CONDUCTANCES MEDIATING INTRINSIC THETA-FREQUENCY MEMBRANE POTENTIAL OSCILLATIONS IN LAYER II PARASUBICULAR NEURONS

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Running Head: Membrane potential oscillations in parasubiclar neurons.

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ABSTRACT

Ionic conductances that generate membrane potential oscillations in neurons of layer II of the parasubiculum were investigated using whole-cell current clamp recordings in horizontal slices from the rat brain. Blockade of ionotropic glutamate and GABA synaptic transmission did not reduce the power of the oscillations, indicating that oscillations are not dependent on synaptic inputs. Oscillations were eliminated when cells were hyperpolarized 6 to 10 mV below spike threshold, indicating that they are mediated by voltage-dependent conductances. Application of tetrodotoxin (TTX) completely eliminated oscillations, suggesting that Na\(^+\) currents are required for the generation of the oscillations. Oscillations were not reduced by blocking Ca\(^{2+}\) currents with Cd\(^{2+}\) or Ca\(^{2+}\)-free ACSF, or by blocking K\(^+\) conductances with either 50 µM or 5 mM 4-aminopyridine (4-AP), 30 mM tetraethylammonium (TEA), or Ba\(^{2+}\) (1-2 mM). Oscillations also persisted during blockade of the muscarinic-dependent K\(^+\) current, I\(_M\), using the selective antagonist XE-991 (10 µM). However, oscillations were significantly attenuated by blocking the hyperpolarization-activated cationic current I\(_h\) with Cs\(^+\) and were almost completely blocked by the more potent I\(_h\) blocker ZD7288 (100 µM). Intrinsic membrane potential oscillations in neurons of layer II of the parasubiculum are therefore likely driven by an interaction between an inward persistent Na\(^+\) current and time-dependent deactivation of I\(_h\). These voltage-dependent conductances provide a mechanism for the generation of membrane potential oscillations that can help support rhythmic network activity within the parasubiculum during theta-related behaviors.
The subicular complex includes the subiculum, presubiculum and parasubiculum, and has recently attracted interest due to the roles that it may play in the modulation of activity in both the hippocampus and entorhinal cortex (Craig and Commins 2006; Hargreaves et al. 2005; Liu et al. 2004; O'Mara 2005). The parasubiculum receives inputs from the CA1 region, medial septum, and anterior thalamus, and has a single major output projection to layer II of the medial and lateral entorhinal cortex (Amaral and Witter 1989; Caballero-Bleda and Witter 1994; 1993; Funahashi and Stewart 1997a; Shibata 1993; Swanson and Cowan 1979; Wouterlood et al. 1990). It is therefore well-positioned to make a substantial contribution to the computational processes of the hippocampal formation. The parasubiculum contains place cells that fire in relation to head-direction, and this suggests that the parasubicular input to the entorhinal cortex may contribute to the specialized place cell representations carried by grid cells in the dorsolateral entorhinal cortex (Cacucci et al. 2004; Hafting et al. 2005; Hargreaves et al. 2005; Taube 1995). Further, layer II of the entorhinal cortex is the major target for cortical sensory inputs to the hippocampal formation, and layer II cells provide much of the processed sensory input received by the dentate gyrus and CA3 regions (Amaral and Witter 1989; Kerr et al. 2007). Stimulation of parasubicular inputs to the entorhinal cortex can facilitate entorhinal cortex responses to subsequent inputs from the piriform cortex (Caruana and Chapman 2004), and the parasubiculum may therefore also modulate transmission of highly processed sensory input to the hippocampal formation.

Neuronal synchronization during theta-frequency (4 to 12 Hz) EEG activity is thought to contribute to the computational functions of the entorhinal cortex and hippocampus (Bland 1986; Bland et al. 1999; Buzsaki 2002; 2005; Buzsaki et al. 1983; Hasselmo 2005; Vertes 2005), and it
is also now clear that the activity of parasubicular neurons is modulated by local theta activity. Low-amplitude theta activity was recorded near the parasubiculum in early mapping studies (Bland and Whishaw 1976), but because this activity might have been volume-conducted from adjacent structures we recently used depth profiles with moving bipolar electrodes in urethanized rats to verify that theta-frequency EEG activity is generated locally within the superficial layers of the parasubiculum (Glasgow and Chapman 2007). The presence of place cells in the parasubiculum suggests that the region contributes to spatial navigation (Hargreaves et al. 2005; Hargreaves et al. 2007; Taube 1995), and a substantial proportion of these cells fire with a consistent phase-relation to theta activity (Cacucci et al. 2004; Taube 1995). This indicates that theta oscillations modulate the firing of parasubicular neurons during theta-related behaviors such as active exploration.

Using whole-cell current clamp recordings in acute brain slices, we previously found that depolarization to near-threshold voltages resulted in theta-frequency membrane potential oscillations in approximately 80% of layer II parasubicular neurons (Glasgow and Chapman 2007). The oscillations persisted in the presence of synaptic blockers and were blocked by hyperpolarization, indicating that they are driven by intrinsic, voltage-dependent conductances. Theta-frequency membrane potential oscillations have been observed in CA1 pyramidal cells in vivo (Bland et al. 2002; Ylinen et al. 1995) and in vitro (Leung and Yim 1991; see also Hu et al. 2002), as well as in interneurons in stratum lacunosum-moleculare (L-M) of the CA1 region (Bourdeau et al. 2007; Chapman and Lacaille 1999b) and in layer II and V entorhinal cortex neurons (Alonso and Llinas 1989; Hamam et al. 2000; Klink and Alonso 1993; Schmitz et al. 1998). However, the ionic conductances that combine to produce subthreshold membrane potential oscillations differ. In L-M interneurons, oscillations result from an interaction between
a persistent sodium current ($I_{\text{NaP}}$), and a 4-AP-sensitive A-type K$^+$-current mediated by Kv4.3 channels (Bourdeau et al. 2007; Chapman and Lacaille 1999b). Oscillations in CA1 pyramidal cells and in entorhinal cortex neurons are also mediated by sodium currents (Klink and Alonso 1993; Leung and Yim 1991; Schmitz et al. 1998) but have been linked to TEA sensitive K$^+$ currents (Leung and Yim 1991), the muscarinic-sensitive K$^+$ current $I_M$ (Hu et al. 2007; 2002; Yoshida and Alonso 2007), and the hyperpolarization-activated cationic current $I_h$ (Dickson et al. 2000; Fransen et al. 2004; Hu et al. 2002).

