be necessary for the generation of population IPSPs in the rhythmic sequence of CA3 PCs [14].

Fig. 6. The effect of benzodiazepines on the oscillatory activity of CA3 PCs and interneurons. (A) Simultaneous intracellular recordings from a CA3 PC (black, upper trace) and a CA3 interneuron (IN, red, lower trace) during MCh-mediated (20 nM) oscillatory activity. Traces are presented at two time scales: 10-s (left) and 2-s (middle), and are accompanied by corresponding spectrograms (10 s). Left-hand spectrograms depict the windowed FFT results of the original signal and the right-hand ones of the subtracted signal (used to evaluate gamma activity [14]). Color bars: windowed FFT power relative to the maximum in a 35-dB scale. The PC exhibited synaptic theta and the interneuron synaptic gamma oscillations (with contributions in the theta range). The response of the PC to midazolam (50 and 100 nM) is similar to that illustrated in Fig. 5. With midazolam (50 nM) the amplitude of excitatory gamma activity in the interneuron diminished. This was associated with the appearance of IPSPs in the interneuron sequence (asterisks). With midazolam (100 nM) further decrease in the amplitude of gamma events was observed, and IPSPs appeared more frequently. Flumazenil (1 μM) blocked these effects. Resting potentials were −59 mV (PC) and −58 mV (IN). Dominate frequencies in the interneuron (following subtraction) were (in Hz, 10 s): 35±9 (MCh), 35±8.5 (MCh, midazolam 50 nM), 35±9 (MCh, midazolam 100 nM), 37.5±9 (MCh, midazolam 100 nM, flumazenil) ($P>0.05$, *t*-test, for each manipulation). Similar results were observed in six such experiments ($P>0.05$, *t*-test, for each manipulation in each experiment). (B) The temporal variation in the dominant frequencies power (squares, original signal; circles, subtracted signal) and the dominant frequencies (bottom, circles, subtracted signal) in the interneuron (for the data presented in A). Mean dominant frequencies as above. Mean dominant frequencies power (in dB); original signal, MCh, 42.11±2.38; MCh and midazolam (50 nM), 39.2±2.43; MCh and midazolam (100 nM), 39.5±4.03; MCh, midazolam (100 nM) and flumazenil, 42.05±2.9; subtracted signal, 31.4±2.55, 27.3±2.7, 26.3±3.7 and 29.9±2.6, respectively. Application of the benzodiazepine midazolam (50 and 100 nM) produced a significant decrease in the signal power (original and subtracted, $P<0.05$, *t*-test). Application of the benzodiazepine antagonist flumazenil reversed the effects of midazolam, the power of the original signal returned to that observed with MCh ($P>0.05$, *t*-test), and that of the subtracted signal increased to 29.9 dB ($P=0.016$).

The temporal relationship between spontaneous action potential discharge and the oscillatory cycles in co-recorded CA3 PCs was used to determine the firing phase of interneuron discharge ($n=8$). Phase analysis has revealed that the firing of individual interneurons was temporally structured to exhibit a unimodal output function that mirrored the time course of the population IPSPs (Fig. 7). Discharge of individual interneurons was largely limited to the first 60° of cycle, where zero-phase was defined as the onset of inhibition, and was maximal during the initial phase of the population IPSPs. All interneurons suitable for such analysis, which included the previously identified



