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Rebound spiking properties of mouse medial entorhinal cortex neurons in vivo

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Abstract

Medial entorhinal cortex is the gateway between cortex and hippocampus, and plays a critical role for spatial coding as represented by grid cell activity. In the medial entorhinal cortex, inhibitory circuits are robust, and the presence of h-current leads to rebound potentials and rebound spiking in in vitro experiments. It has been hypothesized that these properties, combined with local field potential oscillations, may contribute to grid cell formation. To examine the properties of in vivo rebound spikes, we performed whole cell patch clamp recordings in medial entorhinal cortex neurons in anesthetized mice. We injected hyperpolarizing inputs representing inhibitory synaptic inputs with sinusoidal oscillations and found that hyperpolarizing inputs injected at specific phases of oscillation had higher probability of inducing subsequent spikes at the peak of the oscillation in some neurons. Also, this effect was prominent in the cells with large sag potential which is a marker of h-current. In addition, larger and longer hyperpolarizing current square pulse stimulation resulted in a larger probability of eliciting rebound spikes. We did not observe a relationship between amplitude or duration of hyperpolarizing current pulse stimulation and the delay of rebound spikes. These results suggest that rebound spikes are observed in vivo and may play a role in generating grid cell activity in medial entorhinal cortex neurons.

Keywords

patch clamp; sag; h-current; rebound potential; oscillation

Introduction

Medial entorhinal cortex (MEC) is the gateway between cortex and hippocampus, and it plays a critical role in spatial coding and memory. The MEC contains a variety of spatially modulated cells, including grid cells (Hafting et al., 2005), conjunctive grid-by-head-direction cells (Sargolini et al., 2006), and border cells (Solstad et al., 2008). Grid cells are of particular interest due to the spatial influences in their response properties. Theta oscillations in medial entorhinal cortex, driven by medial septum cholinergic and GABAergic neurons, may be essential for generating grid cell activities (Brandon et al.,

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2011; Koenig et al., 2011). These theta oscillations may interact with inhibitory input, as stellate cells are mostly connected to each other via inhibitory interneurons, making a recurrent inhibitory network (Couey et al., 2013). Stellate cells in MEC layer II have characteristic sag potentials and resonant frequency due to h-current (Alonso & Llinas, 1989; Alonso & Klink, 1993; Haas & White, 2004; Giocomo et al., 2007; Fernandez et al., 2013), and this h-current can also elicit rebound potentials after inhibition (Alonso & Klink, 1993; Dickson et al., 2000; Shay et al., 2012). Pyramidal cells in MEC also show sag potentials (Richter et al., 1997; Richter et al., 2000), though they are much smaller than sag potentials in stellate cells (Alonso & Klink, 1993). Thus, inhibitory input to MEC cells may elicit subsequent spikes due to rebound depolarizations that cause rebound spikes.

Rebound spikes may be integrated into a mechanism for generating theta cycle skipping (Jeffery et al., 1995; Brandon et al., 2013) and grid cell activity as demonstrated in a recent model (Hasselmo, 2014). The relationship between amplitude or duration of hyperpolarizing current stimulation and the temporal delay of rebound spikes after stimulation has been described in in vitro recordings of MEC neurons (Ferrante et al., 2014), suggesting a possible mechanism for regulating rebound spike timing by running speed. Recent studies also suggest that there is a relationship between the oscillation phase of hyperpolarizing current stimulation and subsequent spike probabilities at the peak of the oscillation in MEC layer II stellate cells in in vitro slice preparations (Shay et al., 2014), suggesting a mechanism for phase precession and for maintaining select subpopulations of neurons active due to inhibition occurring at a specific phase of theta rhythm oscillations.

Thus, it is critical to examine properties of rebound spikes of MEC neurons in vivo. In this study, we performed current clamp recordings in MEC neurons in vivo from ketamine and xylazine anesthetized mice. To test phase specificity of rebound spiking, we injected hyperpolarizing current stimulation superimposed on an artificial sine wave oscillation to mimic theta oscillations. We also used hyperpolarizing current square pulses with various amplitudes and durations to examine the relationships with the temporal delay of rebound spikes after stimulation. This study helps fill the gap between the knowledge of slice experiments and the data on intact animals relevant to grid cell generation in freely moving animals.

