Local Generation of Theta-Frequency EEG Activity in the Parasubiculum

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Glasgow SD, Chapman CA. Local generation of theta-frequency EEG activity in the parasubiculum. J Neurophysiol 97: 3868–3879, 2007. First published March 28, 2007; doi:10.1152/jn.01306.2006. The parasubiculum is a major component of the hippocampal formation that receives inputs from the CA1 region, anterior thalamus, and medial septum and that projects primarily to layer II of the entorhinal cortex. Hippocampal theta-frequency (4–12 Hz) electroencephalographic (EEG) activity has been correlated with sensorimotor integration, spatial navigation, and memory functions. The present study was aimed at determining if theta is also generated locally within the parasubiculum versus volume conducted from adjacent structures. In urethan-anesthetized rats, the phase-reversal of theta activity between superficial and deep layers of the parasubiculum was demonstrated using differential recordings from movable bipolar electrodes that eliminate the influence of volume-conducted activity. Parasubicular theta was abolished by atropine, and was in phase with theta in stratum radiatum/lacunosum-moleculare of the CA1 region. Whole cell current-clamp recordings in brain slices were then used to determine if parasubicular theta may be generated in part by membrane potential oscillations in layer II neurons. Membrane potential oscillations occurred in most layer II neurons, including four putative interneurons, when cells were held at near-threshold voltages using current injection. The frequency of oscillations increased from 3.2 to 6.1 Hz when bath temperature was raised from 22 to 32°C, and oscillations persisted in the presence of blockers of fast ionotropic glutamatergic and GABAergic synaptic transmission. Oscillations are therefore likely generated by intrinsic, voltage-dependent ionic conductances. These results indicate that theta field activity is generated locally within the parasubiculum and that intrinsic membrane potential oscillations, synchronized by local inhibitory inputs, may contribute to the generation of this activity.

INTRODUCTION

The parasubiculum (PaS) is a major component of the hippocampal formation and may play a substantial role in the sensory and mnemonic functions of the hippocampal formation (Amaral and Witter 1995; Bland 1986; Buzsaki 2002; Caballero-Bleda and Witter 1993). It may also contribute to spatial navigation and the formation of specialized place cell representations in entorhinal cortex (Hafting et al. 2005; Hargreaves et al. 2005; Liu et al. 2001; Taube 1995b). It receives major inputs from the CA1 region and medial septum with weaker inputs from the subiculum, anterior thalamus, and basolateral amygdala, and it sends its major output to layer II of the entorhinal cortex (Caballero-Bleda and Witter 1993, 1994; Funahashi and Stewart 1997a; Swanson and Cowan 1979; Wouterlood et al. 1990). Layer II of the entorhinal cortex carries highly processed multimodal sensory input to the dentate gyrus and CA3 region of the hippocampus, and the large projection to this layer from the PaS suggests that the PaS may have powerful effects on sensory processing in the temporal lobe (Amaral and Witter 1989; Jones 1990). Previously we showed that stimulation of the PaS enhances subsequent responses of the entorhinal cortex to inputs from the piriform cortex at intervals up to ~150 ms, indicating that the PaS can increase entorhinal responses to cortical afferents (Caruana and Chapman 2004). Further, the PaS contains place cells that are dependent on head-direction (Cacucci et al. 2004; Taube 1995b) and may therefore play an important role in providing the dorsal entorhinal cortex with spatial inputs required to support the activity of “grid” cells (Hafting et al. 2005; Hargreaves et al. 2005).

The cellular and network mechanisms through which the PaS modulates processing within the hippocampal formation are likely regulated in part by theta-frequency EEG activity. The theta rhythm is a large-amplitude, sinusoidal EEG rhythm (4–12 Hz) that is observed in entorhinal cortex, dentate gyrus, CA1, and CA3 regions of the hippocampal formation (Bland and Oddie 2001; Buzsaki 2002; Buzsaki et al. 1983; Green and Arduini 1954; Petsche et al. 1962). It has been linked to computational processes that mediate sensorimotor integration, spatial navigation, and memory formation (Bland 1986; Buzsaki 2005; Hasselmo 2005; Vertes 2005). The entorhinal cortex and the hippocampus express robust theta activity that is generated by network and cellular mechanisms including cholinergic and GABAergic inputs from the medial septum, rhythmic inhibitory inputs from local interneurons, and oscillations in membrane potential dependent on intrinsic voltage-dependent conductances (Alonso and Garcia-Aust 1987a,b; Buzsaki 2002; Chapman and Lacaille 1999a,b; Gioveli et al. 2001, 2005; Klink and Alonso 1993; Leung and Yim 1991; Mitchell and Ranck 1980; Soty et al. 2003). Hippocampal theta is also thought to be driven in part by synaptic inputs from the superficial layers of the entorhinal cortex that project to the dentate gyrus, CA3, and stratum lacunosum-moleculare of the CA1 region (Leung and Shen 2004; Leung et al. 1995; Vanderwolf et al. 1985; Wu and Leung 1998). The PaS receives cholinergic inputs from the septum and is tightly linked to structures in the hippocampal formation that display theta-frequency activity (Amaral and Witter 1989; Caballero-Bleda and Witter 1993, 1994; Kohler et al. 1985; van Groen and Wyss 1990b). However, it has not yet been demonstrated that theta field activity is generated locally within the PaS. Initial mapping studies showed low-amplitude theta activity near the PaS (Bland and Whishaw 1976), and a subdivision of PaS cells is known to fire in relation to the phase of theta activity (Cacucci et al. 2004; Taube 1995b), but these activities could
be volume conducted or driven by extrinsic synaptic inputs to the PaS.