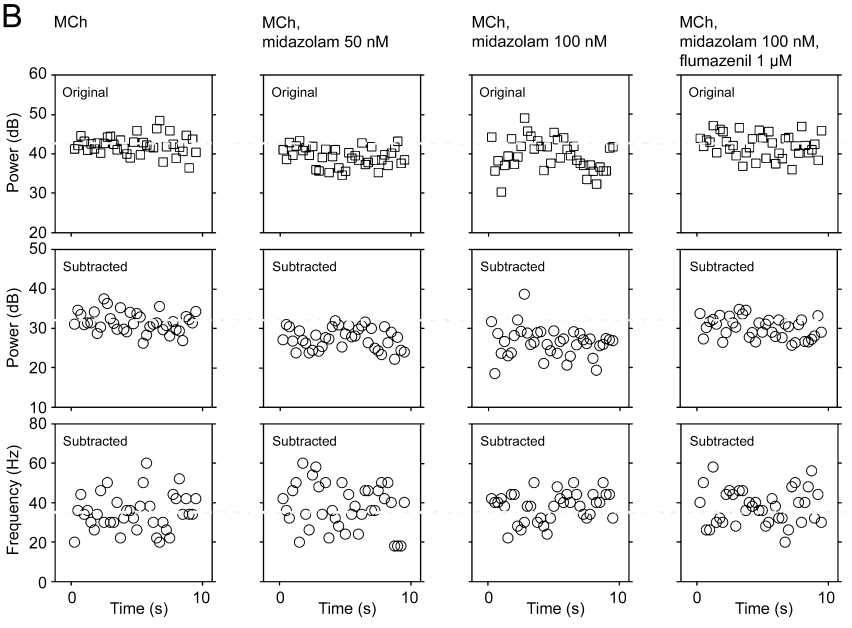


Fig. 6. (*continued*)

basket, str. lucidum-specific and multi-subfield cells [14], exhibited such firing phase profiles, giving a median firing-phase of 18±5.8° (n=8), and demonstrating synchronous co-activation of several interneurons. Thus, the unimodal phasic firing of several interneurons underlies the population IPSPs recorded in CA3 PCs during theta cycles.

4. Discussion

Of the original findings described in this report are: (a) interneuron discharge is secondary to the activation of the intrinsic oscillator, (b) once activated, to discharge, interneurons participate in the dynamic regulation of the apparent patterns of oscillatory activity, however, this regulation is not decisive toward the determination of the oscillatory frequencies, even under conditions of increased inhibitory weight, and (c) phase analysis showed that interneurons discharge in synchrony; however, in a manner that was incompatible with providing a direct clock function. The ability to obtain these findings was a direct result of the experiments and analytical procedures we developed. Such procedures are necessary, since the commonly used fixed statistical models fail to adequately describe the elementary reactions underlying the oscillatory network response, which are inherently time-variable. A more appropriate description is obtained by time-variable analysis, such as the windowed FFT and the immediate firing phase.

Interneurons of area CA3 are the major cell-class to exhibit action potential firing during the hippocampal oscillatory mode of activity, where 40–60% of the population spontaneously discharges [8,14]. We found that during intrinsically generated oscillations CA3 interneurons form a kernel of action potentials that provides a total of 10–80 action potentials per theta cycle, given the fraction of discharging cells (~50%), population size (400 cells), and mean firing rates (1–4 Hz) (see Ref. [14]). We investigated the relation of this kernel to the initiation, pattern and frequency regulation, synchronization, and pacing of the oscillatory activity.

We found that interneuron discharge was not mediated by activation of their muscarinic receptors [21]. We therefore suggest that the activity of the principle cells and of glutamatergic synaptic network interactions is critical toward the initiation. This discharge might still contribute to the above-mentioned functions, but not to the initiation. As to the source of the small inhibitory events seen in CA3 PCs, we speculate that direct activation of the muscarinic receptors located at GABAergic terminals [21] may elicit their release, alternatively, activation of unidentified class of interneuron (excluding basket, bistratified and multi-subfield cells (identified previously [14])) may underlie this response, but is insufficient to produce theta or gamma. Moreover, there might exist scenarios where, during blockade of receptors that mediate fast glutamatergic transmission, the autonomous interneuron network would express oscillatory activity. For example through activation of metabotropic glutamate receptors [36] or through other theoretical models [3,34]; however, under what physiological conditions these scenarios would materialize is unknown. We can only say that those conditions do not occur at our experimental system.

Thereafter, we tested whether, once activated, interneuron discharge would determine the pattern and/or frequency of oscillatory activity. Through the use of