Materials and Methods

Animal surgery

Experiments were performed on 14 young adult C57BL6 male mice (age 4–14 weeks, 15–30 g; Charles River Laboratories) from which 16 successful neuronal recordings were used for analysis. Animals were anesthetized with an intraperitoneal injection of ketamine (75 mg/kgbw, kilograms body weight), xylazine (15 mg/kgbw), or ketamine (75 mg/kgbw), xylazine (15 mg/kgbw), acepromazine (2.0 mg/kgbw). Buprenorphine (0.05 mg/kgbw) was injected before craniotomy in some animals. Additional doses of ketamine and xylazine were given as needed (1/4 of initial dose) via injection cannula placed intraperitoneally. Body temperature was maintained to 37 °C with a heating pad. The depth of anesthesia was monitored by tail pinch and the rhythm of breathing was also monitored during the entire experiment. Before surgery, bupivacaine (0.25%, 0.15mL) was injected subcutaneously at

the site of the incision. Each mouse was placed in a stereotaxic apparatus (SR-8N; Narishige) and prepared for acute electrophysiological recording from MEC. Lubricant eye drops were placed on animals' eyes. After the incision, the surface of the skull was wiped with hydrogen peroxide and saline. Bregma and lambda were set at the same height, and a dental cement sheet was made on the surface of the skull with vetbond and metabond. The positions for craniotomies were marked for hippocampus (posterior 2.2mm, lateral 2.0mm from bregma) and MEC (posterior 4.0mm, lateral 3.7mm from bregma). A craniotomy for the hippocampal electroencephalogram (EEG) recording was made, and twisted teflon-insulated stainless steel electrodes (#790900, A-M Systems) were implanted vertically in the stratum radiatum of dorsal CA1 hippocampus (posterior 2.2mm, lateral 2.0mm from bregma; depth 1.4mm from the surface) and secured in place using dental cement. A chamber was made around the position of craniotomy for MEC recording with dental cement. An aluminum bar was implanted onto the skull by dental cement. A craniotomy (450–800 micrometer diameter) was made for patch clamp recording from MEC. A needle was used to remove Dura. 1% Agarose was put on the surface of the brain above the MEC to reduce pulsation, drying, and instability.

Ethical approval

All experiments were performed in accordance with experimental guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University and The Animal Welfare Act.

Electrophysiology

In vivo patch clamp recordings (Margrie et al., 2002; Hahn et al., 2012; Domnisoru et al., 2013; Schmidt-Hieber & Häusser, 2013) were performed with patch electrodes created from glass capillaries (#BF150-86-10, Sutter Instruments) on an electrode puller (P-97, Sutter Instruments). All recordings were done in current clamp mode. Pipettes were filled with an internal solution containing (in mM): 130 K-gluconate, 10 HEPES, 4 Mg-ATP, 7 KCl, 0.3 Na₂-GTP, 10 Na₂-Phosphocreatine (pH adjusted to 7.25 with KOH, 285 mOsm). In addition, 0.5% biocytin was included in the internal solution for labeling. The filled pipette resistances were 3.8–7.5 MΩ. An Ag-AgCl reference electrode was placed outside the craniotomy, submerged in extracellular saline containing, (in mM) 150 NaCl, 2.5 KCl, 10 HEPES, pH 7.4 (Domnisoru et al., 2013). The liquid junction potential (14.0 mV) was corrected. Electrodes were angled at 14 degrees from vertical, pointing to posterior. The electrode was lowered to the MEC (posterior 4.0mm, lateral 3.7mm from bregma, angled 14 degrees to posterior, depth 1.9–2.6mm from surface) by a micromanipulator (MP-225, Sutter). The manual blind patch recording was performed with the method described previously (Domnisoru et al., 2013; Schmidt-Hieber & Häusser, 2013; Kodandaramaiah et al., 2012). Briefly, high positive pressure more than 200 mbar was used for the penetration going just above the target, and then the positive pressure was reduced to 25 mbar. Pipettes went down with 2-micrometer steps. When the resistance increased more than 0.3 M ohm within 4 micrometers, the positive pressure was released. Then, suction pressure of –20 mbar was applied, and membrane potential was held at –30mV. The potential was decreased to –40mV, and suction pressure was released. The potential was slowly lowered to –70mV, and a gigaseal was formed. Then, suction pressure of –20 mbar and an electronic buzz were

applied to rupture the cell membrane. If the break-in was successful, capacitance compensation and bridge balance were set, and membrane potential recording began. The membrane potential and hippocampal EEG signal were amplified by Multiclamp 700B (Molecular Devices) and stored via an analog-to-digital (A/D) converter (Digidata 1440A, Molecular Devices) with pCLAMP software (Sampling rates: 10kHz). Mechanical noise was reduced by use of an air table. Only recordings with resting membrane potentials more hyperpolarized than -54mV ($-68.9 \pm 5.2\text{ mV}$, mean $\pm$ SD) and spike peaks higher than -34mV ($7.7 \pm 25.2\text{ mV}$) were used for analysis. 9 out of 16 cells showed overshooting spikes above zero. All spike amplitudes were greater than 20mV ($53.7 \pm 21.9\text{ mV}$). During recording, respiration rhythm was monitored by pressure sensor (MLT 1010, AD Instruments). In most cases, tonic negative current was injected to reduce spontaneous firing and set membrane potential to peri-threshold (-50pA – -200pA ; $-103 \pm 56\text{ pA}$, mean $\pm$ SD). By injecting this negative tonic current, resting membrane potentials were slightly hyperpolarized from $-68.9 \pm 5.2\text{ mV}$ to $-73.8 \pm 7.2\text{ mV}$, and spontaneous firing rate decreased from $5.7 \pm 5.5\text{ Hz}$ to $0.4 \pm 1.0\text{ Hz}$ (mean $\pm$ SD). Resting membrane potentials were measured at the trough of slow wave oscillation, if slow wave oscillations were observed. The duration of successful recordings was $26.4 \pm 9.8\text{ minutes}$ (mean $\pm$ SD). If a drift of the baseline membrane potential occurred, actual membrane potential was estimated by the assumption of a linear drift.