The current study investigated ionic conductances responsible for the generation of voltage-dependent membrane potential oscillations in layer II cells of the parasubiculum using whole-cell current clamp recordings. Results indicate that oscillations are generated by mechanisms similar to those that drive oscillations in principal neurons of the entorhinal cortex (Dickson et al. 2000), and likely rely on an interaction between $I_{\text{NaP}}$ and $I_h$.

**METHODS**

*Slice preparation*

Methods used for in vitro recordings were similar to those in previous reports (Chapman and Lacaille 1999b; Glasgow and Chapman 2007) and were conducted in accordance with guidelines of the Canadian Council on Animal Care. Acute brain slices were obtained from 4 to 6 week old male Long Evans rats (Charles River, Montreal, QC). The rat was deeply anesthetized with halothane and decapitated. The brain was quickly removed and submerged in cold ACSF (4 °C) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH$_2$PO$_4$, 2 MgSO$_4$, 2 CaCl$_2$, 26 NaHCO$_3$, and 10 dextrose saturated with 95% O$_2$ and 5% CO$_2$ (pH ~7.3; 300-310 mOsm). Horizontal brain slices (300 µm) containing the parasubiculum were cut with a vibratome
(WPI, Vibroslice NVSL), and allowed to recover at room temperature for ~1 h. Individual slices were then transferred to a recording chamber, and superfused with oxygenated ACSF at room temperature (22-24° C) at a rate of 1.5-2.0 ml/min. Cells of the superficial layers of the parasubiculum were visualized using an upright microscope (Leica, DM-LFS) equipped with a long-range water immersion objective (40x), differential interference contrast optics, and a near-infrared camera (COHU). The borders of the superficial layers of the parasubiculum were delineated using criteria described previously (Funahashi and Stewart 1997b; Glasgow and Chapman 2007). Layer II of the parasubiculum contains relatively large principal neurons, and is diffuse and disorganized compared to the relatively compact superficial layers of the medial entorhinal cortex and presubiculum (Amaral and Witter 1989; Funahashi and Stewart 1997a; b). Layer II parasubicular cells can also be distinguished from deep layer cells by lack of burst firing in response to current injection (Funahashi and Stewart 1997a; Jones and Heinemann 1988).

**Whole cell recordings**

Intracellular patch pipettes were pulled using a horizontal puller (Sutter Instruments, P-97) and contained (in mM) 140 K-gluconate, 5 NaCl, 2 MgCl₂, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 0.5 ethylene glyco-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 2 ATP-Tris, and 0.4 GTP-Tris (pH calibrated to 7.20 - 7.26 using KOH; 270-280 mOsm). The patch pipette (4-8 MΩ) was lowered under visual guidance to contact with the soma of the target parasubicular cell, and gentle suction was applied. After achieving a tight seal (>1 GΩ) under voltage-clamp, strong suction was applied to obtain whole-cell configuration. The cells were allowed to recover for approximately five minutes before recordings proceeded. Whole-cell current clamp recordings of membrane potential (DC-10 kHz) were amplified using an Axoclamp 200B amplifier (Axon Instr.), monitored using a digital
oscilloscope, and digitized at 20 kHz (Axon Instr., Digidata 1322A) for storage on hard disk using the software package Clampex 8.2 (Axon Instr.). Recordings were accepted if the series resistance was below 30 MΩ (mean, 11.10 ± 0.75).

The majority of layer II parasubicular neurons demonstrated membrane potential oscillations when depolarized to near-threshold voltage levels using steady current injection (Glasgow and Chapman 2007). Ten-sec duration recordings were obtained at a range of voltages relative to action potential threshold by varying the level of constant current injection. Oscillation frequency is temperature dependent (Glasgow and Chapman 2007), but the amplitude is similar at both room temperature and 32° C, and the recordings reported here were therefore obtained at room temperature to reduce the metabolic demands on the cells. After initial baseline tests, recordings were repeated at the same voltages in the presence of pharmacological agents.

The effects of drugs on action potentials and voltage responses to hyperpolarizing and depolarizing current steps were monitored regularly throughout the experiment.

Pharmacological manipulations

All drugs were stored in frozen stock solutions and were added to ACSF just prior to recordings. Sodium currents were blocked by tetrodotoxin (TTX; 0.5 µM). Calcium- and Ca^{2+}-dependent currents were blocked by application of CdCl_{2} (50 µM) or by perfusion of Ca^{2+}-free ACSF in which Ca^{2+} was replaced with Mg^{2+}. Potassium channels were blocked using 4-aminopyridine (4-AP; 50 µM and 5 mM), tetraethylammonium (TEA; 30 mM) or Ba^{2+} (2 mM) in the presence of the ionotropic glutamate receptor antagonist kynurenic acid (KYNA; 1 mM) and the GABA_{A} receptor antagonist bicuculline methiodide (BIC; 25 µM). The GABA_{B} receptor antagonist CGP 55845 (1 µM) was added to the bath when TEA and high doses of 4-AP were used. Normal osmolarity was maintained in control ACSF when testing high doses of 4-AP and
TEA by reducing Na⁺, and replaced it with equimolar choline. When assessing the effects of 
Ba²⁺ and Cd²⁺, PO₄ and SO₄ were removed. The muscarinic-sensitive K⁺ current Iₘ was assessed 
using the selective Kv7.2/3 channel blocker XE-991 (10 µM). The hyperpolarization-activated 
inward-rectifying current Iₕ was blocked using 1 mM CsCl, 2 mM CsCl in the presence of 
synaptic blockers kynurenic acid (1 mM) and bicuculline (25 µM), or the Iₕ blocker ZD7288 
(100 µM; Dickson et al. 2000, see also Chevaleyre and Castillo 2002). All drugs were purchased 
from Sigma (St. Louis, MO), except for ZD7288 (Tocris, Bristol, UK) and XE-991 (Ascent 
Scientific, Weston, UK).