benzodiazepines, we found that, in spite of their effectiveness in prolonging the unitary IPSP [27], the frequencies of theta and gamma oscillations were not changed. Although in accordance with similar experiments preformed on different cholinergic in vitro models [10,28], these finding are contradicting the conclusion obtained by Fisahn et al. [12]. Possible explanations are: (a) the use of barbiturates rather than benzodiazepines, i.e., an agent known to be more potent and to have a different mechanism of action [29], and (b) the use of horizontal instead of transverse hippocampal slices. Nevertheless, the enhancement of the phasic inhibitory output by benzodiazepines had consistent effects on the oscillatory activity. It lead to depression of the amplitude of the synchronized excitatory input to PCs (CA1 and CA3) and interneuron (CA3), to the transient blockade of the intrinsic oscillator output to area CA1, to the enhancement of IPSPs weight in CA3 PCs, and to the appearance of IPSPs in CA3 interneurons. We refer to all those effects as a reflection of interneuron function to dynamically regulate the oscillatory activity. This, since such effects were time- and magnitude-variable. However, these regulatory functions are not decisive toward the pacing function of the intrinsic oscillator, and therefore secondary with respect to the mechanism.

In spite of the findings that interneuron discharge does not initiate or determine the frequency of oscillations, it could still play an important role with respect to pacing and synchronizing of the oscillations, by providing a reference temporal signal that by an unknown mechanisms would lead to the synchronization of the synaptic patterns to produce theta and gamma oscillations. For this purpose we determined the firing phase. We confirmed our previous suggestion [14] that the synchronized discharge of different interneurons produces the pattern of population IPSP observed in CA3 PCs. The phase of interneuron discharge mirrored the population IPSP, indicating that they are mainly activated during the falling-phase of the population IPSP. However, we cannot explain the synchronization of interneuron discharge by the activity of any of the interneuron classes we previously identified [14], since all of these cells showed a similar phase relationship. Moreover, synchronization of interneuron discharge did not result from an intrinsic process, as their membrane potential was neither depolarized nor temporally modulated during oscillations, and as action potentials were not generated, when the excitatory synaptic interactions of the network were eliminated. Therefore, we conclude that such synchroniza-