The objective of the present study was to determine if theta-frequency EEG activity is generated locally within the PaS and to assess whether membrane potential oscillations may contribute to the generation of theta in the PaS. Differential recordings from a moving bipolar electrode were used to localize theta to the superficial layers of the PaS in urethane-anesthetized rats, and whole cell current-clamp recordings were used to characterize voltage-dependent membrane potential oscillations in layer II PaS neurons in vitro.

METHODS

Recordings from anesthetized animals

Surgery. Data were obtained from seven male Long-Evans hooded rats (300–500 g) in accordance with guidelines of the Canadian Council on Animal Care. Recordings were also obtained from three rats with electrodes that passed either rostral or dorsal to the PaS, but theta activity was not observed in these cases (not shown). Animals were anesthetized with an oxygen/isoflurane mixture (98.5/1.5%), and a catheter was inserted into the jugular vein. The rat was then switched to anesthesia using urethan (ethyl carbamate, 0.8 g/ml iv), which allowed for the fine regulation of level of anesthesia for the remainder of the procedures. Body temperature was monitored using a digital rectal thermometer and maintained at 36–37°C with a heating lamp. Chemicals were obtained from Sigma.

Animals were prepared for stereotaxic surgery in a standard manner and secured in a grounded Kopf stereotaxic apparatus with Bregma and lambda leveled. A stainless-steel jeweler’s screw was placed in the right frontal bone as a reference electrode. A monopolar recording electrode made from Teflon-coated stainless-steel wire (200 μm diameter) was placed in the CA1 region of the hippocampus (P, 4.5 mm; L, 3.0 mm relative to Bregma; and V, 2.1–2.7 mm from dura) (Paxinos and Watson 1998). The depth of the electrode was determined by maximizing the amplitude and clarity of theta-frequency activity (Bland et al. 1975; Robinson 1980). To obtain recordings from the PaS, a small opening was drilled in the side of the skull to allow the recording electrode to enter the brain at an angle of 30° above horizontal. Mineral oil was applied to the exposed dura. The electrode was constructed from two tungsten electrodes (10–50 μm tip diameter; Frederick Haer) with one tip staggered 500–700 μm back from the other. The active, leading tip of the electrode was aimed at the surface of the PaS at a point 1.0 mm anterior to the interaural line, 3.8 mm from midline, and 5.0 mm above the interaural line (Caballero-Bleda and Witter 1993; Paxinos and Watson 1998). Differential recordings from the bipolar electrode were used to minimize the contribution of volume-conducted activity from the hippocampal formation on parasubicular recordings.

Depth profile recordings. Recordings were obtained from the bipolar electrode in 100-μm increments as it was moved along a 4-mm track from a position 2 mm away from the target. Each recording began with an initial 5-s baseline period with no theta activity in the hippocampus followed by a tail-pinch to induce theta. Field activity was recorded simultaneously in hippocampus and PaS and was analog filtered (0.1 Hz to 10 kHz passband), amplified (1,000 times; A-M Systems, Model 1700), and monitored using a digital oscilloscope. Recordings were digitized at 20 kHz (12-bit) and recorded to hard disk using the program Experimenter’s Workbench (Datawave Technology).

Theta activity in the hippocampus and entorhinal cortex of urethane-anesthetized rats is dependent on cholinergic receptors and is disrupted by systemic administration of the nonselective muscarinic antagonist atropine (Dickson et al. 1994; Kramis et al. 1975). To determine if parasubicular theta is dependent on cholinergic inputs, atropine sulfate (10 mg/kg iv) (Natsume et al. 1999) was administered after the depth profile was completed. Recordings were obtained before and after atropine administration at the depth where the largest parasubicular theta activity was observed.

Histochemistry. After recordings were completed, a cathodal current (100 μA for 8 s) was passed through the leading tip of the parasubicular electrode to mark the tip location at the depth of the PaS and at the beginning of the electrode track. The animal was then killed with urethan and perfused transcardially with 0.9% saline followed by 10% formalin. Brains were stored in 10% formalin, and transferred to 30% sucrose solution 3 day before sectioning. Coronal sections (40 μm thick) were obtained and stained with cresyl violet (0.1%). Coronal sections containing hippocampal and parasubicular electrode tracks were photographed using a Sony XC-77 camera, a Scion LG-3 frame grabber, and Image SXM (v1.6, SD Barrett, http://www.imag-esXM.org.uk). Electrophysiological recordings were matched to locations on the recording track using the location of target lesions. The thicknesses of cortical layers were determined according to the convention of Amaral and Witter (1995) and Funahashi and Stewart (1997b).

Data processing. Two-second samples of EEG activity were selected at each depth along the recording electrode track to construct profiles of the power of theta activity. Profiles were constructed by calculating the total power between 3.1 and 10.4 Hz at each depth using the software package, Origin 7.0 (OriginLab). Absolute power in the EEG varied across animals, and power was plotted as a proportion of maximal theta-band power along the recording track. The phase relationship between theta activity in the superficial layers of the PaS and in the CA1 region was determined using cross-correlations of simultaneously recorded 2-s EEG samples and the phase of the cross spectrum (Alonso and Garcia-Austt 1987a). Because the phase of theta within the CA1 depends on laminar position (Leung 1984), and the location of the CA1 reference electrode varied across different animals, the phase of the cross-spectrum was plotted as a function of the position of the CA1 reference electrode.