Analysis

Ten-sec samples of membrane potential at near-threshold voltages were prepared for 
spectral analysis by reducing the effective sampling rate to 1 kHz. A 2.048-s segment that 
contained no action potentials was chosen and passed through a Blackman window prior to 
computing the power spectral density. The power spectrum was calculated as the squared 
magnitude of the fast Fourier transform (Clampfit 8.2, Axon Instr.), and was averaged across 
three samples of membrane potential. The power of oscillations between 1.5 and 5.9 Hz was 
expressed as a percent of total power. Paired t-tests and repeated measures ANOVA were used 
to analyze alterations in peak frequency and theta-band power.

Electrophysiological characteristics of layer II parasubicular neurons were analyzed using 
the Clampfit 8.2 software package (Axon Instr.). Action potential height was measured from 
resting membrane potential, and action potential width and fast and medium 
afterhyperpolarizations (fAHP and mAHP) were measured from action potential threshold. Input 
resistance was calculated from the peak voltage response to a 500 ms, –200 pA current step, and
inward rectification was quantified by expressing the peak voltage response as a proportion of the steady-state response (rectification ratio; Chapman and Lacaille, 1999a).

RESULTS

Membrane potential oscillations and electrophysiological characteristics of layer II parasubicular neurons were similar to those observed previously using whole cell recordings (Funahashi and Stewart 1997a; b; Glasgow and Chapman 2007). Mean resting membrane potential in oscillatory cells was –60.1 ± 0.5 mV and a peak input resistance was 120.1 ± 4.0 MΩ, with moderate inward rectification during hyperpolarizing current pulses observed in all cells (rectification ratio: 1.15 ± 0.01). Action potentials (amplitude, 120.2 ± 1.0 mV; duration, 3.7 ± 0.1 ms) were typically followed by fast and medium duration afterhyperpolarizations (fAHP, amplitude: 8.7 ± 0.3 mV; mAHP, amplitude: 6.2 ± 0.3 mV). Oscillatory cells displayed relatively high action potential thresholds (-42.8 ± 0.5 mV; Glasgow and Chapman 2007), and holding the cell near spike threshold using positive current injection resulted in 2 to 5 Hz membrane potential oscillations (mean peak frequency: 2.65 ±0.06 Hz) and intermittent repetitive spiking in almost all PaS neurons recorded (Fig. 1; 75 of 83 cells, or 90.3%). The average theta-band power was 0.42 ± 0.02 mV²/Hz between 1.5 and 5.9 Hz, and accounted for 53.8 ± 1.3% of total power. Non-oscillatory cells displayed comparable electrophysiological properties, except for larger fAHPs (10.6 ± 0.9 mV, $t_{81}= 2.17, p < 0.05$) and mAHPs (8.1 ± 0.7 mV, $t_{81}= 2.10, p < 0.05$).

Blockade of glutamate- and GABA$_A$-mediated synaptic transmission with kynurenic acid (1 mM), and bicuculline (25 μM) was used to verify that oscillations were intrinsic and did not require ionotropic glutamate or GABA synaptic input from other cells (Glasgow and Chapman
2007). Bath application of the synaptic antagonists (n= 15) failed to disrupt oscillations in membrane potential (percent of total power: 50.0 ± 3.2 % in blockers vs. 45.5 ± 3.4 % in control ACSF, \( p > 0.05 \); frequency: 2.6 ± 0.2 in blockers vs. 2.4 ± 0.1 Hz in control ACSF, \( p > 0.05 \)), indicating that synaptic inputs are not necessary for oscillations (Fig. 1). However, oscillations were eliminated by membrane hyperpolarization to 6 to 10 mV below threshold, suggesting that they are generated by intrinsic voltage-dependent conductances.

**Oscillations are Dependent on Sodium Currents.** The role of Na\(^+\) currents in generating membrane potential oscillations was tested using the Na\(^+\) channel blocker tetrodotoxin (TTX). Bath application of TTX (0.5 \( \mu \text{M} \)) both eliminated Na\(^+\)-dependent action potentials evoked by depolarizing current pulses, and also entirely blocked membrane potential oscillations (n= 7). Application of TTX reduced power in the theta band from 55.7 ± 4.2% to 33.8 ± 5.8% of total power (\( t_6 = 3.79, p < 0.01 \); Fig. 2), and raw power values were reduced from 0.58 ± 0.07 to 0.04 ± 0.01 mV\(^2\)/Hz (\( t_6 = 7.80, p < 0.001 \)). Membrane potential oscillations in layer II parasubicual neurons are therefore dependent on inward Na\(^+\) currents. Oscillations are observed at subthreshold membrane potentials in the absence of sustained repetitive spiking in control ACSF, suggesting that oscillations do not require Na\(^+\) currents that drive action potentials, but rather may depend on a persistent non-inactivating Na\(^+\) current (Klink and Alonso 1993; Magistretti et al. 1999).

**Oscillations do not Require Ca\(^{2+}\) Currents.** The potential role of Ca\(^{2+}\) and Ca\(^{2+}\)-dependent K\(^+\) conductances in the generation of oscillations was tested through bath application of the Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (50 \( \mu \text{M}; n= 5 \)) and Ca\(^{2+}\)-free ACSF (n= 5) (Fig. 3). Amplitudes of afterhyperpolarizations, which are dependent on Ca\(^{2+}\)-activated K\(^+\) conductances, were reduced by Cd\(^{2+}\) (fAHP, 4.1 ± 1.9 mV in Cd\(^{2+}\) vs. 7.8 ± 1.8 mV in control ACSF, \( t_4 = 3.03, p < 0.05 \);
mAHP, 2.0 ± 1.3 mV vs. 5.0 ± 1.6 mV, \( t_4 = 2.44, p < 0.05 \) and by Ca\textsuperscript{2+}-free ACSF (fAHP, 3.6 ± 1.3 mV in Ca\textsuperscript{2+}-free ACSF vs. 7.8 ± 1.3 mV in control ACSF, \( t_4 = 3.92, p < 0.01 \); mAHP, 1.7 ± 0.9 vs. 5.8 ± 0.9 mV, \( t_4 = 2.88, p < 0.05 \)) (Fig. 3B). The reduced fAHP was also associated with a moderate increase in action potential duration in both Cd\textsuperscript{2+} (5.7 ± 0.9 ms vs. 3.6 ± 0.4 ms in control ACSF, \( t_4 = 2.83, p < 0.05 \) and Ca\textsuperscript{2+}-free ACSF (6.4 ± 0.5 ms vs. 3.9 ± 0.3 ms in control ACSF, \( t_4 = 4.04, p < 0.01 \)). The frequency and power of oscillations, however, were unaffected by either Cd\textsuperscript{2+} (frequency: 3.1 ± 0.3 Hz in Cd\textsuperscript{2+} vs. 3.2 ± 0.3 Hz in control ACSF; power: 53.9 ± 6.3 % vs. 54.9 ± 4.2 %) or Ca\textsuperscript{2+}-free ACSF (frequency: 3.0 ± 0.2 Hz in Ca\textsuperscript{2+}-free ACSF vs. 3.0 ± 0.2 Hz in control ACSF; power: 65.8 ± 4.7 % vs. 68.2 ± 4.6 %) (Fig. 3C), indicating that Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-mediated K\textsuperscript{+} currents are not necessary for the generation of subthreshold membrane potential oscillations in layer II parasubicular neurons.

