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In vivo contribution of h-channels in the septal pacemaker to theta rhythm generation

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Abstract

One of the most intriguing network-level inferences made on the basis of *in vitro* and modelling data regarding the role of I_h current was that they participate in rhythmogenesis in different parts of the brain. The nature of I_h contribution to various neuronal oscillations is far from uniform however, and the proper evaluation of the role of I_h in each particular structure requires *in situ* investigations in the intact brain. In this study we tested the effect of I_h blockade in the medial septum on hippocampal theta rhythm in anaesthetized and freely behaving rats. We could not confirm the recent report of elimination of theta by septal injection of ZD7288 [C. Xu *et al.* (2004) *Eur. J. Neurosci.*, 19, 2299–2309]; the observed effects were more subtle and more specific. We found that I_h blockade in the medial septum substantially decreased the frequency of hippocampal oscillations without changing the context in which theta occurred, i.e. specific behaviours in freely moving rats and spontaneous switching and brainstem stimulation under anaesthesia. Septal injection of ZD7288 eliminated atropine-resistant theta elicited by high intensity electrical stimulation of the reticular formation in anaesthetized rats but was ineffective in combination with the muscarinic agonist, carbachol. Thus, functional I_h was necessary for the septum to generate or transmit high frequency theta rhythm elicited by strong ascending activation, whereas low frequency theta persisted after I_h blockade. These results suggest that I_h plays a specific role in septal theta generation by promoting fast oscillations during exploratory behaviour and rapid eye movement sleep.

Introduction

Hyperpolarization-activated cation currents (I_h) have been implicated in rhythmogenesis in both heart (DiFrancesco, 1993) and brain (Pape, 1996; Robinson & Siegelbaum, 2003). In the central nervous system, the pacemaker capacity of I_h has been extensively investigated *in vitro* (Soltesz *et al*., 1991; Luthi & McCormick, 1998; Santoro *et al*., 1998) and in modelling studies (McCormick & Huguenard, 1992; Kopell & LeMasson, 1994; Hasselmo *et al*., 2000; Gillies *et al*., 2002) but its contribution to neuronal oscillations in preparations, preserving all synaptic and modulatory connections, remains poorly understood. *In vivo* investigations are specifically important for studying distributed networks where neuronal pacemakers and network oscillators are functionally coupled to produce synchronized rhythmic activity.

Theta rhythm, a prominent feature of hippocampal activity involved in memory processing, is present in numerous limbic structures (Vertes & Kocsis, 1997) which are synchronized during specific behaviours, such as awake exploration and rapid eye movement (REM) sleep. Hippocampal theta is driven by major inputs from entorhinal cortex and medial septum/diagonal band (MS) (Kocsis *et al*., 1999; Buzsaki, 2002). H-channels are expressed in projecting neurons of both these structures (Griffith, 1988; Dickson *et al*., 2000; Sotty *et al*., 2003). In the MS, which is considered the most important theta pacemaker, I_h was found in bursting GABAergic neurons (Griffith, 1988; Morris *et al*., 1999; Sotty *et al*., 2003; Xu *et al*., 2004). Sustained rhythmicity of these cells, however, was not observed in slice indicating that they might not necessarily pace hippocampal theta by themselves but may transfer rhythmic burst discharges coming from other structures (Sotty *et al*., 2003). GABAergic septohippocampal neurons are indeed part of a network of rhythmically firing cells, which include both ascending rhythmic input from the supramammillary nucleus (SUM) (Kirk & McNaughton, 1991; Kocsis & Vertes, 1994) and descending feedback from the hippocampus (Toth *et al*., 1993). They also receive excitatory drive from local cholinergic neurons (Wu *et al*., 2000) which are known to play an important role in hippocampal theta generation (Vanderwolf, 1969; Stewart & Fox, 1990; Vertes & Kocsis, 1997; Buzsaki, 2002).

The role of I_h on hippocampal theta rhythm *in vivo* was addressed in two recent studies which, however, lead to conflicting conclusions. Buhl *et al*. (2002) reported that in mice in which one of the HCN-channels had been deleted, theta was present in the hippocampal electroencephalogram (EEG) and appeared in the proper behavioural context. In the second study by Xu *et al*. (2004) I_h was specifically blocked in the MS of rats after which theta was found completely eliminated. Theta rhythm in this study, however, was elicited by acoustic stimuli, which has limited effect in rats and depends on normal startling reaction.

Selective I_h blockers were widely used in examinations of the properties and role of h-channels in slice. The effect of caesium and ZD7288 (Harris & Constanti, 1995) on pacemaker activity was found similar in isolated sinoatrial node and various nerve cells and the cardiac effect of I_h blockade (i.e. bradycardia) has been also verified *in vivo* (Rouse *et al*., 1994). On the other hand, activators of adenylyl cyclase, such as forskolin, were shown to enhance I_h activity (Brown *et al*., 1979; Pape, 1996; Robinson & Siegelbaum, 2003). In the

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present study we tested the effect of pharmacological suppression and enhancement of I_h in the MS on hippocampal theta oscillations. As GABAergic septohippocampal neurons, i.e. the carriers of I_h in the MS, are responsible for a fast (6–9 Hz) atropine-resistant component of the theta rhythm but may also participate in atropine-sensitive slow (3–5 Hz) theta, we also asked whether I_h blockade completely disrupts theta rhythm or selectively eliminates its faster component.