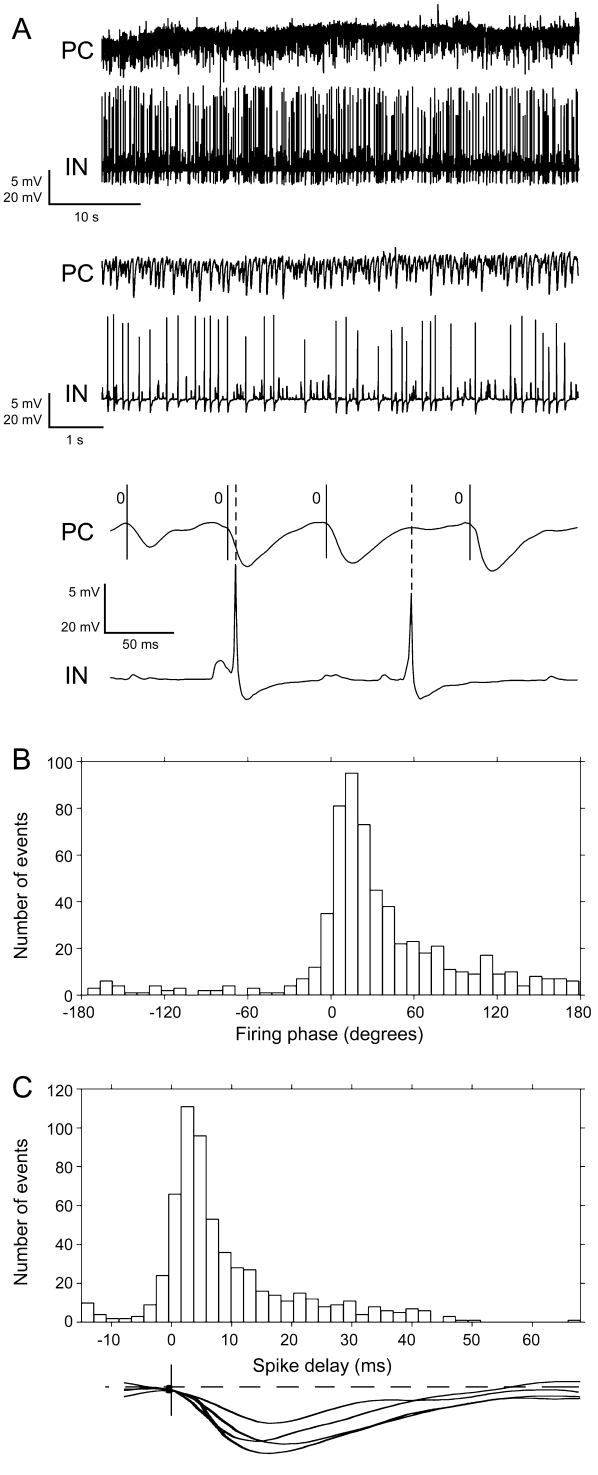


Fig. 7. Phase relationship of interneuron discharge and theta oscillations in PCs. (A) Simultaneous intracellular recordings from a CA3 PC (upper) and a CA3 interneuron (IN, lower) during oscillatory activity (MCh, 20 nM). Solid lines: cycles onset in the PC (taken as zero-phase). The distance between each pair of solid lines determines the local cycle length. The ratio between the local spike-delay (action potential delay to the cycle onset) and the local cycle-length provide the firing-phase for a particular action potential. A total of 609 action potentials, detected during 200-s of network oscillations, were used for phase analysis. Appearance of action potentials of varying amplitude was mainly due to sampling at 1-kHz (some interneurons have action potentials with a half-width shorter than 1 ms) (see also Section 2). (B) The firing phase histogram shows a phasic distribution of action potential firing, which peaks at positive delays, mainly within the first 60°. (C) Most action potentials occurred after the start of the cycle (IPSP onset), mainly within the first 10-ms, and mirror the time course of the population IPSPs recorded in the PC. Four IPSPs (illustrated in A) are temporally aligned with the spike-delay histogram. Resting potentials were −58 mV (IN) and −58 mV (PC).

tion depends on non-interneuronal network interactions, and suggest the principal cells as the major candidate. Also here there might exist conditions where the interaction between interneurons would lead to synchronized hippocampal responses, suggested to be mediated by gap junctions [9,17,18,31,32]. We cannot exclude a critical role for gap junction interactions in the synchronization of the network activity; however, our results indicate that such interactions just at the interneuron–interneuron level are insufficient.

Moreover, although interneuron discharge was phasic, in relation to the cycle, we cannot assign to it a clock function. The unimodal phasic discharge of interneurons could in theory serve as a time reference for the oscillatory activity. If it were to serve the clock function, we could have expected the main mode of firing to occur before the cycle onset. However, the unimodal discharge occurred preferentially after the onset of the population IPSP, i.e., the cycle onset, and therefore indicates that it is incompatible with that function. Therefore, we assume that pacing is achieved by other cells and interactions of the network, and suggest that the principle cells are more critical for this function.

To date a large body of work exist, which suggests that the activity of interneurons can be critical to the initiation, regulation and pacing of hippocampal oscillation. Among which: the setting of oscillatory frequencies by the duration of the IPSP [7,12,28]; the setting of oscillatory frequencies by interneuron discharge [7,12]; the initiation of oscillation by interneuron discharge [7,30,36]; the synchronization of oscillations by interactions among interneurons [9,17,18,31,32]; and a large set of theoretical studies [3,31,32,34,35].

Although these studies show the capability of the interneuronal network to produce patterns similar to those observed during hippocampal oscillation, it is not clear at all when the suggested experimental conditions are to be met. Indeed these studies help us to understand and speculate the scope of potential interactions. However, the consistent results obtained in our system strongly indicate that alternative solutions must be explored as well.

At present we cannot explain the oscillatory activity, i.e., initiation, frequency-control, synchronization and pacing, by the spontaneous patterns of discharge in the interneuron population. This since interneuron discharge did not result from direct cholinergic activation, the duration of individual interneurons synaptic output did not determine the frequencies of oscillatory activity, the time at which action potentials occurred was incompatible with pacing or triggering of the oscillations, and their firing activity was governed by non-interneuronal network interactions. We therefore suggest the function of interneurons is secondary with respect to these functions, and that other types of cells and network interactions serve them.

Consequently, we suggest that excitatory network interactions, in the putative intrinsic oscillator [13,24] (located in area CA3), determine both the synchronization of interneuron discharge and the expression of theta and gamma oscillations in the hippocampus.

Acknowledgements

We gratefully acknowledge the comments of Professor B.H. Gähwiler, Dr. M. Mori and Dr. N. Savic, and the technical assistance of L. Heeb, E. Hochreutener, L. Rietschin, and R. Schöb. Supported by the Dr. Eric Slack-Gyr and Swiss National Science Foundations.