In vitro slice recordings

Slice preparation. Methods for in vitro recordings were similar to those reported previously (Chapman and Lacaille 1999b). Acute brain slices were obtained from 4- to 6-wk-old male Long-Evans rats. After anesthetization with halothane, the animal was decapitated, and the brain was quickly removed and submerged in cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, and 10 dextrose saturated with 95% O2-5% CO2 (pH ~7.3; 300–310 mOsM). Horizontal brain slices (300 μm) were allowed to recover for ~1 h at room temperature. Individual slices were transferred to a recording chamber and visualized with an upright microscope (Leica, DM-LFS) equipped with a long-range water immersion objective (~40), differential interference contrast optics, and a near-infrared camera (COHU). Slices were superfused with oxygenated ACSF at room temperature at a rate of 1.5–2.0 ml/min. The location of layer II PaS cells was determined using criteria similar to Funahashi and Stewart (1997b). The diffuse layer of disorganized cells that make up layer II of the PaS is distinct from the compact layer II cells of the medial entorhinal cortex and presubiculum (Amaral and Witter 1989; Funahashi and Stewart 1997a,b). Further, in contrast to deep layer cells in MEC and subiculum, none of the parasubicular neurons recorded here showed burst firing in response to current injection (Funahashi and Stewart 1997a; Jones and Heinemann 1988; Stewart and Wong 1993).

Whole cell recordings. Patch pipettes were prepared from borosilicate glass (1.0 mm OD, 5–10 MΩ) using a horizontal puller
of synaptic inputs to the generation of oscillations at both

...zine-2-ethanesulfonic acid (HEPES), 0.5 ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.20–7.26 with KOH; 270–280 mOsM). Electrodes were visually guided to contact the soma of layer II parasubiculur neurons, and tight seals (>1 GΩ) were obtained under voltage clamp with gentle suction. Increasingly strong suction was applied until whole cell configuration was established. Current-clamp recordings were begun after 3–5 min. Membrane potential recordings (DC to 10 kHz) were obtained with an Axoclamp 200B amplifier (Axon Instruments), monitored on a digital oscilloscope, and digitized at 20 kHz (Axon Instruments, Digidata 1322A) for storage on computer hard-disk using Clampex 8.1 software (Axon Instruments).

Recordings were accepted if series resistance was <35 MΩ [mean, 19.7 ± 2.6 (SE) MΩ], cells displayed overshooting action potentials, and if the input resistance and resting potential were stable. Series resistance was monitored repeatedly throughout recordings, and recordings were discontinued if there was a change of >20%.

Membrane potential oscillations in hippocampus and entorhinal cortex are observed at membrane potentials close to action potential threshold (Alonso and Klink 1993; Chapman and Lacaille 1999b; Dickson et al. 2000). To determine if membrane potential oscillations in PaS neurons are voltage-dependent, 5- to 10-s-duration recordings of membrane potential were obtained at a range of voltages relative to action potential threshold. Membrane potential was altered by varying the amount of steady current injection.

Recordings were obtained routinely at 22°C to reduce metabolic demands on slices. To determine the frequency of oscillations at more physiological temperatures, the temperature of the recording bath was increased to 32 ± 0.5°C in nine cells. Bath temperature was regulated using an automated temperature controller (Warner Instruments, Model TC-324B) and recordings at a range of voltages relative to threshold were obtained at each temperature.

To determine whether oscillations require synaptic inputs or are generated intrinsically, ionotropic glutamate receptors were blocked using 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide disodium (NBQX, 50 μM) and GABAA receptor-mediated synaptic transmission was blocked using bicuculline methiodide (10 μM). After control tests in normal ACSF, recordings were obtained at the same range of voltage levels after application of pharmacological agents at either 22°C (n = 4) or 32°C (n = 4) to assess the contribution of synaptic inputs to the generation of oscillations at both temperatures.

DATA ANALYSIS. Samples of membrane potential were prepared for spectral analysis by reducing the effective sampling rate to 1 kHz and isolating 2,048 s segments of recordings that contained no action potentials. The dominant frequency of membrane potential oscillations was calculated by computing the power spectra after passing samples through a Blackman window (Clampfit 8.2, Axon Instruments). Changes in the peak frequency of oscillations were assessed using matched samples t-test.

Electrophysiological characteristics of parasubiculur neurons were analyzed using the software program Clampfit 8.2. Action potential height was measured from resting potential. Action potential width and fast and medium afterhyperpolarizations were measured from action potential threshold. Input resistance was calculated by measuring the peak voltage response to a −200-pA current step (500 ms). Inward rectification was quantified by expressing the peak input resistance as a proportion of the steady-state resistance measured at the end of the negative current pulse (rectification ratio) (Chapman and Lacaille 1999b). Data were expressed as means ± SE.