**Potassium Currents.** The potential role of K\textsuperscript{+} conductances in oscillations was tested using several K\textsuperscript{+} channel blockers. Parasubicular neurons have relatively high action potential thresholds which suggests that the voltage-gated outward-rectifying current I\textsubscript{D} and the voltage-dependent potassium conductance I\textsubscript{A}, which activate close to –40 and –50 mV respectively, may play roles in the repolarizing phase of oscillations (Wu and Barish 1992). The slowly inactivating K\textsuperscript{+} current I\textsubscript{D} is sensitive to low doses of 4-AP, whereas high doses of 4-AP block the transient K\textsuperscript{+} current I\textsubscript{A} (Storm 1990). Because theta-frequency oscillations in hippocampal L-M interneurons require the voltage-dependent potassium conductance I\textsubscript{A} mediated by Kv4.3 channels sensitive to high doses of 4-AP, we tested the effects of both low (50 μM, n= 6) and high doses (5 mM, n= 5) of 4-AP on oscillations (Bourdeau et al. 2007; Chapman and Lacaille 1999b). Both low and high doses of 4-AP had strong effects on electrophysiological properties of parasubicular cells, but did not have a significant effect on oscillations. Bath application of 4-
AP significantly increased spike duration (50 µM, 6.7 ± 0.8 ms in 4-AP vs. 4.0 ± 0.1 in control ACSF ms, t5 = 2.52, p < 0.05; 5 mM, 11.5 ± 4.0 vs. 4.0 ± 0.4 ms, t4 = 2.12, p = 0.05). Fast and medium AHPs were also significantly reduced by 50 µm 4-AP (fAHP: 2.2 ± 1.2 mV in 4-AP vs. 8.2 ± 1.4 mV in control ACSF, t5 = 5.48, p < 0.01; mAHP: 5.9 ± 1.7 to 1.4 ± 0.6 mV, t5 = 3.06, p < 0.05; Fig. 4B), and were completely abolished by 5 mM 4-AP (Fig. 4F). However, there were no significant changes in either the frequency (50 µM, 2.9 ± 0.1 Hz in 4-AP vs. 2.4 ± 0.1 Hz in control ACSF; 5 mM, 2.8 ± 0.3 vs. 2.6 ± 0.2 Hz) or power of oscillations (50 µM, 50.0 ± 1.7 % in 4-AP vs. 51.5 ± 6.4 % in control ACSF; 5 mM, 50.3 ± 6.8 % in 4-AP vs. 42.4 ± 5.6 % in blockers; Fig. 4), suggesting that neither I_A nor I_D are necessary for the generation of subthreshold membrane potential oscillations in parasubicular neurons.

Tetraethylammonium (TEA) blocks delayed rectifier potassium channels (Beck et al. 1992; Chikwendu and McBain 1996) that could contribute to oscillations (Klink and Alonso 1993; Leung and Yim 1991) and the effect of high doses of TEA on oscillations was therefore tested in parasubicular neurons. Bath application of TEA (30 mM) in the presence of synaptic blockers (n= 4; 1 mM kynurenic acid, 25 µM bicuculline, and 1 µM CGP-55845) resulted in spike broadening, long repolarization periods, and completely abolished fast and medium AHPs (Fig. 5B). However, membrane potential oscillations were not significantly affected in either frequency (2.2 ± 0.1 Hz in TEA vs. 2.3 ± 0.1 Hz in control ACSF) or power (57.7 ± 3.6 % in TEA vs. 54.0 ± 6.3 % in blockers; Figs. 5A and C). Oscillations in layer II parasubicular neurons therefore do not require K+ currents sensitive to TEA.

The potential contribution of K+ channels to oscillations was further tested by application of the widely acting K+ channel blocker Ba2+ in the presence of kynurenic acid, bicuculline, and CGP-55845 (2 mM; n= 6; Fig. 5). Application of Ba2+ resulted in greatly increased action
potential duration (10.7 ± 2.3 ms in Ba\textsuperscript{2+} vs. 3.7 ± 0.3 ms in blockers, \(t_5 = 3.32, p < 0.05\)), decreased action potential amplitude (107.6 ± 5.4 vs. 120.1 ± 4.2 mV, \(t_5 = 8.11, p < 0.001\)), and abolished both the fAHP and mAHP. Application of Ba\textsuperscript{2+} also increased peak and steady state input resistance (249.1 ± 67.1 M\textOmega\ in Ba\textsuperscript{2+} vs. 98.3 ± 21.2 M\textOmega\ in blockers, \(t_5 = 3.65, p < 0.05\), and 159.6 ± 44.0 vs. 85.7 ± 15.9, \(t_5 = 2.81, p < 0.05\), respectively). However, barium did not significantly reduce oscillation power (48.0 ± 4.5 % in Ba\textsuperscript{2+} vs. 55.4 ± 4.5 % in blockers) or frequency (3.0 ± 0.4 Hz in Ba\textsuperscript{2+} vs. 2.8 ± 0.3 Hz in blockers). The hyperpolarizing phase of oscillations therefore appears not to require activation of Ba\textsuperscript{2+}-sensitive K\textsuperscript{+} channels.