Materials and methods

Electrophysiological recordings

Experiments were performed on 46 male Sprague–Dawley rats (220–420 g, Charles River Laboratories, MA) treated in accordance with NIH guidelines. All procedures were approved by the Institutional Animal Care and Use Committees of Harvard Medical School and Beth Israel Deaconess Medical Center. Five rats for chronic experiments were prepared for standard sleep recordings. Electrodes for neck muscle EMG, frontal cortical EEG, and hippocampal EEG and a guide cannula with diameter of 0.58 mm for MS injections were implanted under ketamine/xylasine (35–45 and 5 mg/kg of body weight, respectively) anaesthesia. The recordings started 5–7 days after surgery and lasted for 4–6 h a day for 3–5 days. The h-current blocker, ZD7288, in doses of 0.2 or 1.5 μg, was administered in 0.5 μL artificial cerebrospinal fluid (ACSF) by pressure injection after which the rat's behaviour and hippocampal EEG was monitored for 1 h in a new environment and for another 4 h in their home cage. Acute experiments were conducted in 41 rats under urethane anaesthesia (1.2–1.5 g/kg of 65–80% solution i.p.). Hippocampal EEG was recorded on both sides and the dynamics of theta and non-theta patterns of activity was monitored and compared in controls and during and after (1 h each) drug administration in the MS using reverse microdialysis (rate 80 μL/h, probe 0.24 mm diameter, 1 mm membrane length, CMA Microdialysis, Acton, MA, USA). Theta was also elicited by electrical stimulation (square wave stimuli of 0.2 ms at 100 Hz) of the nucleus pontis oralis (8 mm caudal from bregma, 1.8 mm lateral, ≈6 mm from skull surface, fine adjusted so the stimulus threshold was below 0.5 mA) at different intensities in control and 20–90 min after the start of drug application. The following drugs were applied: ZD7288 and NKH477 from TOCRIS (Ellisville, MO, USA), caesium from Fluka (Switzerland), carbamylcholine chloride (carbachol) and atropine sulphate from Sigma-Aldrich (St. Louis, MO, USA).

To verify the placement of electrodes and the microdialysis probe after the experiments the rats were deeply anaesthetized and perfused through the aortic arch with 0.9% NaCl followed by a fixative solution containing 4% paraformaldehyde (Sigma-Aldrich, Germany), and 15% (V/V%) saturated picric acid (Sigma-Aldrich, Germany) in 0.1 M phosphate buffer (PB, pH 7.4, 0.1 M) and their brains removed and stored. Sixty-micron sections were taken with a freezing microtome and stained with cresyl violet. The traces of the injection guide cannula in chronic experiments were found above the MS at 5.5 mm from the surface (see Fig. 1A). The 32-gauge injection cannula protruded from the guide cannula by 0.5 mm so as to minimize the damage of MS cells. The microdialysis probe was also aimed at the MS but the tip was placed deeper (7 mm) so the membrane (1 mm in length) covered much of the MS. Figure 2A shows probe placements in two experiments that gave similar results. In the first rat (Fig. 2, A2) the probe was placed at the midline. The damage caused by the metal part of the probe was above the MS in the area where vessels are usually found. The membrane was also at the midline in the MS (note narrow light band along track on both sides). In the second rat (Fig. 2, A3) the probe was deflected to the side and thus spared all neurons in the midline. Hippocampal electrodes were verified (see Fig. 2, A1) in the hippocampus at the level of the fissure whereas the reference electrode was placed above the CA1 layer (interelectrode distance ≈1 mm). This arrangement was shown (Bland *et al*., 1975) to give the largest theta signal.

Data analysis

Sleep-wake states were identified using standard criteria as active waking/exploratory behaviour, quiet waking, grooming, slow wave sleep and rapid-eye-movement sleep. Brain electrical activity in anaesthetized rats was identified on the basis of hippocampal field potentials as theta or non-theta activity. For quantitative analysis, hippocampal EEG recordings were filtered between 1 and 70 Hz and sampled at 256 Hz. Power spectra were calculated using Fast Fourier Transform on 4 s windows as described previously in detail (Kocsis & Vertes, 1994; Kocsis *et al*., 1999). The spectral peaks corresponding to theta oscillations (frequency between 3 and 10 Hz, power at least four times larger than background) were identified and subjected to the following statistical analyses. Peak frequency and power of EEG segments recorded during control and drug administrations (time windows of equal lengths) were compared using paired, two-tailed Student's *t*-test. The dose–response of a drug, and the difference between the effects of different drugs were tested by one-way analysis of variance (ANOVA) with dose or drugs as factors. An effect was considered significant if $P < 0.05$. Values are expressed as mean ± SEM.

Results

In unanaesthetized freely moving rats, hippocampal theta rhythm is known to selectively occur during waking exploration, voluntary movements and rapid-eye-movement sleep, observed also in our rats for 3–5 days before drug injection. Examples of such control recordings are shown in Fig. 1. After septal injection of 0.2 μg ZD7288 the rats appeared somewhat depressed; gentle pushing or auditory stimuli, which normally makes them move were ineffective and, with absent startle response, did not elicit theta in the hippocampus either (Xu *et al*., 2004). When placed in an unfamiliar environment, however, the rats showed normal exploratory behaviour accompanied with strong theta synchronization in the hippocampus. Approximately one hour after injection and after returning to their home cage, natural sleep-wake cycles were observed with prominent theta during episodes of waking motor activity and rapid-eye-movement sleep. The frequency of theta significantly changed, however. Instead of the usual range of 8–10 Hz in control recordings the frequency of theta peak dropped to 6–8 Hz after ZD7288 injection (Fig. 1). The average decrease in the group reached 17.0% during REM sleep ($P < 0.01$, $n = 5$) and 17.4% during awake exploration ($P < 0.001$, $n = 5$). The changes were irreversible; theta remained of low frequency until the end of the recording session and even on the following day. Theta rhythm also persisted in two rats after injection of a larger dose (1.5 μg) of ZD7288.