RESULTS

 Theta activity in the anesthetized animal

Differential recordings from bipolar electrodes were used to localize theta activity in the PaS of urethan-anesthetized rats. Monopolar recordings from individual tips of the bipolar electrode contained theta activity along the entire electrode track that was phase-locked to theta in the CA1 region. Monopolar recordings largely reflect volume conduction from the hippocampus, and only data from bipolar recordings are shown here. To localize the layers in which theta activity is generated, recordings were obtained along a 4-mm track, starting 2 mm from the surface of the PaS (n = 7). Theta was not observed in response to tail-pinch at sites near the beginning of the electrode track but was observed at locations in which the bipolar electrode spanned the superficial cortical layers (Fig. 1, A, 2 and 3, and B). Because locally generated oscillations reverse in phase across the layers that generate them, oscillations have opposite polarities in deep versus superficial layers (e.g., Alonso and Garcia-Austt 1987a), and this greatly enhances differential recordings. The amplitude of theta activity was greatly reduced as the electrode passed out of the PaS, whereas theta at the stationary hippocampal electrode was maintained (Fig. 1A6). Histological analysis showed that electrodes had passed through the PaS whenever theta had been recorded (Fig. 1B2). Theta was not observed in three additional animals (not shown) in which electrodes passed either rostral or dorsal to the PaS.

Changes in the power of theta along the electrode track were quantified using power spectral analysis of representative recordings at each depth (Fig. 1C). Both hippocampal and PaS recordings showed clear peaks in the power spectrum that correspond to the low-end frequencies (~3–6 Hz) that are also typical of theta activity in the hippocampus and entorhinal cortex of urethan-anesthetized rats (Blumberg 1986; Dickson et al. 1994). The maximal amount of theta-band power (3.1–10.4 Hz; mean: 0.007 ± 0.002 mV^2; n = 7) was always observed as the electrode passed through the superficial layers of the PaS when the leading electrode tip was 100–250 μm from the surface of the cortex in layers I or II/III.

Cross-correlational analyses were used to investigate the phase relationship between parasubicular and hippocampal theta activity. We found that the phase relationship was dependent on the depth of the hippocampal reference electrode (Fig. 2) in a way that is related to the known change in phase of theta as a function of laminar depth within the CA1 region (Buzsaki et al. 1986; Leung 1984). Theta in the PaS was in phase with hippocampal theta recorded in s. radiatum and lacunosum-moleculare (mean phase: −17°, n = 4; e.g., Fig. 2, A and B) but was more out of phase with theta recorded in s. oriens (phases of −140 and 113° in 2 animals). An intermediate recording site in s. pyramidale had a phase delay of −71°.

Theta activity in the hippocampus of the urethan-anesthetized rat is paced by GABAergic and cholinergic neurons in the medial septum (Bland and Oddie 2001; Bland 1986; Denham and Borisyuk 2000; Sotty et al. 2003; Vertes and Kocsis 1997) and is blocked by systemic administration of the muscarinic antagonist atropine sulfate (Kramis et al. 1975). Similarly, systemic administration of atropine blocked theta-frequency activity in both the CA1 and PaS (n = 4; Fig. 3). Theta activity elicited by tail-pinch was reduced from 45.0 ± 5.6 to 15.7 ±
3.3% of total power in the CA1 ($t_3 = 3.79$, $P < 0.01$) and from 51.8 ± 2.4 to 15.5 ± 5.0% in the PaS ($t_3 = 10.10$, $P < 0.01$). Theta activity in the PaS is therefore dependent on muscarinic receptor activation.

**Theta-frequency membrane potential oscillations in layer II neurons**

Whole cell current-clamp recordings were obtained from 21 layer II PaS cells. The superficial layers of the PaS contain both stellate and pyramidal neuron cell types with similar basic electrophysiological characteristics (Funahashi and Stewart 1997a), and 17 of the recorded cells fit into this group. Four cells demonstrated electrophysiological properties characteristic of interneurons (Chapman and Lacaille 1999b; Lacaille and Schwartzkroin 1988) and were analyzed separately.

Principal cells had a mean resting potential of $-55.7 ± 1.8$ mV and input resistance of $114.0 ± 9.7$ MΩ. All cells demonstrated some delayed inward rectification in response to hyperpolarizing current steps (mean of rectification ratio: $1.17 ± 0.03$; range: 1.04–1.46) (Funahashi and Stewart 1997a). Action potentials (amplitude: $105.4 ± 3.0$ mV;
were typically followed by fast and medium afterhyperpolarizations (7.5 \( \pm \) 0.5 and 6.1 \( \pm \) 0.4 mV, respectively). Action potential threshold was \(-46.4 \pm 1.5\) mV.

**INTRINSIC VOLTAGE-DEPENDENT OSCILLATIONS.** In recordings in normal ACSF at 22°C, PaS cells typically did not fire at resting membrane potential. However, when depolarized to near-threshold voltage levels (from 0.034 \( \pm \) 0.006 to 0.103 \( \pm \) 0.024 mV/Hz; \( t_5 = 3.07, P < 0.05 \)), similarly, the peak-to-peak amplitude of oscillations also increased from 1.13 \( \pm \) 0.10 to 2.42 \( \pm \) 0.38 mV (\( t_5 = 3.22, P < 0.05 \)). The frequency of oscillations, however, was not significantly affected by depolarization (3.2 \( \pm \) 0.4 Hz at 6 mV below threshold vs. 3.4 \( \pm \) 0.4 Hz at threshold).