Stimulation

To measure phase specificity of the capability of inhibitory inputs to elicit subsequent spikes we superimposed a realistic hyperpolarizing synaptic current on a sinusoidal oscillatory input within MATLAB. Synaptic inputs were constructed with two exponential terms to mimic the time course of synaptic current where fast and slow time constants are $\tau_1=1\text{ms}$ and $\tau_2=5\text{ms}$, respectively.

$$I=e^{(-t/\tau_1)}-e^{(-t/\tau_2)}$$

The hyperpolarizing current was scaled so that the maximum amplitude was equal to twice the peak-to-peak voltage of the oscillatory signal. Hyperpolarizing current stimulation occurred at intervals of 3 cycles of the sinusoidal oscillation, and had 16 different phases (Figs. 1B, 2A). All cells were run with either 8Hz or 10Hz sinusoids, which is based on the resonance frequency of stellate cells in MEC layer II in vivo (Tsuno et al., 2013). Each cell was also run with peak-to-peak amplitudes of either 100, 200, or 400pA, depending on the input resistance of each cell. Each recording trial was 40 seconds long and contained 30 seconds of sinusoid oscillations with hyperpolarizing input pulses, including 5 or 6 repeats of each set of 16 phases of hyperpolarizing input pulses. 1–6 recording trials were performed. Hyperpolarizing step currents were generated in pCLAMP. For the analysis of overall firing frequency, sinusoidal oscillation input without hyperpolarizing input pulses was performed. In these tests, the recording trial was 20 seconds long and contained 15 seconds of sinusoid oscillation without hyperpolarizing input pulses.

Histology

After the completion of each experiment, animals were deeply anesthetized by ketamine and xylazine, and perfused with saline and 10% formalin. The brains were extracted and postfixed with formalin over 24 hours, then placed in 30% sucrose for cryoprotection. The brains were sliced parasagittally into 80 μm thick sections by a cryostat (Leica). Biotin labeled cells were visualized by avidin biotin complex (VECTASTAIN Elite ABC kit (Standard), #PK-6100, Vector) and DAB reactions (DAB Peroxidase Substrate Kit, #SK-4100, Vector). The sections were mounted on gelatin-coated slides and counterstained with Neutral Red.

We used histological information to determine the layer of the recorded cells in MEC. However, cell type was not determined by histology because of the lack of labeling of dendrites in our experimental condition in most neurons. Out of 16 recorded cells, soma and dendrites were labeled in 2 cells. In 6 cells, soma was labeled, though dendrites were not labeled. In 7 cells, multiple cells were labeled probably because of the leak of internal solution. In this case, the layer of the recorded cell was estimated by the position of the multiple labeled cells. In 1 cell, no cell was labeled, though the track trace was visible. From the track trace, the layer of the recorded cell was estimated. Out of 16 recorded cells, 6 cells were in MEC layer II, 4 cells were in layer II or III, 4 cells were in layer III, 1 cell was in layer III or V, and 1 cell was in layer V. Five out of 6 layer II cells showed characteristic large amplitude sag potential. These cells are considered to be putative stellate cells in MEC layer II, though we do not exclude the possibility that they are non-stellate cells.

Sag Analysis

To examine each cell's sag amplitude, hyperpolarizing square current injection was delivered. The amplitude of the hyperpolarizing current was -100pA , -200pA , -500pA , or -1000pA , and the duration was 500ms or 1000ms. 1–15 traces were averaged and used for the sag analysis. Raw sag amplitude was calculated by the difference between the voltage of the peak hyperpolarization elicited by hyperpolarizing square current and the steady-state membrane potential during the hyperpolarizing current. Normalized sag amplitude was calculated as the raw sag amplitude divided by peak hyperpolarization amplitude. Input resistance was calculated as peak hyperpolarization amplitude divided by the amplitude of hyperpolarizing square current. Membrane potential just before the hyperpolarizing square current was between -56 and -78mV .