Theta frequency in freely behaving rats depends on a number of factors including the level of arousal, the speed of the animal's movements, its motivation to explore, etc. Thus, for a more detailed analysis of the effect of I_h blockade on theta frequency we used anaesthetized rats, a more controllable preparation that lacks the confounding effect of behaviour but has all network connections intact. Although anaesthesia introduces other confounds related to the level of anaesthesia, under urethane, slow theta (3–5 Hz) appears in

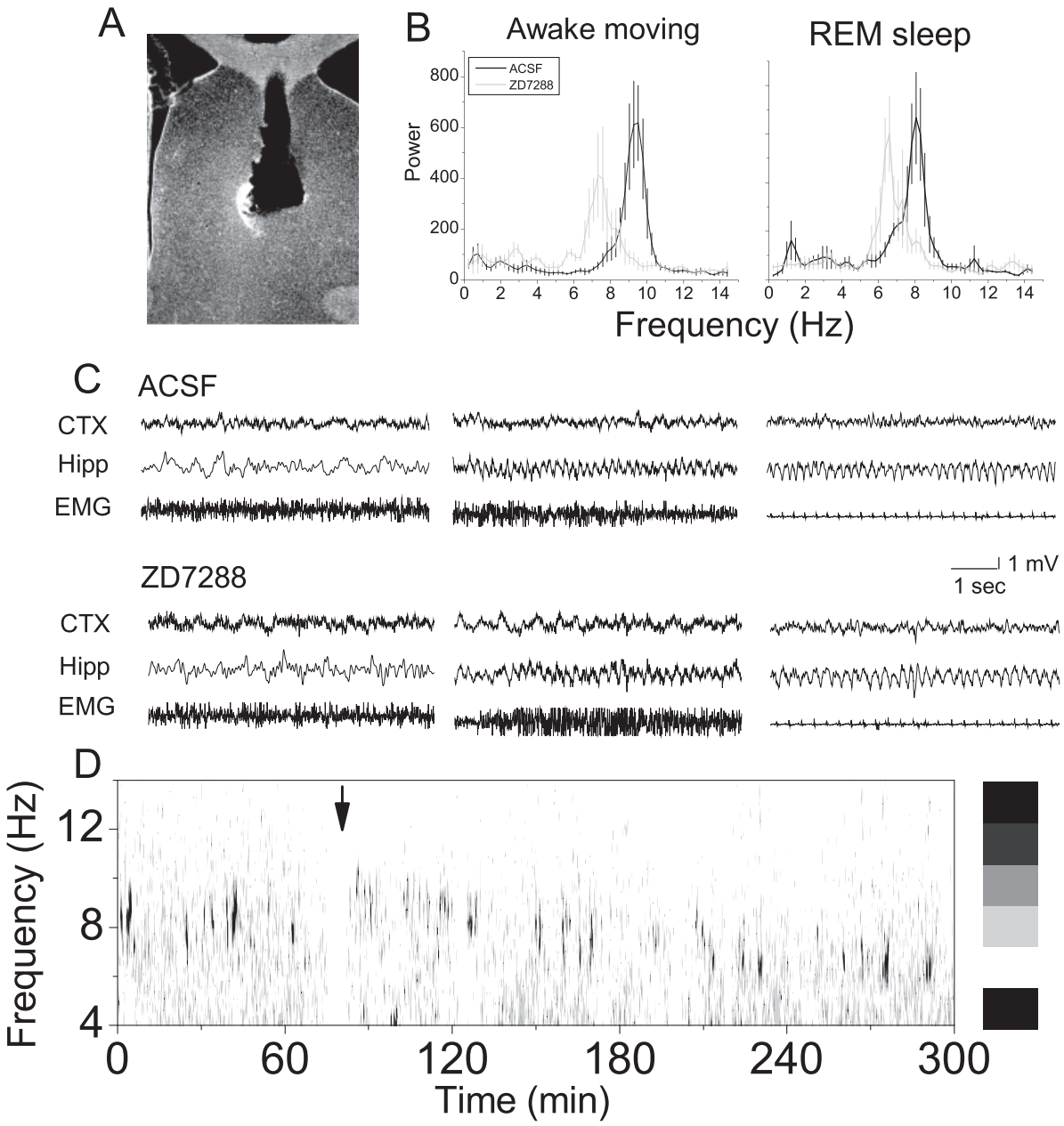
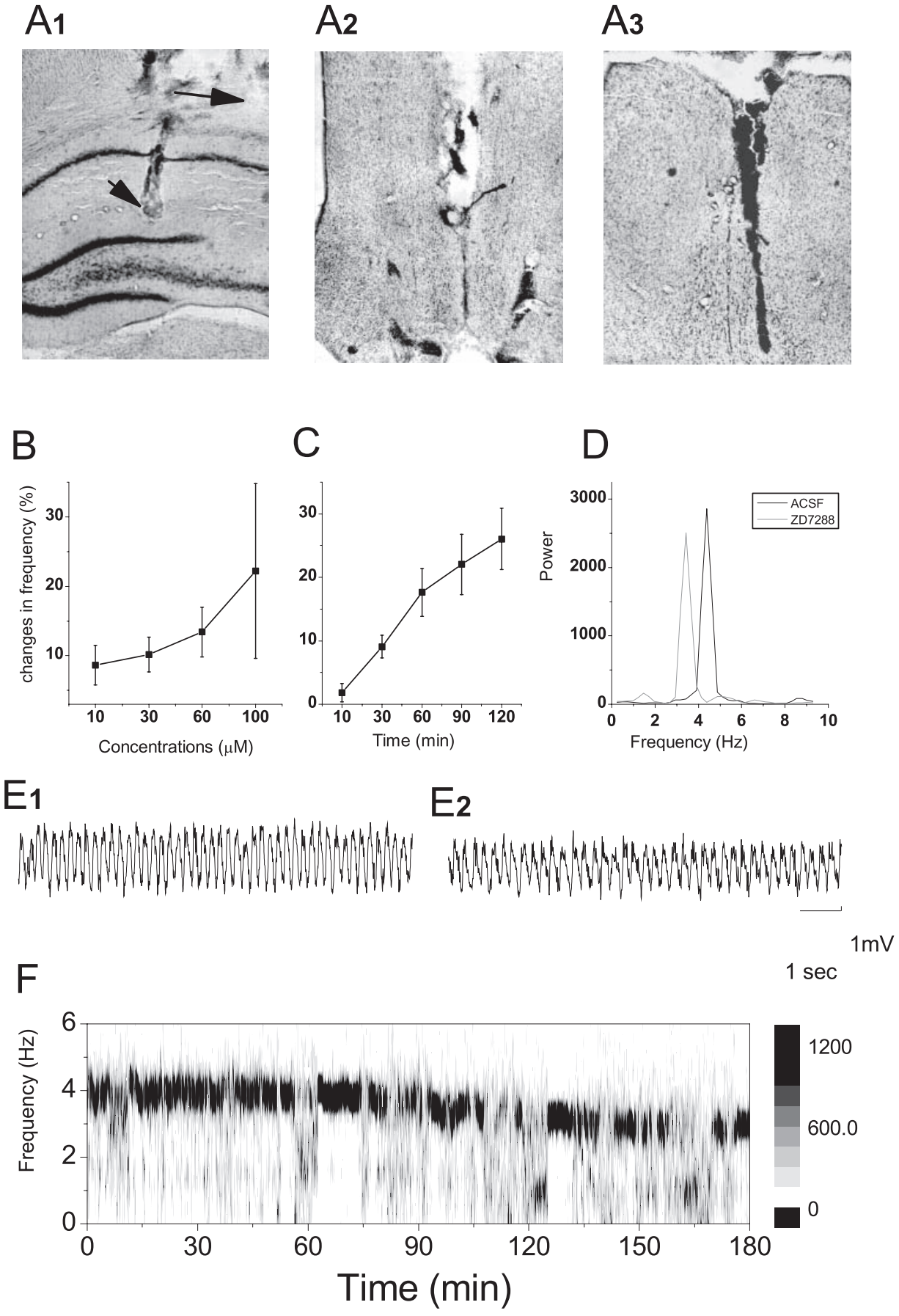