Recent evidence has indicated that the muscarinic-sensitive outward K\textsuperscript{+} current I\textsubscript{M} modulates intrinsic neuronal excitability and may play a significant role in the generation of subthreshold theta-frequency membrane potential oscillations in both CA1 pyramidal neurons and in layer V entorhinal neurons (Hu et al. 2007; 2002; Shalinsky et al. 2002; Yoshida and Alonso 2007). Bath application of the selective Kv7.2/3 channel antagonist, XE-991 (10 µM), resulted in a moderate decrease in fast AHP (5.8 ± 1.2 mV in XE-991 vs. 7.9 ± 0.7 mV in control ACSF, \(t_4 = 2.09, p = 0.052\)) (Yoshida and Alonso 2007), but failed to disrupt oscillations (62.7 ± 2.1 % in XE-991 vs. 60.8 ± 3.4 % in control ACSF; Fig. 6). This indicates that I\textsubscript{M} is not required for oscillations in superficial parasubicular neurons.

**Hyperpolarization-activated current I\textsubscript{h}.** The hyperpolarization-activated cationic current I\textsubscript{h} contributes to theta-frequency oscillations in CA1 neurons and stellate cells of the entorhinal cortex through time-dependent activation and deactivation (Dickson et al. 2000; Hu et al. 2002). The parasubiculum shows high levels of HCN1 protein expression (Notomi and Shigemoto 2004), suggesting that I\textsubscript{h} may play a substantial role in regulating the excitability of parasubicular neurons, and contribute to the generation of subthreshold oscillations. Therefore,
its contribution to oscillations in parasubicule neurons was tested using the Ih blockers Cs+ (1 mM, n= 4; 2 mM, n= 5) and ZD7288 (100 µM, n=10). The inward rectifying sag in voltage responses to hyperpolarizing current steps was eliminated by bath application of Cs+ (rectification ratio, 1 mM: 0.98 ± 0.02 in Cs+ vs. 1.14 ± 0.04 in control ACSF; 2 mM: 1.02 ± 0.01 vs. 1.15 ± 0.02), and this increased steady-state input resistance (1 mM: 226.3 ± 103.5 MΩ in Cs+ vs. 115.8 ± 17.6 MΩ in control ACSF; 2 mM: 136.8 ± 20.3 MΩ vs. 99.2 ± 3.1 MΩ). In addition, Cs+ also significantly attenuated the power of membrane potential oscillations. Theta-band power was reduced from 57.1 ± 5.0% to 34.5 ± 2.3% in 1 mM Cs+ (t3= 3.24, p < 0.05; 0.28 ± 0.05 mV²/Hz in Cs+ vs. 0.37 ± 0.06 mV²/Hz in control ACSF, t3= 3.93, p < 0.05), and was reduced from 52.3 ± 5.5% to 31.5 ± 2.2% in 2 mM Cs+ (t4= 3.06, p < 0.05; 0.20 ± 0.05 mV²/Hz in Cs+ vs. 0.44 ± 0.04 mV²/Hz in blockers, t4= 3.23, p < 0.05; Fig. 7). The peak frequency of oscillations was not significantly affected by Cs+ (1 mM: 3.3 ± 0.3 Hz in Cs+ vs. 3.2 ± 0.4 Hz in control ACSF; 2 mM: 3.7 ± 0.4 vs. 2.9 ± 0.3 Hz).

A residual Ih current can sometimes be observed in the presence of Cs+ (Dickson et al. 2000), and the role of Ih in generating oscillations was therefore tested further using the more potent Ih blocker, ZD7288 (100 µM). Bath application of ZD7288 eliminated inward rectification in response to –200 pA current pulses (n= 10; rectification ratio: 1.00 ± 0.01 in ZD7288 vs. 1.11 ± 0.03 in control ACSF; t9= 4.17, p < 0.01) and also eliminated inward rectification in response to larger, –400 pA, pulses (n= 4; rectification ratio: 0.99 ± 0.01 in ZD7288 vs. 1.17 ± 0.04 in control ACSF). In addition, ZD7288 also almost completely blocked oscillations, and oscillation power was reduced from 55.8 ± 2.8% in control ACSF to 30.6 ± 2.6% in ZD7288 (t9= 6.92, p < 0.001; 0.14 ± 0.02 mV²/Hz in ZD7288 vs. 0.57 ± 0.06 mV²/Hz in control ACSF, t9= 7.25, p < 0.001; Fig. 7). Application of ZD7288 also resulted in a reduction
in the fast afterhyperpolarization (from 6.9 ± 0.3 to 2.2 ± 0.8 mV; $t_9 = 7.14$, $p < 0.001$) and an increase in action potential duration (from 3.9 ± 0.2 to 5.6 ± 0.5 ms; $t_9 = 3.55$, $p < 0.01$), but the block of oscillations by ZD7288 was highly effective, even in cells that showed smaller changes in spike repolarization. The block of oscillations by Cs+ and by the more potent Ih blocker ZD7288 indicate that oscillations in parasubicular neurons are likely mediated by the hyperpolarization activated cationic current $I_h$.

**DISCUSSION**

The present study has identified intrinsic voltage-dependent conductances that drive theta-frequency membrane potential oscillations in putative pyramidal and stellate neurons in layer II of the parasubiculum. Oscillations persisted during blockade of ionotropic glutamate and GABA transmission and are therefore not dependent on synaptic inputs from other neurons (Glasgow and Chapman 2007). Further, parasubicular neurons express membrane potential oscillations at near-threshold voltages and are eliminated by hyperpolarization, suggesting that the oscillations are mediated by voltage-dependent conductances. Here, we have found that membrane potential oscillations in layer II cells of the parasubiculum are primarily generated by an interplay between a sodium conductance and the hyperpolarization-activated mixed cationic current $I_h$ (*Figs. 2 and 7*). These currents have also been shown to underlie the generation of theta-frequency membrane potential oscillations in layer II stellate neurons of the entorhinal cortex (Dickson et al. 2000; Fransen et al. 2004; Hamam et al. 2000) and mediate membrane potential resonance to sinusoidal current injection at hyperpolarized potentials in CA1 pyramidal neurons (Hu et al. 2002). This mechanism contrasts with oscillations in hippocampal inhibitory
interneurons that are dependent on Na\(^+\) and a transient A-type K\(^+\) current (Chapman and Lacaille 1999a; Bordeau et al. 2007).