The temperature of the recording bath was increased from 22 to 32 °C to determine if the frequency of membrane potential oscillations would increase to within the range of frequencies of endogenous theta activity (4–12 Hz) at more physiological temperatures (Bland 1986; Chapman and Lacaille 1999b; Petsche et al. 1962; \( n = 11 \); Fig. 5). Consistent with increased potassium channel kinetics in whole cell recordings at physiological temperatures (Shen and Schwartzkroin 1988; Thompson et al. 1985), there was a reduction in spike amplitude (from 108.5 \( \pm \) 3.9 to 99.4 \( \pm \) 5.4 mV; \( t_{10} = 2.38, P < 0.05 \)) and a decrease in input resistance (from 122.1 \( \pm \) 11.4 to 72.9 \( \pm \) 7.6 M\( \Omega \); \( t_{10} = 3.77, P < 0.01 \)). Raising temperature also significantly increased the frequency of oscillations from 3.5 \( \pm \) 0.2 to 6.1 \( \pm \) 0.3 Hz (\( t_{10} = 8.00, P < 0.001 \)) without significantly affecting the amplitude of oscillations (0.107 \( \pm \) 0.022 mV/Hz at 22°C vs. 0.069 \( \pm \) 0.014 mV/Hz at 32°C; Fig. , B and C).

**FIG. 3.** Blockade of muscarinic cholinergic receptors abolishes theta-frequency EEG activity in both the PaS and the CA1 region. A: samples of EEG activity recorded simultaneously from the CA1 reference electrode and the parasubiculum. B: cross-correlation of the records shown in A reflects strong theta activity in both sites. The peak in the cross-correlation function at a lag close to 0 (\( \rightarrow \)) indicates the signals were nearly phase-locked. C: phase of cross-correlation functions in different animals depended on the laminar location of the reference electrode within the CA1 region (\( n = 7 \)). PaS theta tended to be in phase with theta activity recorded in s. radiatum and was more out of phase with theta in s. oriens. □, phase for the traces shown in A and the location of the reference electrode in s. radiatum.

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<th>Width (ms)</th>
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<td>1.30 ± 0.06</td>
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**FIG. 2.** Theta activity in the PaS is phase-locked to simultaneously recorded theta activity in stratum radiatum of the CA1 region. A: samples of EEG activity recorded simultaneously from the CA1 reference electrode and the parasubiculum. B: cross-correlation of the records shown in A reflects strong theta activity in both sites. The peak in the cross-correlation function at a lag close to 0 (\( \rightarrow \)) indicates the signals were nearly phase-locked. C: phase of cross-correlation functions in different animals depended on the laminar location of the reference electrode within the CA1 region (\( n = 7 \)). PaS theta tended to be in phase with theta activity recorded in s. radiatum and was more out of phase with theta in s. oriens. □, phase for the traces shown in A and the location of the reference electrode in s. radiatum.
Receptor blockers were used to determine if oscillations are dependent on extrinsic glutamatergic or GABAergic synaptic inputs. Recordings in normal ACSF were repeated during co-application of the N-methyl-D-aspartate glutamate receptor blocker AP-5, the AMPA/kainate receptor blocker NBQX, and the GABA<sub>A</sub> receptor blocker bicuculline (Fig. 6). The frequency of membrane potential oscillations was not significantly affected by application of receptor antagonists (3.5 ± 0.4 Hz in ACSF and 3.8 ± 0.8 Hz in antagonists; n = 4), and the power of oscillations was also not significantly reduced by the antagonists (0.078 ± 0.020 mV<sup>2</sup>/Hz in ACSF and 0.061 ± 0.031 mV<sup>2</sup>/Hz in antagonists). Note the reduction in the amplitude of the afterhyperpolarization (Fig. 6A2) attributable to a bicuculline-mediated inhibition of calcium-activated K<sup>+</sup> (SK) channels (Grunnet et al. 2001). Increasing the temperature of the bath can increase spontaneous synaptic activity. However, just as for recordings at 22°C, antagonists did not affect the frequency (6.5 ± 0.6 Hz in ACSF and 7.8 ± 1.1 Hz in antagonists; n = 4) or power (0.036 ± 0.017 mV<sup>2</sup>/Hz in ACSF and 0.042 ± 0.012 mV<sup>2</sup>/Hz) of oscillations recorded at

**FIG. 4.** Neurons in layer II of the PaS display voltage-sensitive oscillations in membrane potential. A: whole cell current-clamp recordings from a representative cell are shown at the membrane potentials indicated at left. When the cell was held near threshold by positive current injection, membrane potential oscillations occurred at a frequency of 2–5 Hz and contributed to the timing of action potentials. Oscillations were not observed at hyperpolarized membrane potentials. Action potentials are truncated in this and subsequent figures. B: membrane potential responses to hyper- and depolarizing current pulses at resting potential in the same cell as in A. Action potentials are truncated. Note the moderate delayed inward rectification in response to strong hyperpolarizing pulses. C: power spectra of recordings at 3 voltage levels from the same cell show that oscillations occur at frequencies of 2–5 Hz (C1). Depolarization significantly affected the power but not the frequency of oscillations in the group of cells tested (n = 6). The power of oscillations is shown in C2 as a function of membrane potential relative to spike threshold for 7 neurons. The power of oscillations increased as membrane potential was raised to near-threshold levels.

**FIG. 5.** The frequency of oscillations increased when the temperature of artificial cerebrospinal fluid (ACSF) was raised from 22 to 32°C. A: recordings from a representative neuron at the indicated voltages at 22 and 32°C. Note that oscillations are abolished by hyperpolarization at both temperatures. B: power spectra of membrane potential recordings just below spike threshold are compared for the same cell at 22 and 32°C. Note the increase in peak frequency with little change in the power of oscillations. C: peak frequency of oscillations increased significantly for the group of cells tested (n = 11; P < 0.05).
32°C (Fig. 6C). The generation and pacing of membrane potential oscillations therefore does not require extrinsic ionotropic glutamate or GABAergic synaptic inputs, and oscillations are likely generated primarily by intrinsic voltage-dependent ionic conductances.