FIG. 1. Effect of septal I_h blockade on hippocampal EEG in freely behaving rats. Theta rhythm associated with exploratory behaviour and rapid-eye-movement sleep remained after septal injection of ZD7288 but its frequency decreased. (A) Location of injection cannula in MS. (B) Autospectra of hippocampal EEG before (ACSF, black traces) and after injection of ZD7288 (grey traces). (C) Four-second segments of cortical (CTX) and hippocampal EEG (Hipp) and neck muscle EMG during grooming (left), awake exploratory behaviour (middle) and REM sleep (right) in control (ACSF) and ZD7288-treated rats. Note prominent theta rhythm in exploration and REM sleep but no theta during grooming, both before and after drug injection. (D) Temporal variations of hippocampal EEG autospectra before and after injection of ZD7288 in MS (arrow). Note short episodes of rhythmic synchronization (spectral peaks) shown in black. Low-frequency (< 4 Hz) components are not shown (scale 4–14 Hz). Calibrations, 1 s, 1 mV.

the rat hippocampus spontaneously (Fig. 2) whereas faster theta (4–9 Hz) can be elicited by graded electrical stimulation of the pontine reticular formation (Vertes, 1981) (Fig. 3).

In anaesthetized rats ZD7288 was administered in the MS at different doses between 10 μM and 300 μM using the reverse microdialysis technique, which allows the maintenance of a certain concentration of the drug in the brain tissue surrounding the probe. Small but significant slowing of spontaneous theta was observed at very low doses of ZD7288. Thirty minutes after the start of perfusion at 10 μM, the frequency of theta decreased by 8.6%. The 30-min fall in theta frequency was larger as the dose increased and reached a maximum of 22.2% at 100 μM (Fig. 2A). A similar degree of frequency reduction was reached at lower doses if the perfusion continued. For example, in the largest group of animals ($n = 15$) the reduction in theta frequency reached 21.3% after 90-min infusion of 30 μM ZD7288 (Fig. 2B). Large doses, i.e. 200–300 μM of ZD7288, which were shown earlier to have nonspecific effects on synaptic transmission (Harris & Constanti, 1995; Dickson *et al.*, 2000) in addition to I_h blockade, resulted in complete elimination of spontaneous theta rhythm.


A1
A2
A3
B
changes in frequency (%)
30
20
10
10
30
60
100
Concentrations (μM)
C
30
20
10
0
10
30
60
90
120
Time (min)
D
3000
2000
1000
0
Power
ACSF
ZD7288
0
2
4
6
8
10
Frequency (Hz)
E1
E2
1mV
1 sec
F
Frequency (Hz)
6
4
2
0
0
30
60
90
120
150
180
Time (min)
1200
600.0
0

Several lines of evidence indicate that hippocampal theta is initiated by tonic activation of the brainstem reticular formation. Neurons in the nucleus pontis oralis are activated during theta states both in waking and REM sleep (Vertes, 1977) and electrical or pharmacological stimulation of this area elicits theta rhythm in the hippocampus in a dose-dependent manner. If the electrode is placed in the precise triggering location in the pons (Vertes, 1981) theta can be elicited using very low currents and the theta frequency that can be achieved in urethane anaesthetized rats nearly equals that in freely moving animals. The relationship between stimulus intensity and the frequency of the evoked theta rhythm is linear (Fig. 3C). Blockade of I_h by ZD7288 in the MS exerted a frequency reduction in evoked theta that was even stronger than in spontaneous theta as demonstrated by the divergence of the stimulus intensity-theta frequency characteristics in control and drug-treated animals (Fig. 3C). Perfusion of the MS with 30 μM of ZD7288 ($n = 15$) resulted in a 13% reduction in low-frequency theta (from 5.12 ± 0.15 Hz to 4.47 ± 0.22 Hz) elicited by 0.4 mA stimulation whereas a significantly ($P < 0.006$) larger, 22% reduction (8.19 ± 0.27 Hz to 6.35 ± 0.29 Hz) was observed at higher stimulation intensities (1.5 mA). Increasing the dose to 200–300 μM led to inconsistent responses, i.e. theta was elicited by some stimulations and not by others with no apparent relationship to time or stimulus intensity (not shown).

Similar frequency reduction of both spontaneous and evoked theta was observed after perfusion of the MS by CsCl ($n = 5$) in doses of 5 or 10 mM (Fig. 3D); 0.5 mM had no effect.

Local perfusion of NKH477, a water-soluble analogue of the adenylyl cyclase activator forskolin had an opposite effect. After 60 or 90 min of NKH477 infusion in concentrations of 0.5 and 2 mM, the highest peak of evoked theta frequency increased by 9.4%. The increase was significant ($P < 0.01$) at stimulations eliciting theta faster than 6 Hz. NKH477 was infused in seven rats in which the maximum of evoked frequency in control was between 6.10 and 7.35 (i.e. lower than in the total population) to avoid the ceiling effect; correlation between control theta peak and the increase in frequency after NKH477 was –0.70.