References

[1] P. Andersen, H.B. Bland, T. Myhrer, P.A. Schwartzkroin, Septo-hippocampal pathway necessary for dentate theta production, Brain Res. 165 (1979) 13–22.
[2] A. Arduini, O. Pompeiano, Attivita elettrica di singole unita dell'ippocampo e sue modificazioni con stimuli afferenti, Arch. Sci. Biol. 39 (1955) 397–406.
[3] M. Bartos, I. Vida, M. Frotscher, A. Meyer, H. Monyer, J.R.P. Geiger, P. Jonas, Fast synaptic inhibition promotes synchronized gamma oscillations in hippocampal interneuron networks, Proc. Natl. Acad. Sci. USA 99 (2002) 13222–13227.
[4] A. Bragin, G. Jandó, Z. Nádasdy, J. Hetke, K. Wise, G. Buzsáki, Gamma (40–100 Hz) oscillations in the hippocampus of the beaving rat, J. Neurosci. 15 (1995) 47–60.
[5] G. Buzsáki, Theta oscillations in the hippocampus, Neuron 33 (2002) 325–340.
[6] G. Buzsáki, J. Czopf, I. Kondákor, L. Kellényi, Laminar distribution of hippocampal rhythmic slow activity (RSA) in the behaving rat: current-source density analysis, effects of urethane and atropine, Brain Res. 365 (1986) 125–137.
[7] S.R. Cobb, E.H. Buhl, K. Halasy, O. Paulsen, P. Somogyi, Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons, Nature 378 (1995) 75–78.
[8] J. Csicsvari, H. Hirase, A. Czurko, G. Buzsáki, Reliability and state dependence of pyramidal cell-interneuron synapses in the hippocampus: an ensemble approach in the behaving rat, Neuron 21 (1998) 179–189.
[9] A. Draguhn, R.D. Traub, D. Schmitz, J.G. Jefferys, Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro, Nature 394 (1998) 189–192.
[10] H.J. Faulkner, R.D. Traub, M.A. Whittington, Disruption of synchronous gamma oscillations in the rat hippocampal slice: a common mechanism of anaesthetic drug action, Br. J. Pharmacol. 125 (1998) 483–492.
[11] J.-M. Fellous, T.J. Sejnowski, Cholinergic induction of oscillations in the hippocampal slice on the slow (0.5–2 Hz), theta (5–12 Hz), and gamma (35–70 Hz) bands, Hippocampus 10 (2000) 187–197.
[12] A. Fisahn, F.G. Pike, E.H. Buhl, O. Paulsen, Cholinergic induction of network oscillations at 40 Hz in the hippocampus in vitro, Nature 394 (1998) 186–189.
[13] Y. Fischer, B.H. Gähwiler, S.M. Thompson, Activation of intrinsic hippocampal theta oscillations by acetylcholine in rat septo-hippocampal cocultures, J. Physiol. (London) 519 (1999) 405–413.
[14] Y. Fischer, L. Wittner, T.F. Freund, B.H. Gähwiler, Simultaneous activation of gamma and theta network oscillations in rat hippocampal slice cultures, J. Physiol. (London) 539 (2002) 857–868.
[15] T.F. Freund, M. Antal, GABA-containing neurons in the septum