Membrane potential oscillations provide a major mechanism that can contribute to the local genesis of theta activity in the parasubiculum, and the oscillations likely modulate neuronal firing in relation to ongoing theta activity within the hippocampal formation (Bland et al. 2002; Glasgow and Chapman 2007; Taube 1995). Many place cells in the parasubiculum fire in relation to the theta rhythm, indicating that theta activity helps determine the firing behavior of parasubicular neurons involved in spatial processing (Cacucci et al. 2004; Hargreaves et al. 2005; Hargreaves et al. 2007; Taube 1995). In addition, the single major output of the parasubiculum is to layer II of the entorhinal cortex, which receive converging inputs from many cortical regions and provides the hippocampus with much of its highly processed sensory input (Caballero-Bleda and Witter 1994; 1993; van Groen and Wyss 1990). Time-dependent stimulation of the parasubiculum can either enhance or suppress entorhinal cortex responses to sensory inputs from other cortical regions, suggesting that coordinated activity in this pathway plays an important role in the modulation of how the entorhinal cortex processes other inputs (Caruana and Chapman 2004). Theta activity, which is prominent during active exploration, is therefore likely to help regulate the manner in which parasubicular efferents combine with extrinsic cortical sensory inputs to the hippocampal formation (Caruana and Chapman 2004; Sejnowski and Paulsen 2006).

**Conductances Generating Oscillations.** Oscillations were completely eliminated by the Na\(^+\) channel blocker TTX, and were also strongly reduced by application of Cs\(^+\) and the potent I\(_h\) blocker ZD7288. This suggests that oscillations in parasubicular neurons are generated by mechanisms analogous to those in stellate neurons of the medial entorhinal cortex and subicular
pyramidal neurons (Dickson et al. 2000; Fransen et al. 2004; Klink and Alonso 1993; Wang et al. 2006). Similar to oscillations in these areas, oscillations in parasubicular cells likely result from sodium-dependent depolarization to near-threshold voltage levels within the activation range of the persistent sodium current $I_{\text{NaP}}$, and a regulation of the frequency of oscillations by the time-dependent activation and deactivation kinetics of $I_h$ (Dickson et al. 2000; Fransen et al. 2004). Coactivation of $I_{\text{NaP}}$ and $I_h$ leads to the depolarizing phase of the oscillations which results in the voltage- and time-dependent deactivation of $I_h$. Subsequent hyperpolarization then leads to the slow reactivation of $I_h$ and promotion of the depolarizing phase of the oscillations (Dickson et al. 2000; Fransen et al. 2004). This interaction between $I_{\text{NaP}}$ and $I_h$ is also thought to contribute to membrane potential resonance in sensorimotor cortex neurons (Hutcheon et al. 1996a; b) and in CA1 neurons at hyperpolarized potentials (Hu et al. 2002). Both $I_{\text{NaP}}$ and $I_h$ currents may also mediate oscillations in pyramidal cells of the subiculum and in layers II/V of perirhinal cortex where neurons show inward rectification during hyperpolarizing current steps (Bilkey and Heinemann 1999; Hamam et al. 2000; Wang et al. 2006). In addition to $I_{\text{NaP}}$, TTX also blocks the transient sodium current, and it is therefore possible that the depolarizing phase of the oscillations may be mediated in part by the activation of transient sodium channels responsible for window currents (Ketelaars et al. 2001).

Cesium blocks $I_h$ currents only partially and, because residual $I_h$ currents can maintain oscillations in the presence of cesium (Dickson et al. 2000), the persistence of some oscillations in $\text{Cs}^+$ cannot be taken to rule-out the involvement of $I_h$ (Klink and Alonso 1993; Dickson et al. 2000). It was initially concluded that $I_h$ does not contribute to oscillations in entorhinal neurons because oscillations persisted during $\text{Cs}^+$ application (Klink and Alonso 1993), but it was later found that $\text{Cs}^+$ reduces $I_h$ by only about 60-75%, and that while $\text{Cs}^+$ can disrupt oscillations,
periods of clear oscillations are still observed (Dickson et al. 2000; Jones 1994). Similarly, bath application of Cs\(^+\) resulted in a significant attenuation of theta-band membrane potential oscillations in layer II parasubicular neurons, but some periods of oscillatory activity were observed intermittently. Periods of oscillatory activity are also observed in entorhinal stellate cells from HCN1 knockout mice in which the I\(_h\) current is greatly reduced (Nolan et al., 2007), and this suggests that the HCN1-mediated component of I\(_h\) may not be required for oscillations in these cells. However, as in stellate cells of the entorhinal cortex (Dickson et al. 2000), complete block of I\(_h\) with ZD7288 eliminated oscillations in parasubicular neurons, indicating that they are likely dependent on I\(_h\) (Fig. 7). ZD7288 has been shown to result in a non-specific, slowly developing suppression of synaptic transmission at mossy fiber synapses (Chevaleyre and Castillo 2002) but we have found that oscillations in parasubicular neurons are not dependent on synaptic transmission and are rapidly blocked by ZD7288 (Figs. 1 and 7). In addition, although ZD7288 can result in a partial block of inward rectifying K\(^+\) channels (Wilson 2005), the block of oscillations is not easily attributable to effects of ZD7288 on I\(_{K_{ir}}\) because oscillations persisted in the presence of Ba\(^2+\) (Fig. 5). The cells tested here showed a reduction in the fast afterhyperpolarization following ZD7288. However, oscillations were blocked effectively in all cells exposed to ZD7288, and the reduction in oscillations was not related to between-cell variability in the effect of ZD7288 on I\(_{K_{ir}}\) because oscillations persisted.

Voltage-gated potassium channels can contribute to oscillations and rhythmic firing activity in a variety of cell types. Oscillations in hippocampal L-M interneurons are generated by an interaction between I\(_{NaP}\) and a A-type potassium current mediated by Kv4.3 channels that
is sensitive to 4-AP (Chapman and Lacaille 1999a; Bordeau et al. 2007). In CA1 pyramidal cells, oscillations are maintained in 200 µM 4-AP, but are disrupted by moderate doses of TEA, suggesting that oscillations in these cells do not depend on activation of I_A but rather on TEA-sensitive delayed rectifying potassium currents (Garcia-Munoz et al. 1993; Leung and Yim 1991). The conclusion that TEA-sensitive currents could contribute to oscillations was made tentatively, however, because of the strong bursting behavior induced by TEA. In the present study, oscillations were not significantly affected either by low or high doses of 4-AP or by TEA, indicating that I_A and delayed rectifier K^+ channels do not play a central role in the oscillations of parasubicular neurons.