Next, we examined the possible contribution of I_h to atropine-resistant and atropine-sensitive components of the theta rhythm. Systemic administration of large doses of atropine has long been known to eliminate slow theta components while sparing high frequency theta bursts in freely behaving animals (Vanderwolf, 1969; Bland, 1986; Buzsaki, 2002). Under urethane anaesthesia, spontaneous theta was completely abolished by atropine (Vanderwolf, 1969; Bland, 1986; Buzsaki, 2002) and low intensity pontine stimulation was in most experiments incapable of triggering theta synchronization in the hippocampus (Fig. 4D). Reliable theta triggering could only be achieved when the stimulus intensity was high enough to induce fast oscillations with frequencies above 5–6 Hz. Twenty-five to sixty minutes after intraperitoneal injection of 50 mg/kg atropine the stimulus threshold to elicit theta at high probability (i.e. failure rate below 30%, Fig. 4D) was 0.7 mA, up from 0.1 mA before injection. The frequencies of theta rhythm elicited by strong stimuli were similar to those measured in control ($P < 0.05$, Fig. 4C). This residual, atropine-resistant, high frequency theta was completely removed (failure rate = 100% at all intensities) by I_h blockade (30 μM ZD7288, $n = 5$, Fig. 4A and D).

On the other hand, local injection of muscarinic agonists in the MS resulted in lasting theta in the hippocampus (Fig. 4A). The frequency of this rhythm was close to 6 Hz and could not be modified with pontine stimulation. ZD7288 administered in the MS reduced the frequency of carbachol-induced theta by 8–10% to an average of 5.3–5.6 Hz ($n = 4$). Thus, at high intensity brainstem activation, the effects of carbachol and ZD7288 were similar ($P = 0.5$), both preventing stimulation-induced accelerations of theta oscillations above 5–6 Hz.