**OSCILLATIONS IN PUTATIVE INTERNEURONS.** GABA-mediated inhibitory responses have been recorded previously from parasubicular neurons (Funahashi and Stewart 1998), and a small group of cells recorded here demonstrated electrophysiological characteristics similar to hippocampal interneurons (n = 4, Fig. 7). Putative inhibitory interneurons were distinguished from principal cells by their high-input resistances (264.3 ± 48.9 MΩ; t_16 = 4.55, P < 0.01) and prominent fast afterhyperpolarizations (13.5 ± 2.0 mV; t_16 = 4.10, P < 0.01; Fig. 7B). Inhibitory cells had a mean resting potential of −57.0 ± 2.5 mV. Three of the four cells showed large delayed inward rectification, but the mean rectification ratio did not differ significantly from other PaS neurons (1.49 ± 0.35 vs. 1.17 ± 0.03).

Parasubicular interneurons showed voltage-dependent, theta-frequency membrane potential oscillations (Fig. 7A). Interneurons in s. lacunosum-moleculare of the hippocampus also show oscillations that may synchronize theta activity by leading to rhythmic inhibition of principal cells (Chapman and Lacaille 1999b). Similar to other PaS cells, hyperpolarization of membrane potential reduced the power of oscillations (from 0.146 ± 0.017 to 0.032 ± 0.008 mV²/Hz at 6 mV below threshold; t_3 = 7.66, P < 0.01), but had little effect on peak frequency (2.4 ± 0.2 Hz at 6 mV below threshold vs. 3.6 ± 0.5 at threshold; t_3 = 3.97, P < 0.05). Bath temperature was raised...

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**FIG. 6.** Membrane potential oscillations persist in the presence of blockers of ionotropic glutamate- and GABA- mediated synaptic transmission. A: recordings at the indicated voltage levels are shown before (1) and after (2) blockade of ionotropic glutamatergic and GABAergic synaptic transmission with 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide disodium (NBQX, 10 µM), (±)-2-amino-5-phosphonopentanoic acid (AP-5, 50 µM), and bicuculline (10 µM). Recordings were conducted at 22°C. B: power spectra of traces in A reflect little change in power or frequency of oscillations in the presence of synaptic blockers. C: blockade of synaptic transmission had no significant effect on mean frequency of oscillations at either temperature (n = 4; P = 0.49). The persistence of voltage-dependent oscillations during blockade of ionotropic synaptic transmission suggests that oscillations are generated by intrinsic conductances.

**FIG. 7.** Putative inhibitory interneurons in layer II of the parasubiculum also display voltage- and temperature-dependent oscillations in membrane potential. A: representative recordings from one of the 4 cells display voltage-dependent oscillations at both 22°C (1) and 32°C (2), which are abolished by hyperpolarization. B: voltage responses to hyperpolarizing current steps reflect high-input resistance (353 MΩ) and marked inward rectification, and putative interneurons also large fast afterhyperpolarizations (n = 4). C: power spectra of traces recorded from the neuron in A show an increase in the peak frequency of oscillations as temperature was increased from 22 to 32°C.
in experiments for three of the cells, and this resulted in an increase in the frequency of oscillations (from 3.5 ± 0.7 to 6.5 ± 0.4 Hz; $t = 6.21, P < 0.05$) with no significant effect on the power of oscillations ($0.160 ± 0.013$ mV$^2$/Hz at 22°C vs. $0.100 ± 0.001$ mV$^2$/Hz at 32°C; Fig. 7, A and C). In one interneuron tested (not shown), oscillations persisted in the presence of NBQX, AP5, and bicuculline, suggesting that oscillations in interneurons are also dependent on intrinsic voltage-dependent conductances.

**Discussion**

Theta-frequency EEG activity in the PaS has been investigated here using both in vivo and in vitro electrophysiological techniques. Theta activity has been recorded previously in the PaS of freely moving animals (Cacucci et al. 2004), and a substantial proportion of place cells in the PaS are known to fire with a consistent phase relationship to theta (Taube 1995b). This suggested that the PaS generates theta field activity, but the hippocampus and entorhinal cortex generate theta activity that is volume conducted across the PaS, and a definitive demonstration of the local generation of theta within the PaS requires that the phase of theta be shown to reverse with cortical depth. Here we have used differential recordings from a movable bipolar electrode in urethan-anesthetized rats to demonstrate that theta is generated locally within the superficial layers of the PaS. Similar to hippocampal theta-frequency activity under urethan anesthesia (Kramis et al. 1975; Vanderwolf 1969), theta activity in the PaS evoked by tail-pinch stimulation was blocked by atropine and is therefore dependent on muscarinic receptor activation. This is the first direct evidence that atropine-sensitive theta field activity is generated within PaS.