Muscarinic receptor activation closes Kv7/KCNQ channels, and can affect neuronal excitability by depolarizing membrane potential, altering spike frequency adaptation and suppressing spike afterpotentials (Gu et al. 2005; Hu et al. 2007; 2002; Lawrence et al. 2006; Womble and Moises 1992; Yoshida and Alonso 2007; Yue and Yaari 2006; 2004). The muscarinic-sensitive inward rectifying potassium current (I_M) is active at near-threshold potentials, and has been linked to membrane potential oscillations in hippocampal neurons (Gutfreund et al. 1995; Hu et al. 2002). Block of I_M suppresses oscillations in layer V entorhinal cells (Yoshida and Alonso 2007), and I_M is also required for theta-frequency resonance responses in CA1 pyramidal neurons at potentials near threshold (Hu et al. 2002). Here, we have used both Ba^{2+}, a wide-acting K^+ channel blocker which blocks leak conductances as well as inward rectifying potassium channels including I_M, and the selective I_M antagonist XE-991 to determine whether oscillations in parasubicular neurons are dependent on I_M (Benson et al. 1988; Hu et al. 2007; Yoshida and Alonso 2007). It was initially found that oscillations in entorhinal cortex neurons were disrupted by Ba^{2+} (Klink and Alonso 1993), but this was later attributed to greatly
increased synaptic inputs (Dickson et al. 2000). When synaptic blockers are present, membrane potential oscillations in entorhinal cortex actually increase in amplitude in the presence of Ba$^{2+}$ due to an increase in membrane resistance (Dickson et al. 2000). Oscillations in CA1 neurons are not disrupted by Ba$^{2+}$ (Leung and Yim 1991), and oscillations in L-M interneurons also persist during blockade of I$_m$ using the selective antagonist XE-991 (Bordeau et al. 2007). Similarly, we have found that oscillations in parasubicular neurons persist in the presence of Ba$^{2+}$ so that oscillations are not dependent on I$_{K_{ir}}$ conductances such as I$_m$. Further, we have also recently observed that bath application of the cholinergic agonist carbachol which acts in part by inhibiting I$_m$ (Womble and Moises 1992), does not disrupt oscillations in layer II cells of the parasubiculum (S.D. Glasgow and C.A. Chapman, personal observations). Finally, our present results show that selective blockade of I$_m$ with XE-991 fails to disrupt oscillations in layer II parasubicular neurons, indicating that I$_m$ is not required for the generation of the oscillations.

Calcium currents and Ca$^{2+}$-dependent K$^+$ currents (Sah 1996) contribute to oscillations in the thalamus, inferior olivary nucleus and mammillary complex (Alonso and Llinas 1992; Jahnsen and Llinas 1984; Llinas and Yarom 1986). However, the present study demonstrated that oscillations in parasubicular neurons were not significantly affected by Ca$^{2+}$-free ACSF or by the Ca$^{2+}$ channel blocker Cd$^{2+}$, suggesting that Ca$^{2+}$ conductances are not required for the generation of this activity.

**Extrinsic Mechanisms Contributing to Theta Activity.** In addition to the ionic conductances described here that drive oscillations in individual neurons, other extrinsic mechanisms are required to synchronize theta-frequency population activity and lead to the associated field potential. Parasubicular neurons recorded here usually required positive constant current injection to depolarize neurons to the subthreshold range where they expressed
oscillations, and we found previously that parasubiclar theta activity in vivo is dependent on cholinergic mechanisms (Glasgow and Chapman 2007). As is the case in the entorhinal cortex (Klink and Alonso 1997b) and CA1 region (Chapman and Lacaille 1999a), it is likely that septal cholinergic projections to the parasubiculum results in muscarinic depolarization to near-threshold voltages during theta activity (Alonso and Kohler 1984; Benson et al. 1988; Hu et al. 2007; 2002; Klink and Alonso 1997a). Cholinergic theta activity likely serves as a mechanism contributing to timing-dependent changes in synaptic responsivity in the parasubiculum, and cholinergic effects on neuronal excitability, spike timing, and synaptic integration need to be assessed further.

In the CA1 in the intact brain, field activity associated with theta activity is generated by rhythmic perisomatic inhibition and excitatory inputs to distal dendrites (Buzsaki 2002). We have found previously that putative inhibitory interneurons in the parasubiculum also display membrane potential oscillations (Glasgow and Chapman 2007), and the parasubiculum contains large numbers of glutamic acid decarboxylase (GAD) and GABA-immunoreactive cells (Kohler et al. 1985). Inhibitory interneurons contact many parasubiclar neurons, and the rhythmic inhibition of large numbers of principal cells can contribute synchronization of theta activity by “rebound depolarizations” via synchronous activation of I_h (Chapman and Lacaille 1999a; Cobb et al. 1995). Rhythmic excitatory synaptic input to the parasubiculum from the CA1 region and other glutamate inputs from theta-related structures such as the subiculum, the anterior thalamus, and the deep layers of the entorhinal cortex (Kohler 1986; 1985; Shibata 1993; van Groen and Wyss 1990; Vertes et al. 2001) might also contribute to neuronal synchronization in the parasubiculum, and to the membrane currents that generate associated field activity. Thus, membrane potential oscillations are likely to combine with extrinsic synaptic and
neuromodulatory inputs in the generation and synchronization of theta activity within the parasubiculum.
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FIGURE CAPTIONS

Figure 1. Membrane potential oscillations in layer II cells of the parasubiculum are voltage-dependent, and persist during blockade of fast ionotropic glutamatergic and GABAA synaptic transmission. **A**: Whole-cell current clamp recordings were taken at a range of membrane potentials relative to action potential threshold during bath application of synaptic antagonists kynurenic acid (KYNA; 1 mM) and bicuculline (BIC; 25 µM). Depolarization of cells to near-threshold voltages using steady positive current injection resulted in membrane potential oscillations at 2 to 5 Hz. Oscillations are abolished by hyperpolarization. Note that action potentials are truncated in this and subsequent figures. **B**: An autocorrelogram reflects the rhythmicity of membrane potential oscillations in the same cell as in panel A at a membrane potential of -52 mV. **C**: Power spectra for recordings at a range of voltages are shown for the same cell as in **A**. The power of oscillations increased as membrane potential was raised to threshold, with no significant change in frequency. **D**: Group data show that the power of oscillations is not affected by blockade of synaptic transmission, indicating that membrane potential oscillations in layer II parasubicular neurons do not require extrinsic synaptic inputs.