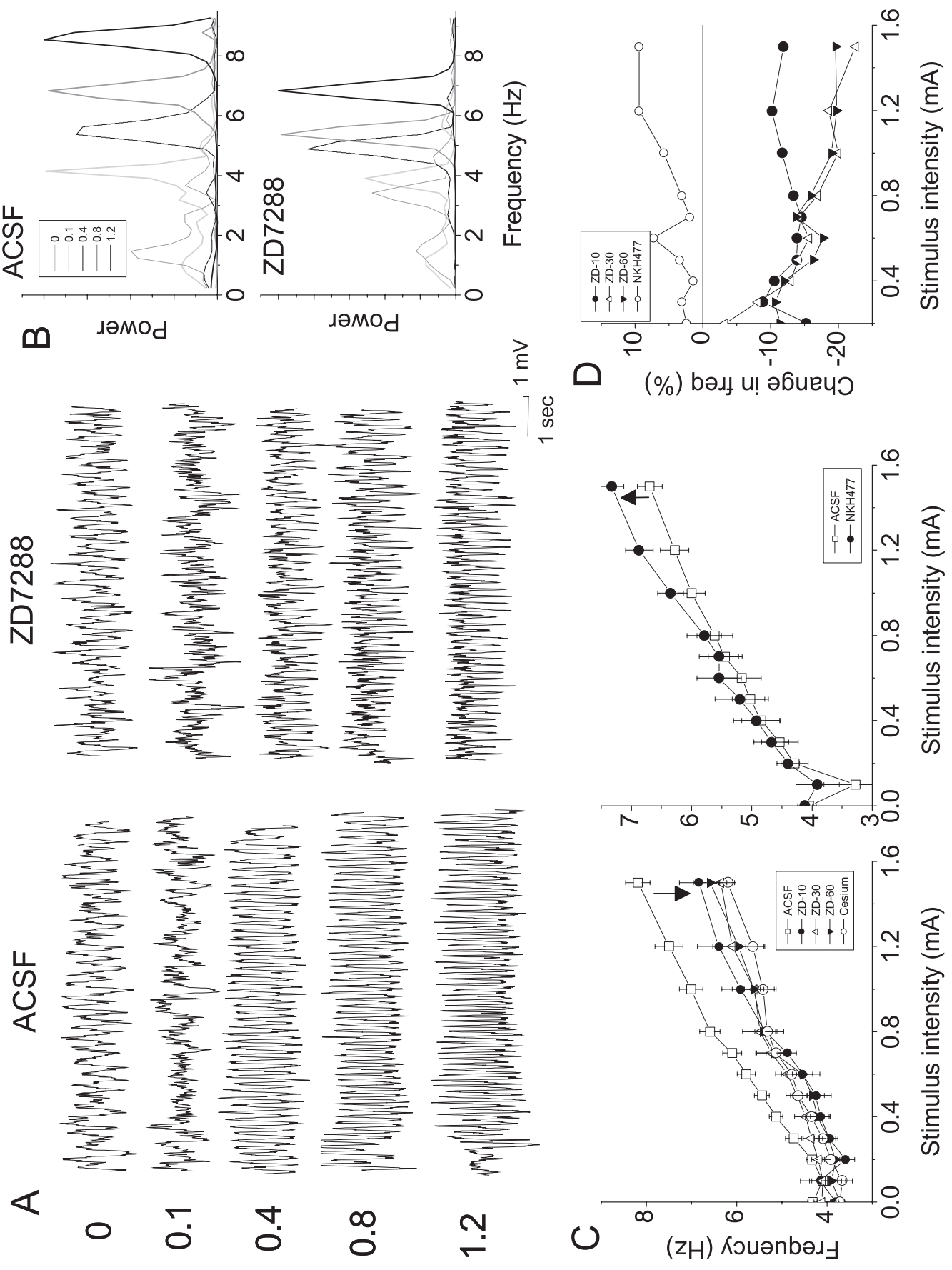
Discussion

In freely behaving rats, hippocampal theta persisted after I_h blockade in the MS and its appearance followed a natural dynamics, as found earlier in mice in which one of the HCN-channels had been deleted (Buhl *et al*., 2002). Thus, theta consistently occurred in all rats during exploratory behaviour and REM sleep even after injections of large doses of ZD7288. This finding does not necessarily contradict to the data obtained in the experiments of Xu *et al*. (2004) in which after MS injection of ZD7288, using a similar protocol, theta could no longer be elicited by acoustic stimuli. Importantly, according to the spectral analysis in this latter report, the response to sensory stimulation was not that of reduced theta oscillations but the acoustic stimulus was unable to set off the reaction (compare sharp spectral peaks in Fig. 1B with wide band delta signal in Fig. 4 in Xu *et al*., 2004). Although not specifically tested, diminished startling, which could explain lack of theta after auditory stimulation, was in accord with our observations of the general behaviour of rats after ZD7288 injection. Xu *et al*. (2004) also mentioned that after septal I_h blockade 'hippocampal EEG waves became large and irregular during voluntary movements'. This was clearly not the case in our experiments, but the two studies are hard to compare as no quantitative data have been presented by Xu and colleagues to support their note except a specimen recording (see Fig. 3 Xu *et al*., 2004) showing irregular hippocampal EEG together with neck muscle activity, which can also appear during grooming or other consummatory types of movements (see, e.g. Fig. 1C left column).

Although it failed to eliminate naturally occurring theta, blockade of I_h in the septal pacemaker was not without effect in freely moving rats. As a sign of altered mechanism of rhythm generation, the frequency of theta oscillations in the hippocampus was significantly reduced, both during awake exploratory behaviour and REM sleep. The decrease in theta frequency was irreversible and the size of reduction (≈17%) was similar to earlier behavioural studies in which ascending activation via the supramammillary nucleus was suppressed (McNaughton *et al*., 1995; Thinschmidt *et al*., 1995). Theta synchronization is important for memory processing and even a small decrease in theta frequency was shown to impair spatial learning (Pan & McNaughton, 1997).

The mechanism of changes in theta generation was further investigated in anaesthetized rats. Under urethane anaesthesia, theta rhythm also occurred after I_h blockade both spontaneously and in response to electrical stimulation of the pontine reticular formation.

FIG. 2. Effect of septal I_h blockade on spontaneous hippocampal theta rhythm in rats anaesthetized with urethane. (A) Placement of hippocampal electrodes (see arrows in A1) and MS microdialysis probe. The latter is shown in two experiments (A2 and A3) which gave similar results. In A2 the probe was placed on the midline in A3 it was deflected to the right. (B) Per cent decrease in theta frequency 30 min after the start of microdialysis application of different doses (10, 30, 60, and 100 μM) of ZD7288 in the MS. (C) Time course of changes in theta frequency during 120-min perfusion with 30 μM of ZD7288. (D) Autospectra and (E) specimen recordings of hippocampal EEG during theta epochs before (E1) and during (E2) I_h blockade. (F) Time–frequency contour plot of changes in hippocampal EEG autospectra before (1 h) and during 10 μM ZD7288 perfusion (2 h; start at 60 min). Time calibration, 1 s.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ACSF
ZD7288
0
0.1
0.4
0.8
1.2
1 mV
1 sec
B
ACSF
ZD7288
Power
Frequency (Hz)
0
0.1
0.4
0.8
1.2
C
Frequency (Hz)
Stimulus intensity (mA)
ACSF
ZD-10
ZD-30
ZD-60
Cesium
NKH477
D
Change in freq (%)
Stimulus intensity (mA)
ZD-10
ZD-30
ZD-60
NKH477