Data Analysis

All data was recorded by pCLAMP software, and exported to MATLAB for analysis. For sinusoidal inputs, action potentials were found as the peak membrane potential in any 20 millisecond period reaching to at least -25mV . Output phases were identified as the phase of the baseline oscillation of the current at the time of the spike potential. Input phases were calculated as the phase of the most recent current input previous to an action potential, with the additional requirement that the input is within two cycles of the oscillation. In the case of bursting activity (Fig. 2B), all spikes of the burst were included for analyses. Phases are defined based on the sinusoid function, putting 90° at the peak of the input, 270° at the trough, 0° at the zero-crossing on the ascending phase, and 180° at the zero-crossing on the

descending phase of the injection. Sinusoid strength was adjusted as in the methods, leading to at least 30 spikes for each cell. The degree of non-uniformity was determined by the mean resultant length (MRL), and preferred angle calculated with the mean resultant angle (MRA) (Berens, 2009). Tests of non-uniformity were performed with the Rayleigh test included in the MATLAB circular statistics toolbox (Berens, 2009), by which z score and p value were calculated. Pearson correlation was used for the analysis of linear regression. To examine the overall firing frequency induced by sinusoidal input without or with hyperpolarizing current input, the probability of more than one spike in each sinusoidal oscillation cycle was calculated. The phases of these spikes in the oscillation were also calculated. A two-sided Wilcoxon signed-rank test was used for comparing paired data, and a Watson-Williams Test was used for comparing circular data.

Results

Difference of subsequent spike probability due to phase of hyperpolarizing current stimulation during sinusoidal input in vivo

In order to determine the feasibility of rebound spiking models of generating grid cells, we performed patch clamp techniques in anesthetized animals. Neurons in this study were confirmed to lie in layer II, III or V of the medial entorhinal cortex, based on biocytin labeling or track traces of recording pipettes (Fig. 1A). Of 16 neurons, 5 were determined as putative MEC layer II stellate cells exhibiting clear large amplitude sag potentials.

We used hyperpolarizing current stimulation with sinusoidal input to elicit subsequent spikes (Figs. 1B, 2A). Hyperpolarizing current stimulation occurred in 16 different phases of the sinusoidal oscillation. Raw traces of membrane potential at specific phases of hyperpolarizing input pulses were shown in Figs. 2B and 2C. We defined input phase (Figs. 3A, 3D) as the phase of hyperpolarizing current stimulation that elicits subsequent spikes, and output phase (Figs. 3B, 3E) as the phase of the occurrence of output spikes. A polar plot of the input phase of this putative MEC layer II stellate cell (normalized sag amplitude = 0.38, Fig. 3C) combined with 5 recording trials showed that most hyperpolarizing input pulses which elicit spikes occurred at the latter descending phase (near the trough) of the oscillation (Fig. 3A, Rayleigh Test, $P = 1.18\text{E-}17$, 552 spikes in total). A polar plot of Output Phase of this cell showed that most spikes occurred at the peak of the sinusoidal oscillation (Fig. 3B, Rayleigh Test, $P = 5.32\text{E-}281$, 552 spikes in total). Another cell, which is in MEC layer II or III with small sag potential (normalized sag amplitude = 0.09, Fig. 3F), showed no clear input phase relationship (Fig. 3D, Rayleigh Test, $P = 0.67$, 6 recording trials, 226 spikes in total), though it showed a clear output phase relationship (Fig. 3E, Rayleigh Test, $P = 7.43\text{E-}103$, 6 recording trials, 226 spikes in total). In total, 6 out of 16 neurons showed significant unimodal deviation from uniformity of input phase (Rayleigh Test with Bonferroni correction for multiple comparison, $P < 0.05/16 = 0.003125$). 5 out of 6 neurons which showed significant input phase relationship (Fig. 3G right, filled circles) had mean resultant angles (MRA) that were between 180° to 270° , which is the latter descending phase of the oscillation. One neuron with significant input phase relationship showed an MRA of 330° . These 6 neurons had mean resultant lengths (MRL) that were larger than 0.2. Of the 5 putative MEC layer II stellate cells recorded in this dataset, 3

neurons showed a significant input phase relationship. In the other 11 cells, including MEC layer II, III, and V neurons without large sag potential (normalized sag potential was less than 0.1), 3 neurons showed a significant input phase relationship. All 16 neurons showed significant unimodal deviation from uniformity of output phase that tended to be at the peak of the oscillation (Fig. 3H, Rayleigh Test with Bonferroni correction, $P < 0.003125$). In evaluating the data from the overall population, the data showed both a significant input phase relationship (Fig. 3I, Rayleigh test, $P = 9.41\text{E-}25$, MRL = 0.16, MRA = 233° , 55 trials, 16 neurons, 2228 spikes in total) and a significant output phase relationship (Fig. 3J, Rayleigh test, $P < 2.23\text{E-}308$, MRL = 0.79, MRA = 109° , 55 trials, 16 neurons, 2228 spikes in total). These results indicate that spikes in all cells tended to occur around the peak of the oscillation, while hyperpolarizing input pulses were most likely to elicit subsequent spikes if injected during the latter descending phase of oscillation.