Figure 2. Oscillations in parasubicular neurons are dependent on voltage-gated sodium channels. **A**: Voltage-dependent oscillations were eliminated by constant bath application of the Na+ channel blocker tetrodotoxin (TTX; 0.5 µM; n= 7). Recordings were obtained at the membrane potentials indicated at left. **B**: Responses of the same cell to positive current pulses at resting membrane potential in normal ACSF and in the presence of TTX show
that TTX blocked Na⁺-dependent action potentials. C: Group data show a significant reduction in percent of total power in the theta-band during bath application of TTX (**: p < 0.01). This was associated with a large reduction in raw power values within the theta band (0.04 ± 0.01 vs. 0.58 ± 0.07 to mV²/Hz, p < 0.001). The block of oscillations by TTX suggests that inward Na⁺ currents contribute to the depolarizing phase of oscillations.

Figure 3. Oscillations are not dependent on calcium currents. A: Voltage-dependent oscillations were not reduced in tests in which Ca²⁺ currents were reduced using Ca²⁺-free ACSF (n= 5). Similar results were obtained using Cd²⁺ (n= 5; 50 µM). B: Superimposed action potentials show reductions in the amplitude of fast afterhyperpolarizations during application of Ca²⁺-free ACSF (B₁) and Cd²⁺ (B₂) at the latency indicated by circles. C: The mean power of oscillations was unaffected by application of either Ca²⁺-free ACSF (C₁) or Cd²⁺ (C₂). Inward Ca²⁺ currents and Ca²⁺-dependent K⁺ conductances are therefore not required for membrane potential oscillations in parasubicular neurons.

Figure 4. Oscillations are not dependent on voltage-dependent potassium currents sensitive to either low (50 µM) or high (5 mM) doses of 4-AP. A: Oscillations were not significantly affected by a low dose of 4-AP (50 µM). B: Superimposed action potentials from the same cell as in A show a reduction in the fast afterhyperpolarization in 50 µM 4-AP. C and D: The reduction in the fast afterhyperpolarization was significant for the group of cells tested (n=6; *: p < 0.05), but there was no significant
change in the mean power of subthreshold oscillations in the presence of 50 µM 4-AP.

**E:** Oscillations recorded in the presence of antagonists for ionotropic glutamate and GABA$_A$ and GABA$_B$ receptors were not significantly reduced during bath application of a high dose of 5 mM 4-AP. **F:** Membrane potential responses to hyperpolarizing and depolarizing current pulses in are shown for the same cell as in E during bath application of synaptic antagonists ($F_1$) and 5 mM 4-AP ($F_2$). Note the multiple spikes and delayed repolarization. **G:** Mean power of oscillations at near-threshold voltages was not significantly affected by the high dose of 4-AP (5 mM; n= 5).

**Figure 5.** Blockade of potassium channels with either TEA or Ba$^{2+}$ does not block oscillations.

**A:** Voltage-dependent oscillations recorded in the presence of antagonists of glutamatergic and GABA$_A$ and GABA$_B$ receptors were not reduced by 30 mM TEA (n= 4). **B:** Membrane voltage responses to hyperpolarizing and depolarizing current pulses in ACSF containing the synaptic antagonists ($B_1$) and with the addition of 30 mM TEA ($B_2$). Note the significant spike broadening, and the elimination of fast and medium afterhyperpolarizations by TEA. **C:** The mean power of oscillations was not significantly affected by TEA. **D:** Voltage-dependent oscillations recorded in the presence of glutamate and GABA$_A$ and GABA$_B$ receptor antagonists were not reduced by the broad-acting K$^+$ channel blocker Ba$^{2+}$ (2 mM; n= 6). **E:** Membrane potential responses to hyperpolarizing and depolarizing current pulses in ACSF containing synaptic antagonists ($E_1$) and with the addition of 2 mM Ba$^{2+}$ ($E_2$). Note the increased spike duration, reduced fAHPs, and increased peak and steady-state input resistance. **F and G:** Peak input resistance was increased during bath application of Ba$^{2+}$ ($*$: $p <$
0.01), but the mean power of oscillations was not affected, indicating that outward K\(^+\) currents sensitive to Ba\(^{2+}\) are not required for oscillations in parasubicular neurons.

**Figure 6.** The muscarine-sensitive potassium current I\(_M\) is not required for the generation of membrane potential oscillations in parasubicular neurons. A: The power and frequency of oscillations were not significantly affected by the selective I\(_M\) antagonist XE-991 (10 \(\mu\)M). B: Superimposed action potentials reflect a reduction in the fAHP following bath application of XE-991. C: Group data show that the power of oscillations in parasubicular cells was not significantly affected by XE-991 (n= 5).

**Figure 7.** Blocking the hyperpolarization-activated cationic current I\(_h\) disrupts voltage-dependent oscillations in parasubicular neurons. A: Voltage-dependent oscillations recorded from a parasubicular neuron in the presence of ionotropic glutamate and GABA receptor antagonists were markedly reduced by Cs\(^+\) (2 mM). B: Responses to hyperpolarizing and depolarizing current pulses are shown before (B1) and during bath application of Cs\(^+\) (B2) for the same cell as in A. Action potentials have been truncated here and in panel F. The voltage- and time-dependent inward rectification typical of parasubicular cells was not observed in the presence of Cs\(^+\), and rebound potential responses were abolished. C: There was a significant reduction in the rectification ratio during bath application of 2 mM Cs\(^+\) (*: \(p < 0.05\)). D: Both 1 mM (n= 4) and 2 mM (n= 5) Cs\(^+\) significantly reduced the power of oscillations (*: \(p < 0.05\)). E: Voltage-dependent oscillations were also blocked by the potent I\(_h\) blocker ZD7288 (100 \(\mu\)M). F: Membrane potential responses to current steps in control ACSF (F1) and ZD 7288 (F2)
show the complete block of inward rectification. Also note the spike broadening and reduction of the fast afterhyperpolarization. **G:** Changes in mean rectification ratio reflect the elimination of inward rectification in cells treated with ZD7288 (n=10; **: p < 0.01). **H:** The block of oscillations by ZD7288 was also reflected in a significant reduction of theta-band power (**: p < 0.001).