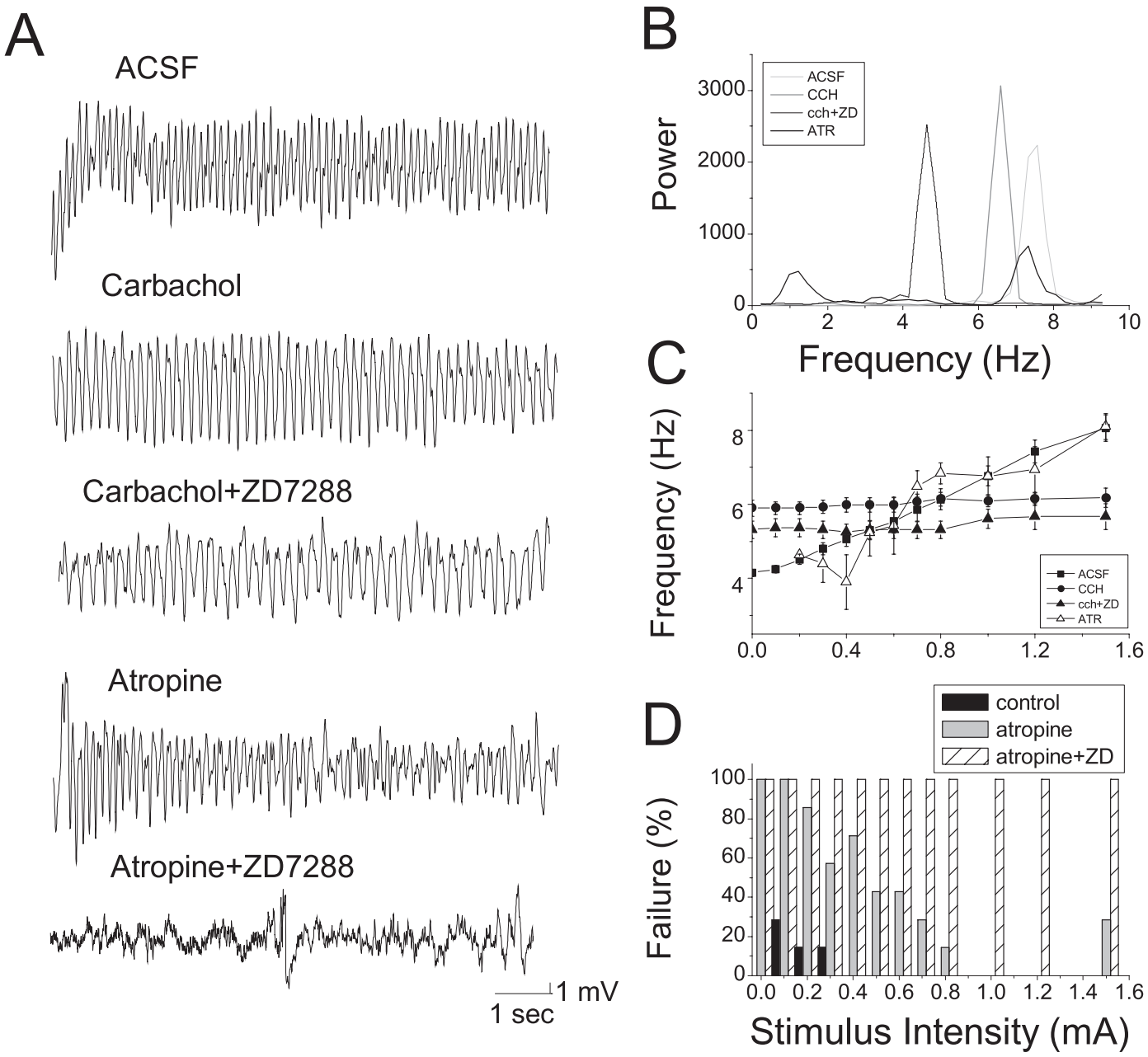

FIG. 4. Effect of septal I_h blockade on atropine-resistant and atropine-sensitive hippocampal theta rhythm. (A) Specimen recordings of hippocampal EEG during 1.0 mA stimulation in control (top trace) and during application of carbachol, a mixture of carbachol and ZD7288, atropine, and ZD7288 on the background of atropine. (B) Autospectra of the traces shown in A. (C) Frequency of theta peaks in the autospectra during stimulation with different intensities in control and after drug treatments. (D) Per cent of stimulation trials that failed to elicit hippocampal theta in control and after atropine.

Here, too, however, its frequency was limited to the lower half of the theta range (i.e. 3–6 Hz). The constraints imposed on theta frequency by ZD7288 injection, at least in doses ≤ 100 μM, were due to selective and irreversible closing of h-channels by ZD7288 as reported in numerous *in vitro* studies in different parts of the brain (Harris & Constanti, 1995; Chevaleyre & Castillo, 2002; Sotty *et al*., 2003). Synaptic depression was also reported recently in hippocampal slices 80–90 min after bath application of 50 μM ZD7288 (Chevaleyre &

FIG. 3. Effect of septal blockade and enhancement of I_h on hippocampal theta rhythm evoked by brainstem stimulation. (A) Specimen recordings of hippocampal EEG during electrical stimulation of the reticular formation with increasing intensity (0.1, 0.4, 0.8, and 1.2 mV; 0-spontaneous theta) before (left column) and during microdialysis administration of 30 μM ZD7288 in the MS (right column). (B) Autospectra of hippocampal EEG segments shown in A. (C) Frequency of theta peaks in the autospectra during stimulation with different intensity in control rats (ACSF) and 30 min after the start of ZD7288 (10, 30, 100 μM) or caesium (5 mM) application (left) and 90 min after injection of 0.5 mM adenylyl cyclase stimulator NKH477 (right). (D) Per cent change in theta frequency after infusion of ZD7288 or NKH477.

Castillo, 2002) but it was negligible when conditions similar to those in our study were used, i.e. 30 min after application or at lower dose (10 μM). Direct comparison of effective doses and time courses of pharmacological interventions in slice and in whole animal is difficult but one would expect drugs to equilibrate with the target tissue and thus to exert their effect on neurons slower and at a higher dose *in vivo* than *in vitro* rather than *vice versa*. Furthermore, brainstem stimulation in our experiments did evoke theta rhythm in the hippocampus after I_h blockade indicating that the ascending drive reached the theta generator. We also verified the effect of I_h blockade on theta frequency by local administration of caesium in the MS, which also reduced the frequency of fast theta oscillations in doses of 5.0 and 10 mM. The action of caesium is less selective on I_h than ZD7288 but it does not alter basal synaptic transmission (DiFrancesco, 1993; Pape, 1996; Robinson & Siegelbaum, 2003).

An important property of HCN channels is that I_h gating can be regulated by intracellular cyclic AMP. This regulation does not require protein phosphorylation (DiFrancesco & Tortora, 1991), rather it is accomplished by conformational changes after direct binding of cAMP to the cytoplasmic nucleotide-binding domain of the channel (Wainger *et al*., 2001; Wang *et al*., 2002). Consequently, changes in cAMP levels induce shifts in the I_h activation curve to more negative or positive potentials (McCormick & Pape, 1990; Pape, 1996). Stimulants of adenylyl cyclase were indeed shown to enhance the activation of I_h in slice and were therefore expected to accelerate rhythmogenesis (Brown *et al*., 1979; Pape, 1996; Robinson & Siegelbaum, 2003). In this study, brainstem-evoked theta rhythm attained the highest frequency after infusion of NKH477, an activator of adenylyl cyclase, into the MS although the effect was limited by a maximum determined by the properties of the theta generator (ceiling effect). Importantly, cyclic AMP is involved in intracellular messenger systems mediating the effect of many neurotransmitters acting on the MS theta generator, which were shown to modify the I_h activation curve in other parts of the brain (Bobker & Williams, 1989; Pape & McCormick, 1989; Pape, 1996). Further studies will be necessary to understand the details of this important mechanism.