Correlation between normalized sag amplitude and input phase relationship

If the input phase relationship is because of the rebound potential and rebound spikes mediated by sag potential and h-current, there should be a correlation between the magnitude of sag potential and induction of input phase relationship. To examine this, normalized sag amplitude and z score of Rayleigh test, which tests unimodal deviation from uniformity of input phase relationship were compared (Fig. 4A). There was a correlation between normalized sag amplitude and z score of Rayleigh test of input phase relationship ($r = 0.57$, $P = 0.021$, Pearson correlation). Thus, it is possible that sag potential accounted for the input phase relationship. Also, stellate cells have large sag potential and relatively low input resistance (Quilichini et al., 2010). To examine the relationship between sag potential and input resistance, they are compared in Figs. 4B1 and 4B2. There was a tendency that larger normalized sag amplitude was associated with lower input resistance. Though there was no significant linear correlation (Fig. 4B1 $r = -0.36$, $P = 0.18$, Pearson correlation), there was significant correlation between the $\log_{10}$ value of input resistance and normalized sag potential (Fig. 4B2 $r = -0.57$, $P = 0.021$, Pearson correlation). Four putative MEC layer II stellate cells which had large normalized sag potentials (more than 0.25, Fig. 4B1) also had low input resistance (less than $60\text{M}\Omega$). Also, 3 out of these 4 cells showed significant deviation from uniformity of input phase (filled circles in Fig. 4B1). However, 3 cells which showed significant deviation from uniformity of input phase had small normalized sag amplitude (less than 0.1, filled circles in Figs. 4A and 4B1).

Overall firing frequency was slightly decreased by hyperpolarizing current stimulation

To examine whether hyperpolarizing input pulses affect the overall firing frequency, we compared the probability of spikes in each oscillatory cycle without or with hyperpolarizing input pulses during sinusoidal oscillatory current stimulation in 11 out of 16 recorded neurons. The probability of more than one spike in each cycle of sinusoidal oscillation was slightly decreased by hyperpolarizing current inputs. As shown in Fig. 5A, the probability was 0.289 ± 0.091 (mean $\pm$ SD) without hyperpolarizing current input, and 0.241 ± 0.119 with hyperpolarizing current input, ($P = 0.042$, two-sided Wilcoxon signed-rank test for paired data, $n=11$). The phases of spike firing were also compared between sinusoidal current stimulation without or with hyperpolarizing current input. There were no significant differences for both MRL and MRA. As shown in Fig. 5B, the MRL was 0.79 ± 0.19 without

hyperpolarizing current input, and 0.83 ± 0.13 with hyperpolarizing current input ($P = 0.067$, two-sided Wilcoxon signed-rank test, $n=11$). As shown in Fig. 5C, the MRA was $102.6^\circ \pm 36.3^\circ$ (mean $\pm$ circular SD) without hyperpolarizing current input, and $111.2^\circ \pm 9.2^\circ$ with hyperpolarizing current input ($P = 0.467$, Watson-Williams Test, $n=11$) for spike phases in the oscillation.

Rebound spikes are elicited by strong and long duration hyperpolarizing stimulation, but not by weak and short duration hyperpolarizing stimulation

We also investigated how the amplitude and duration of hyperpolarizing input affected rebound spikes, using various durations and amplitudes of square pulse currents. 4 out of 16 recorded neurons, including 3 putative stellate cells in MEC layer II, showed rebound spikes with hyperpolarizing current square pulse stimulation in some cases. Figures 6A and 6B show one neuron in which -500pA , 500ms hyperpolarizing square pulse stimulation elicited rebound spikes in 2 out of 5 trials (Fig. 6A). -1000pA , 500ms hyperpolarizing square pulse stimulation elicited rebound spikes in 4 out of 5 trials (Fig. 6B). In all 4 neurons, there is a tendency that larger amplitude hyperpolarizing square current stimulation has higher rebound spiking probability (Fig. 6C). Figure 7A shows that short durations of hyperpolarizing stimulation (40ms , 50ms , and 80ms) do not often elicit subsequent spikes, though rebound potentials were observed. In contrast, longer duration of hyperpolarizing stimulation (500ms and 1000ms) elicited subsequent spikes with higher probabilities (Fig. 7A). In all 4 neurons, there is a tendency that longer duration hyperpolarizing square current stimulation has higher rebound spiking probability (Figs. 7B, and 7C). We did not observe any trend between the amplitude or duration of the inhibition and the temporal delay to rebound spiking at the end of the square current injection pulse.