Intrinsic membrane potential oscillations contribute to the generation of theta activity in the hippocampus, entorhinal cortex, and basolateral amygdala (Alonso and Klink 1993; Bland et al. 2002; Bonansco and Buno 2003; Chapman and Lacaille 1999b; Pape et al. 1998). Using visually guided whole cell current-clamp recordings, we have found that layer II cells of the PaS also express intrinsic, voltage-dependent, oscillations in membrane potential. This is a major mechanism that can contribute to the generation of theta-frequency EEG activity in the PaS and to the timing of synaptic outputs to layer II of the entorhinal cortex. Further the presence of oscillations in putative inhibitory interneurons indicates that local rhythmic inhibitory inputs to principal neurons may also help synchronize theta activity in the PaS (Chapman and Lacaille 1999b).

**Local generation of theta-frequency activity in the PaS**

Differential recordings from movable bipolar electrodes have demonstrated the local generation of parasubicular theta activity. This method ensures that volume-conducted theta activity from adjacent structures, which has a common phase at both tips, is rejected from recordings. The method also accentuates locally generated oscillations which have an opposite polarity at each tip due to the phase-reversal of oscillations between superficial and deep cortical layers. As the bipolar electrode passed through the superficial layers of the PaS, peak power values were observed when the leading tip of the electrode was positioned 100–250 μm from the surface of the cortex. This corresponds to layers I–III, with the trailing tip of the electrode in layers V–VI (Funakashi and Stewart 1997a).

Maximal theta activity was observed at slightly different positions within the superficial layers in different animals, and the distribution was sometimes asymmetric with larger theta observed as the leading tip of the electrode exited layer I of the PaS, compared with when it began to enter layer III. Active currents associated with membrane potential oscillations or extrinsic synaptic inputs may be more concentrated near the soma and proximal dendrites of neurons in layers I and II. Excitatory synaptic inputs from the CA1 terminate in layers I and II of the PaS (Kohler 1985; van Groen and Wyss 1990a,b) and could accentuate field oscillations in the superficial layers. This could lead to larger power values when the leading tip has exited the PaS and the lagging tip is still within layers I–II.

We have shown here that theta activity in the PaS is approximately in-phase with theta activity in the CA1 near the dentate fissure. In the CA1, the phase of theta gradually shifts such that theta in s. radiatum and lacunosum-moleculare is approximately phase reversed with respect to s. oriens (Bland et al. 1975; Buzsaki et al. 1986; Kowalczyk and Konopacki 2002; Leung 1984). Accordingly, we have found here that the phase difference between PaS and CA1 theta depends on the dorsoventral position of the CA1 reference electrode; PaS theta was phase-locked to CA1 recordings when the reference electrode was near the fissure or in s. radiatum, and it was most out of phase when the reference electrode was in s. oriens (Fig. 2).

Theta in layer I and in upper layer II of the entorhinal cortex is also in-phase with theta in s. radiatum (Alonso and Garcia-Aust 1987a; Buzsaki 2002; Dickson et al. 1994), and this suggests that theta in the PaS and entorhinal cortex are also closely synchronized. This is consistent with the possibility that excitatory synaptic inputs from the PaS may contribute to entorhinal cortex theta.

Cholinergic and GABAAergic inputs from the medial septum are critical for theta activity in the hippocampus and entorhinal cortex (Alonso and Klink 1994; Baisden et al. 1984; Bland and Oddie 1998; Bland et al. 1999; McNaughton et al. 1977; Petsche et al. 1962; Sotty et al. 2003; Vertes and Kocsis 1997), and theta activity in the urethan-anesthetized rat is blocked in these areas by the muscarinic antagonist atropine (e.g., Dickson et al. 1994; Kramis et al. 1975). We have found here that theta activity in the PaS is also blocked by atropine (Fig. 3).

The large septal projection to the PaS (Amaral and Witter 1987a; Buzsaki et al. 1986; Kowalczyk and Konopacki 1975). We have found here that theta activity in the PaS is also blocked by atropine (Fig. 3). The large septal projection to the PaS (Amaral and Witter 1987a; Buzsaki et al. 1986; Kowalczyk and Konopacki 1975). We have found here that theta activity in the PaS is also blocked by atropine (Fig. 3).
and repetitive firing in different brain areas (e.g., Llinas 1988). Interactions between the persistent sodium current ($I_{\text{NaP}}$) and a potassium conductance contribute to theta-frequency membrane potential oscillations in pyramidal cells and L-M interneurons in the CA1 region (Chapman and Lacaille 1999b; Garcia-Munoz et al. 1993; Leung and Yim 1991). In entorhinal cortex stellate cells, membrane potential oscillations are mediated by an interplay between activation of $I_{\text{NaP}}$ and inactivation of the time-dependent inward rectifying conductance $I_h$ (Dickson et al. 2000; Fransen et al. 2004). The conductances that generate oscillations in PaS neurons have not been determined but likely depend on an interaction between $I_{\text{NaP}}$ and $I_h$ or a potassium conductance (Hu et al. 2002; Schreiber et al. 2004; Wang et al. 2006). The $I_h$ current also contributes to membrane potential oscillations in deep layer neurons of the entorhinal cortex (Egorov et al. 2002; Gloveli et al. 2001; Schmitz et al. 1998) and in layers III/V of the perirhinal cortex (Bilkey and Heinemann 1999).

Active membrane currents that generate theta field activity in the CA1 region include strong excitation in distal dendrites as well as rhythmic perisomatic inhibition (Buzsaki 2002). Our intracellular recordings show somatic membrane potential oscillations, but the depth profile data (Fig. 1) are not sufficient to discriminate the relative contribution of somatic and apical dendritic currents to the field activity. Determining the location of active currents in the PaS will require current-source density analysis using simultaneous recordings from multi-site probes or intracellular recordings in dendrites.