H-channels are expressed not only in the MS but other structures of the limbic system, as well (Maccaferri & McBain, 1996; Dickson *et al*., 2000; Sotty *et al*., 2003), and the present findings draw further attention to the diverse roles of h-channels (Robinson & Siegelbaum, 2003; Santoro & Baram, 2003) even in such closely related structures as hippocampus, entorhinal cortex, and MS, all coupled by theta rhythmicity during specific behavioural states. In entorhinal cortex and hippocampal slices I_h plays a role of pacemaker current and activates at –50 to –60 mV (Maccaferri & McBain, 1996; Dickson *et al*., 2000). In the MS, I_h is activated at more negative potentials (less than –80 or –90 mV; Sotty *et al*., 2003) and does not show autorhythmicity in slice (Sotty *et al*., 2003; Xu *et al*., 2004). I_h conductance was found, however, to facilitate burst firing, to shorten the delay of rebound-firing after hyperpolarization and to increase its instantaneous frequency (Sotty *et al*., 2003), thus enabling these cells to resonate to or accurately transmit rhythmic input in a relatively wide theta range. Resonant properties of neurons are particularly important in networks in which synaptic inputs to neurons in certain states are oscillatory rather than transient (Hutcheon *et al*., 1996; Hutcheon & Yarom, 2000). Resonance at theta frequencies was reported in different neurons of the hippocampus (Leung & Yu, 1998; Pike *et al*., 2000; Hu *et al*., 2002). In CA1 pyramidal cells theta resonance appeared in both subthreshold depolarized and hyperpolarized states due to activation of two different mechanisms; h-current contributed to theta resonance at membrane potentials negative to –80 mV (Hu *et al*., 2002). Theta resonance is also present in certain types of interneurons in the stratum oriens (Pike *et al*., 2000) which are also endowed with h-channels (Gillies *et al*., 2002) but not in others (e.g. basket cells; Pike *et al*., 2000). The results of Sotty *et al*. (2003) indicate that I_h in the MS more likely supports resonance than pacemaker activity although the resonant properties of septal neurons have not been specifically investigated. This should be relatively easy to test *in vitro* using subthreshold sinusoidal current injections (Hutcheon *et al*., 1996) and would be important in light of the position of MS between the brainstem and forebrain structures. Previous studies demonstrated that brainstem stimulation produced fast theta in the supramammillary nucleus (Kirk & McNaughton, 1993) which is a major source of afferents to the MS. We have shown recently that the supramammillary nucleus (SUM) drives theta selectively during epochs of theta acceleration (Kaminski & Kocsis, 2003).

Although the primary observation of this study concerned theta frequency the underlying mechanisms may be more complex than a shift in the actual working frequency of a master theta oscillator. It is known that theta rhythm is not generated by one single oscillator; multiple oscillators may even exist within the same structure, such as in the MS. Traditionally, at least two theta components were identified by pharmacological manipulations of the cholinergic system (Vanderwolf, 1969; Bland *et al*., 1975). MS is the primary source of cholinergic input to the hippocampal formation and cholinergic neurons play a fundamental role in theta generation (Vanderwolf, 1969; Buzsaki, 2002). Muscarinic activation results in lasting theta in freely moving and anaesthetized animals (Kramis *et al*., 1975; Monmaur & Brenton, 1991). Nevertheless, large doses of atropine failed to eliminate theta rhythm indicating the existence of an atropine resistant component (Vanderwolf, 1969; Kramis *et al*., 1975). In intact rats the two theta components occur coincidentally (Buzsaki *et al*., 1983; Vertes & Kocsis, 1997; Bland & Oddie, 2001; Buzsaki, 2002). Although its mechanism remains uncertain it was suggested that GABAergic septohippocampal neurons are critically involved (Freund & Antal, 1988; Lee *et al*., 1994; Buzsaki, 2002). We have found in this study that MS cholinergic activation exerts a dual effect by driving low frequency theta oscillations and at the same time eliminating the capability of the septal pacemaker to accelerate the rhythm in response to ascending drive from the brainstem. This latter finding is consistent with the known capability of carbachol to depress the activation of I_h by direct action on h-channels through cAMP down-regulation (DiFrancesco & Tromba, 1988; DiFrancesco & Tortora, 1991; Pedarzani & Storm, 1995) and/or by membrane depolarization (Wu *et al*., 2000). In the MS, both h-channels (Sotty *et al*., 2003; Xu *et al*., 2004) and muscarinic receptors (Wu *et al*., 2000) are preferentially located on septohippocampal GABAergic neurons. The atropine-resistant component of theta was completely eliminated by septal I_h blockade.

It is worth noting that a link between atropine resistant theta and I_h was also reported in hippocampal slice where metabotropic glutamate agonist elicited theta oscillation in stratum oriens interneurons in the presence of AMPA blocker. This rhythm was not sensitive to atropine but was completely eliminated by ZD7288 (Gillies *et al*., 2002). On the other hand, theta oscillations of muscarinic origin in another type of hippocampal interneurons in the lacunosum-moleculare layer (Chapman & Lacaille, 1999a) were not sensitive to I_h blockers (Chapman & Lacaille, 1999b). This suggests that, at least in the hippocampal interneuron network, the two 'classic' theta generating mechanisms, i.e. those of atropine-sensitive (i.e. carbachol-elicited) and atropine-resistant theta (the latter being also I_h -dependent), can appear structurally separated. In the MS the two mechanisms may be more closely related as the underlying membrane machineries coexist in the same population of GABAergic septohippocampal interneurons.

Their characteristics are significantly different, however. Injection of carbachol in the MS activated a theta pacemaker, which resulted in lasting rhythmic activity in the hippocampus. The presence of I_h or even its enhancement did not generate spontaneous theta by itself (see also Sotty *et al*., 2003) but allowed the modulation of theta frequency according to the level of ascending drive from the brainstem. The frequency maximum of this component was higher than that of the carbachol-elicited rhythm and could reach the level (7–8 Hz) characteristic for unanaesthetized rats. This is not to suggest, however, that the two components can be separated solely on the basis of their frequency. Even in the reduced preparation of urethane-anaesthetized rats, the two mechanisms were active in overlapping frequency bands. Carbachol applied in the MS, for example increased the frequency of theta oscillations up to 5–6 Hz and part of this shift was dependent on I_h. In freely behaving rats both mechanisms are most likely present and active at the same time and their separation is even more difficult if not impossible.

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Abbreviations

ACSF, artificial cerebrospinal fluid; EEG, electroencephalogram; I_h, h-current; MS, medial septum; REM, rapid eye movement.

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