Discussion

In the present study, we examined the property of rebound potentials and spikes in vivo using two stimulation methods, hyperpolarizing current stimulation with sinusoidal oscillation and hyperpolarizing current square pulse stimulation with various amplitudes and duration. By using hyperpolarizing current stimulation with sinusoidal oscillations, we found that there is a relationship between the phase of hyperpolarizing current stimulation and the probability of spikes at the peak of the oscillation after the stimulation. This indicates that hyperpolarizing input pulses at specific phases of the oscillation increases the probability of subsequent spikes. This can be explained by the rebound potential after the hyperpolarizing current stimulation. By using hyperpolarizing current square pulse stimulation with various amplitudes and durations, we found that larger and longer duration hyperpolarizing stimulation has a higher probability to elicit rebound spikes in vivo. We did not observe a relationship between amplitude and duration of hyperpolarizing current pulse stimulation and the temporal delay of rebound spikes at the end of stimulation. These results contribute to experimental evaluation of a model of grid cell activity incorporating oscillatory interference, resonance frequency, and theta cycle skipping (Hasselmo, 2014; Hasselmo and Shay, 2014).

Rebound spikes in vivo

Because of the predominance of inhibitory network interactions within layer II of the MEC (Dhillon & Jones, 2000; Couey et al., 2013) and the h-current present in stellate cells (Dickson et al., 2000; Heys et al., 2010; Heys & Hasselmo, 2012; Tsuno et al., 2013), it was proposed that inhibition enhances the excitation and firing of entorhinal neurons in a timely manner via rebound spikes (Ferrante et al., 2014; Hasselmo, 2014; Shay et al., 2014). This possibility is supported by the current study of hyperpolarizing current stimulation with sinusoidal input in vivo. This study is the first report of the relationship between hyperpolarizing current input and subsequent spike probability during oscillatory input in vivo.

Although overall firing frequency was slightly decreased by hyperpolarizing current stimulation, the cells that did not show an input phase relationship for inducing spikes showed a larger decrease of overall firing frequency (open circles in Fig. 5A) than the cells that showed an effect of input phase for inducing spikes (filled circles in Fig. 5A). Rebound potentials and rebound spikes probably increase firing frequency and compensate for the decrease of overall firing frequency in the neurons which showed an effect of input phase for inducing spikes. It is also possible that hyperpolarizing input pulses on later ascending phases (between 0° and 90°) decrease subsequent spike probability.

Spontaneous membrane potential fluctuations are observed in anesthetized animals and in awake head-restrained animals (Domnisoru et al., 2013; Schmidt-Hieber & Häusser, 2013; personal observation). These fluctuations of membrane potentials made it difficult to set the membrane potential to a peri-threshold region in vivo, whereas the membrane potential is more easily controlled during in vitro experiments. Also, large fluctuations, including large intrinsic oscillations called slow waves, can make it difficult to observe rebound spikes. These difficulties in observing rebound spikes in vivo might be the reasons for the lack of observation of a relationship between the amplitude or duration of hyperpolarizing current square pulse stimulation and the temporal delay of the subsequent rebound spike, which was clearly shown in our in vitro study (Ferrante et al., 2014).

We also observed an influence of input phase on spike induction in putative non-stellate cells. Some non-stellate cells in layer III and V of MEC showed small sag potentials. Thus, small sag potentials generated by h-current might affect the probability of rebound spikes and the influence of input phase on spike induction in these neurons. Another possible cause of the influence of input phase on spike induction is the effect of some currents in which inactivation is released by hyperpolarization, such as the low-threshold Ca^{2+} current (McCormick & Huguenard, 1992; Lüthi & McCormick, 1998; Izhikevich, 2007). It is also possible that hyperpolarizing input pulses in the ascending phase of the oscillation inhibit subsequent spike firing and thereby affects the input phase relationship.

Function of rebound spikes

Our study suggests that the probability of rebound spikes increases if the hyperpolarizing input pulses comes at specific phases of oscillation in the membrane potential. Stellate cells show intrinsic subthreshold membrane potential oscillations (Dickson et al., 2000; Yoshida

et al., 2011), and the entorhinal cortex also receives oscillatory input from the medial septum (King et al., 1998; Varga et al., 2008) whose GABAergic projections regulate the activity of inhibitory interneurons in the entorhinal cortex (Gonzales-Sulser et al., 2014). These properties of rebound spikes and oscillation will help MEC cells to code spatial and temporal information in a timely manner. It was reported that there is a relationship between theta input and spike timing (Fernandez et al., 2013), which is the output phase in our definition. Although this study analyzed the influence of voltage fluctuations, they did not focus on induction of rebound spikes with hyperpolarizing input pulses.