control inhibitory interneurons in the hippocampus, Nature 336 (1988) 170–173.
[16] B.H. Gähwiler, S.M. Thompson, R.A. McKinney, D. Debanne, R.T. Robertson, Organotypic slice cultures of neuronal tissue, in: G. Banker, K. Goslin (Eds.), Culturing Nerve Cells, Vol. 2, MIT Press, Cambridge, MA, 1998, pp. 461–498.
[17] M. Galarreta, S. Hestrin, A network of fast-spiking cells in the neocortex connected by electrical synapses, Nature 402 (1999) 72–75.
[18] J.R. Gibson, M. Beierlein, B.W. Connors, Two networks of electrically coupled inhibitory neurons in neocortex, Nature 402 (1999) 75–79.
[19] J.A. Gray, Medial septal lesions, hippocampal theta rhythm and control of vibrissal movement in freely moving rat, Electroencephalogr. Clin. Neurophysiol. 30 (1971) 189–197.
[20] J.D. Green, A.A. Arduini, Hippocampal electrical activity in arousal, J. Neurophysiol. 17 (1954) 533–557.
[21] N. Hájos, E.C. Papp, L. Acsády, A.I. Levey, T.F. Freund, Distinct interneuron types express m2 muscarinic receptor immunoreactivity on their dendrites or axon terminals in the hippocampus, Neuroscience 82 (1998) 355–376.
[22] J.G. Jefferys, R.D. Traub, M.A. Whittington, Neuronal networks for induced '40 Hz' rhythms, Trends Neurosci. 19 (1996) 202–208.
[23] R. Jung, A.E. Kornmüller, Eine methodik der ableitung lokalisierter potential schwankungen aus subcorticalen hirngebeiten, Arch. Psychiatr. Nervenkrankh. 109 (1938) 1–30.
[24] B. Kocsis, A. Bragin, G. Buzsáki, Interdependence of multiple theta generators in the hippocampus: a partial coherence analysis, J. Neurosci. 19 (1999) 6200–6212.
[25] M.G. Lee, J.J. Chrobak, A. Sik, R.G. Wiley, G. Buzsáki, Hippocampal theta activity following selective lesion of the septal cholinergic system, Neuroscience 62 (1994) 1033–1074.
[26] B.A. MacVicar, F.W.Y. Tse, Local neuronal circuitry underlying cholinergic rhythmical slow activity in area CA3 of rat hippocampal slices, J. Physiol. (London) 417 (1989) 197–212.
[27] J.-C. Poncer, R. Dürr, B.H. Gähwiler, S.M. Thompson, Modulation of synaptic GABAA receptor function by benzodiazepines in area CA3 of rat hippocampal slice cultures, Neuropharmacology 35 (1996) 1169–1179.
[28] K. Stenkamp, J.M. Palva, M. Uusisaari, S. Schuchmann, D. Schmitz, U. Heinemann, K. Kaila, Enhanced temporal stability of cholinergic hippocampal gamma oscillations following respiratory alkalosis in vitro, J. Neurophysiol. 85 (2001) 2063–2069.
[29] R.E. Study, J.L. Barker, Diazepam and (–)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of gamma-aminobutyric acid responses in cultured central neurons, Proc. Natl. Acad. Sci. USA 78 (1981) 7180–7184.
[30] K. Tóth, T.F. Freund, R. Miles, Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum, J. Physiol. (London) 500 (1997) 463–474.
[31] R.D. Traub, M.A. Whittington, I.M. Stanford, J.G. Jefferys, A mechanism for generation of long-range synchronous fast oscillations in the cortex, Nature 383 (1996) 621–624.
[32] R.D. Traub, N. Koppel, A. Bibbig, E.H. Buhl, F.E. LeBeaue, M.A. Whittington, Gap junctions between interneuron dendrites can enhance synchrony of gamma oscillations in distributed networks, J. Neurosci. 21 (2001) 9478–9486.
[33] W.G. Walter, in: The Living Brain, W.W. Norton & Company, New York, 1953.
[34] X.J. Wang, G. Buzsáki, Gamma oscillation by synaptic inhibition in a hippocampal interneuronal network model, J. Neurosci. 16 (1996) 6402–6413.
[35] J.A. White, M.I. Banks, R.A. Pearce, N.J. Kopell, Networks of interneurons with fast and slow γ-aminobutyric acid type A ($GABA_A$) kinetics provide substrate for mixed gamma-theta rhythm, Proc. Natl. Acad. Sci. USA 97 (2000) 8128–8133.
[36] M.A. Whittington, R.D. Traub, J.G. Jefferys, Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation, Nature 373 (1995) 612–615.
[37] J.H. Williams, J.A. Kauer, Properties of carbachol-induced oscillatory activity in rat hippocampus, J. Neurophysiol. 78 (1997) 2631–2640.
[38] A. Ylinen, I. Soltész, A. Bragin, M. Penttonen, A. Sik, G. Buzsáki, Intracellular correlates of hippocampal theta rhythm in identified pyramidal cells, granule cell, and basket cells, Hippocampus 5 (1995) 78–90.