The principal neurons recorded here were not identified morphologically but likely include both stellate and pyramidal neurons of layer II. Layers II and III are not easily distinguished in slices, but cells were obtained near the layer I border to ensure they were located in layer II. Morphologically identified stellate and pyramidal neurons in layer II do not differ markedly in basic electroresponsiveness, and distinctions are most apparent between cells of different layers (Funahashi and Stewart 1997a,b). It is likely that we sampled both stellate and pyramidal neurons in this study, and most neurons showed oscillations. Both cell types therefore likely contribute to theta activity through the expression of membrane potential oscillations, but this will have to be verified in future using anatomical techniques. In addition, evaluating the resonance of membrane potential responses to frequency-varying current injection (Erchova et al. 2004; Schreiber et al. 2004) could be used to help assess the potential contributions of these cell types to rhythmic network activity.

**Oscillations in interneurons**

Membrane potential oscillations were recorded from four putative interneurons characterized by high-input resistance, short spike-widths, and prominent fast afterhyperpolarizations. The superficial layers of the PaS contain numerous GABAergic neurons (Kohler et al. 1985; van Vliet et al. 2004). These interneurons may play a central role in synchronizing theta activity among principal cells by providing synchronous rhythmic inhibition to large numbers of neurons (Funahashi and Stewart 1998). In the CA1 region, cholinergic depolarization of interneurons in s. lacunosum-moleculare leads to membrane potential oscillations (Chapman and Lacaille 1999a), and the resulting rhythmic inhibitory input to pyramidal neurons paces theta-frequency rebound firing of pyramidal cells (Chapman and Lacaille 1999b; see also Rotstein et al. 2005). Hippocampal pyramidal neurons are synchronized by inhibitory synaptic inputs (Cobb et al. 1995), and GABAergic septal inputs can phasically inhibit local interneurons that normally provide tonic inhibition of pyramidal cells, and this can result in the rhythmic disinhibition of pyramidal cells during theta activity (Toth et al. 1997). Further, a population of interneurons in s. oriens also shows intrinsic membrane potential oscillations during metabotropic glutamate receptor activation, and synaptic inputs from these cells may play a central role in synchronizing atropine-resistant theta activity (Gillies et al. 2002). In the CA3 region, O-LM interneurons that show membrane potential oscillations also appear to synchronize theta activity along the longitudinal hippocampal axis (Gloveli et al. 2005). Parasubicular interneurons likely receive cholinergic and GABAergic inputs from the septum, and the prominent membrane potential oscillations in these cells observed here suggest that they play a substantial role in the local synchronization of parasubicular theta activity. This idea is consistent with the finding that IPSPs in the PaS do not decrement but remain stable in response to theta-frequency stimulation (Funahashi and Stewart 1998).

**Functional significance**

Because the PaS sends its major output to layer II of the entorhinal cortex (Amaral and Witter 1989, 1995; Caballero-Bleda and Witter 1994; Swanson and Cowan 1979; van Groen and Wyss 1990b), theta activity in the PaS may play an important role in modulating responses of the entorhinal cortex to multimodal inputs from other cortical regions (Caruana and Chapman 2004). The theta rhythm is thought to play a central role in synchronizing neural mechanisms that mediate sensorimotor integration, memory formation, and spatial navigation (Bland and Oddie 2001; Buzsaki 2005; Cacucci et al. 2004; Caruana and Chapman 2004; Hasselmo 2005; Vertes 2005). Both hippocampal and parasubicular place cells fire in relation to the theta rhythm, and it is likely that the theta rhythm helps regulate the manner in which PaS place cells contribute to spatial processing by interacting with the CA1 region and entorhinal cortex. Unlike hippocampal place cells, which discharge preferentially in certain locations of an environment, or head-direction cells, which code for directional heading (Blair et al. 1999; Taube 1995a), “place-by-direction cells” in the PaS encode allocentric information about both location and orientation (Cacucci et al. 2004). These cells appear to integrate information about spatial location from hippocampal place cells and information about head direction from the anterodorsal thalamus (Blair and Sharp 1995; van Groen and Wyss 1990b). This could provide much of the information necessary to support representations carried by spatial “grid cells” in the dorsomedial entorhinal cortex (Hargreaves et al. 2005). Further, in addition to grid cells in layer II, the deeper layers of the medial entorhinal cortex also contain grid cells, head-direction cells, and conjunctive grid by head-direction cells (Sargolini et al. 2006). In addition to hippocampal inputs to deep layers and local synaptic interactions between layers (Sargolini et al. 2006), the activity of directionally selective layer III neurons could be modulated by parasubicular inputs to the dendrites of these cells within layer II (Caballero-Bleda and Witter 1994).
Theta in the PaS may also help determine how layer II cells in medial and lateral entorhinal cortex respond to extrinsic multimodal sensory inputs from the piriform and perirhinal cortex (Burwell and Amaral 1998). The output of the PaS is known to cause a rapid short-term facilitation of responses of layer II entorhinal neurons to inputs from piriform cortex (Caruana and Chapman 2004), and the output of the PaS might facilitate entorhinal cortex responses during theta. This could enhance transmission of volleys that arrive in phase with theta and could also promote heterosynaptic potentiation effects leading to lasting alterations in how multimodal sensory information is processed within the EC (Chapman and Racine 1997).

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