Although there is evidence to support the possibility that MEC layer II stellate cells are grid cells (Burgalossi et al., 2011; Schmidt-Hieber & Häusser, 2013; Domnisoru et al., 2013), it was proposed that most of the grid cells are pyramidal cells in MEC layer II, and most border cells are stellate cells (Tang et al., 2014; Ray et al., 2014; see preview Savelli & Knierim, 2014). In the Tang et al. 2014 study, the proportion of observed grid cells was low (19% in putative pyramidal cells and 3% in putative stellate cells) relative to other studies (50%, Sargolini et al., 2006; Boccara et al., 2010), indicating that there are some discrepancies. Also, a recent study using Ca^{2+} imaging in freely moving mice showed that grid cell responses appeared in both stellate cells and pyramidal cells in similar proportions (Sun et al., 2015). Although we need additional experimental evidence to make a conclusion, we also observed an input phase relationship for inducing subsequent spikes in some putative non-stellate cells. Rebound spikes may also have a critical role in pyramidal cells. It was reported that sag potential mediated by h-current was also observed in CA1 pyramidal cells in the hippocampus (Zemankovics et al., 2010), and HCN1 knock out mice showed a significant change of place cell activity (Hussaini et al., 2011). Also, a relationship between rebound spikes and synchronization was previously proposed in the hippocampus (Cobb et al., 1995). It may be possible that the relationship between the phase of hyperpolarizing current input and the probability of subsequent spikes would also be observed in the hippocampal pyramidal cells, and affect the generation of place cell activity.

MEC layer II excitatory neurons interact with each other primarily via inhibitory interneurons (Dhillon & Jones, 2000; Couey et al., 2013), and rebound spikes might help generate theta cycle skipping (Jeffery et al., 1995; Deshmukh et al., 2010) that is maintained as synchronous or anti-synchronous firing in different sets of neurons (Brandon et al., 2013). Our study of single cell electrophysiology in vivo fills the gap between intrinsic properties shown by in vitro slice electrophysiology and grid cell activity in freely moving animals. Although we are unable to say that recorded cells are grid cells because of the use of anesthetized mice, this result shows the insight that rebound spikes in vivo may contribute to the spatial coding in MEC. Further experiments are needed to show the relationship between rebound spikes and grid cell activity with activation of the inhibitory network of MEC in freely moving animals.

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Abbreviations

EEG	electroencephalogram
MEC	medial entorhinal cortex
MRA	mean resultant angle
MRL	mean resultant length

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Figure 1. Example of a biocytin labeled cell and schematic diagram of the hyperpolarizing current stimulation in the oscillation

A. One example of a recorded cell (parasagittal section). The biocytin labeled cell is a stellate cell in layer II of the medial entorhinal cortex (MEC).

B. Schematic diagram of the hyperpolarizing current stimulation superimposed with sinusoidal current input. Hyperpolarizing current stimulation is performed at 16 different phases of the oscillation, and subsequent spikes after the current stimulation are detected. The peak of the oscillation is 90°, and the trough is 270°.

Figure 2. Probability of subsequent spikes depended on the phase of the hyperpolarizing current stimulation in the oscillation

A. Experimental design of hyperpolarizing current stimulation superimposed with sinusoidal current input. Hyperpolarizing current stimulation was performed on every third cycle of the sinusoidal current input shifting across 16 different individual phases of the oscillation.

Upper trace: membrane potential. Lower trace: injected current. From left, the phases are 157.5°, 225°, 292.5°, 360° (= 0°), 67.5° (with arrow C), 135°, 202.5°, 270°, 337.5°, 45°, 112.5°, 180°, 247.5° (with arrow B), 315°, 22.5°, 90°. By using this stimulation, the effect of input phase of hyperpolarizing current stimulation on induction of subsequent spikes was evaluated.

B. Six examples of subsequent spikes induced in response to a specific input phase (247.5° , arrow B in Fig. 2A) of hyperpolarizing current injection. Counted spikes are (from top): 4, 2, 2, 0, 3, 0 (11 spikes in total).

C. Six examples of subsequent spikes induced in response to another specific input phase (67.5° , arrow C in Fig. 2A) of hyperpolarizing current injection. Counted spikes are (from top): 0, 0, 0, 2, 0, 3 (5 spikes in total).

Figure 3. Hyperpolarizing current stimulation at specific input phases of the oscillation increased the probability of subsequent spikes at the peak of the oscillation in some neurons

A. Polar plot of the input phase of hyperpolarizing current stimulation which elicited subsequent spikes of a putative MEC layer II stellate cell. We defined this as input phase for elicited spikes. Thick line indicates mean resultant length (MRL, 0.26) and mean resultant angle (MRA, 256°). Hyperpolarizing inputs which occur at the later descending phase of the oscillation (180° to 270°) tend to elicit subsequent spikes in this cell. This cell showed significant non-uniformity of input phase for inducing spikes (Rayleigh Test, $P = 1.18\text{E-}17$, including 5 recording trials, 552 spikes in total).

B. Polar plot of the output phase where spikes occur in the same neuron with Fig. 3A. We defined this as output phase of elicited spikes. Thick line indicates MRL (0.91) and MRA (109°). Spikes tend to occur at the peak of the oscillation. This cell showed significant non-uniformity of output phase (Rayleigh Test, $P = 5.32\text{E-}281$, 5 recording trials, 552 spikes in total).

C. Averaged trace of the response to hyperpolarizing current square pulse ($n=5$) of the cell in Fig. 3A and 3B. Normalized sag amplitude was 0.38.

D. Another example of input phase which did not show input phase relationship for inducing subsequent spikes. This cell was in MEC layer II or III with small sag potential. This cell did not show significant non-uniformity of input phase (MRL=0.04, MRA=218°, Rayleigh Test, $P = 0.67$, 6 recording trials, 226 spikes in total).

E. Output phase of the neuron in Fig. 3D. This cell showed significant non-uniformity of output phase (MRL=0.88, MRA=119°, Rayleigh Test, $P = 7.43\text{E-}103$, 6 recording trials, 226 spikes in total).

F. Averaged trace of the response to hyperpolarizing current square pulse ($n=10$) of the cell in Fig. 3D and 3E. Normalized sag amplitude was 0.09.

G. Plots of MRL and MRA of input phase for eliciting spikes in all 16 neurons. Filled circles indicate the data which showed significant non-uniformity of input phases that induced subsequent spikes (Rayleigh Test with Bonferroni correction for multiple comparison, $P < 0.05/16 = 0.003125$). Open circles indicate the data which did not show significant nonuniformity.

H. Plots of MRL and MRA of Output Phase in all 16 neurons. All data showed significant nonuniformity of output phase (Filled circles, Rayleigh Test with Bonferroni correction for multiple comparison, $P < 0.003125$).

I. Input phase of overall population. MRL = 0.16, MRA = 233°, Rayleigh Test, $P = 9.41\text{E-}25$, 55 trials, 16 neurons, 2228 spikes in total.

J. Output phase of overall population. MRL = 0.79, MRA = 109°, Rayleigh Test, $P < 2.23\text{E-}308$, 55 trials, 16 neurons, 2228 spikes in total.

Figure 4. Sag amplitude was correlated with input phase relationship

A. The relationship between normalized sag amplitude and z score of Rayleigh test of input phase in each cell ($n=16$). Filled circles indicate the data which showed significant nonuniformity of input phase in Fig. 3G. Open circles indicate the data which did not show significant non-uniformity of input phase.

B. The relationship between normalized sag amplitude and input resistance (linear scale in B1 and log scale in B2) in each cell ($n=16$). Filled and Open circles indicate same properties as in Fig. 4A.

Figure 5. Hyperpolarizing input pulses slightly decreased overall firing frequency and had almost no effect on output phases

A. Probability of more than one spike in each sinusoidal oscillation cycle without or with hyperpolarizing current stimulation (n=11). Filled circles indicate the data which showed significant non-uniformity of input phase in Fig. 3G. Open circles indicate the data which did not show significant non-uniformity of input phase.

B. MRL of phases of firing in sinusoidal oscillatory input without or with hyperpolarizing current input (n=11). Filled and Open circles indicate same as Fig. 5A.

C. MRA of phases of firing in sinusoidal oscillatory input without or with hyperpolarizing current input (n=11). Filled and Open circles indicate same as Fig. 5A.

Figure 6. Larger amplitude of hyperpolarizing current square pulse stimulation had higher probability to elicit rebound spikes

A. Examples of rebound spikes with long (500ms) and medium (-500pA) hyperpolarizing current square pulse stimulation in an entorhinal cortex neuron. In this example, 2 out of 5 hyperpolarizing current injections elicited rebound spikes. Membrane potential was between -59mV and -69mV.

B. Examples of rebound spikes with long (500ms) and large (-1000pA) hyperpolarizing current square pulse stimulation in the same neuron with A. 4 out of 5 hyperpolarizing current injections elicited rebound spikes. Membrane potential was between -59mV and -69mV.

C. Rebound spiking probability plot with different amplitude of hyperpolarizing current square pulse stimulation in 4 cells. Duration of hyperpolarizing current stimulation is fixed to 500ms.

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Figure 7. Longer duration of hyperpolarizing current square pulse stimulation had higher probability to elicit rebound spikes

A. Examples of rebound spikes with different duration (From top: 40 ms, 50ms, 80ms, 500ms, 1000 ms) of hyperpolarizing current square pulse stimulation (-1000pA) in an entorhinal cortex neuron. Note that hyperpolarizing stimulation with short duration (40ms, 50ms, and 80 ms) did not elicit rebound spikes, and hyperpolarizing stimulation with long duration (500ms and 1000ms) did elicit rebound spikes. Membrane potential was between -59mV and -69mV .

B. Rebound spiking probability plot with different duration of hyperpolarizing current square pulse stimulation in 4 cells. Amplitude of hyperpolarizing current stimulation is fixed to -500pA .

C. Rebound spiking probability plot with different duration of hyperpolarizing current square pulse stimulation in 3 cells. Amplitude of hyperpolarizing current stimulation is fixed to -1000pA .