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Research report

Cholinergic receptor activation supports persistent firing in layer III neurons in the medial entorhinal cortex

Arthur Jochems[a], Antonio Reboreda[b,c], Michael E. Hasselmo[d], Motoharu Yoshida[a,e,*]

[a] International Graduate School of Neuroscience, Ruhr-University Bochum, Bochum, Germany
[b] Department of Functional Biology, University of Vigo, CINBIO – IBIV, Vigo, Spain
[c] Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada
[d] Faculty of Psychology, Center for Memory and Brain, Department of Psychology and Graduate Program for Neuroscience, Boston University, 2 Cummington Street, Boston, MA, USA
[e] Faculty of Psychology, Mercator Research Group – Structure of Memory, Ruhr-University Bochum, Bochum, Germany

HIGHLIGHTS

- We report persistent firing in MEC layer III cells *in vitro*.
- Cholinergic stimulation increases the fraction of cells that show persistent firing.
- Repeated stimulation gradually suppresses persistent firing.
- Persistent firing in MEC layer III may help temporal association tasks.

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ABSTRACT

Medial temporal lobe (MTL) areas are crucial for memory tasks such as spatial working memory and temporal association memory, which require an active maintenance of memory for a short period of time (a few hundred milliseconds to tens of seconds). Recent work has shown that the projection from layer III neurons in the medial entorhinal cortex (MEC) to hippocampal region CA1, the temporoammonic (TA) pathway, might be specially important for these memory tasks. In addition, lesions to the entorhinal cortex disrupt persistent firing in CA1 which is believed to support active maintenance of memory. Injection of cholinergic antagonists and group I mGlu receptor antagonists to the MEC impairs spatial working memory and temporal association memory. Consistent with this, we have shown that group I mGlu receptor activation supports persistent firing in principal cells of the MEC layer III *in vitro* (Yoshida et al. [39]). However, it still remains unknown whether cholinergic receptor activation also supports persistent firing in MEC layer III neurons. In this paper, we tested this in MEC layer III cells using both ruptured and perforated whole-cell recordings *in vitro*. We report that the majority of cells we recorded from in MEC layer III show persistent firing during perfusion of the cholinergic agonist carbachol (2–10 μM). In addition, repeated stimulation gradually suppressed persistent firing. We further discuss the possible role of persistent firing in memory function in general.

1. Introduction

Much evidence supports the involvement of medial temporal lobe (MTL) structures in tasks that require active maintenance of memory (duration: a few hundred milliseconds to tens of seconds) such as working memory, delayed match to sample and trace conditioning both in humans [1–4] and animals [5–10]. Active maintenance of memory may help association of temporally separated information which is thought to be necessary for the formation of long-term episodic and sequential memory [11–13]. *In vivo* recordings in animals [14–16] and fMRI in humans [14,15] have revealed persistent activity during the trace or delay period of memory tasks when information retention is necessary (reviewed in [16]). These indicate that persistent neural firing may support temporal association by providing a means to retain necessary information across the temporal gap. However, the specific MTL areas of importance and underlying mechanisms for persistent firing still remain unknown.

Persistent firing can be supported either by synaptic networks or the mechanisms within individual neurons (reviewed in [17]).

* Corresponding author at: Faculty of Psychology, Ruhr-University Bochum, GA04/49, Universitätsstraße 150, Bochum 44801, Germany. Tel.: +49 234 32 27138; fax: +49 234 32 14463.
E-mail address: motoharu.yoshida@rub.de (M. Yoshida).

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Network based persistent firing was introduced by classical theoretical work which dates back to Donald Hebb's theory of cell assemblies [18] or to David Marr's theory of memory [19]. In these theories, synaptic excitation maintains neural activity. Another line of studies supports the importance of mechanisms in individual cells to support persistent firing. This view is supported by the experimental observations of persistent firing *in vitro* in multiple areas in the MTL including the entorhinal cortex [20,21], postsubiculum [22] and perirhinal cortex [23]. In these areas, persistent firing is supported in individual cells by cholinergic and group I mGlu receptor activation which leads to the activation of the CAN current (reviewed in [24]).

Persistent firing *in vivo* has been shown in the EC [25,26], and hippocampal regions CA1 and CA3 [27–31] both in DMS tasks and trace conditioning. It has also been clear that cholinergic [10,32–34] and/or group I mGlu [35–37] receptor activation are necessary for performance in these memory tasks. These pharmacological studies are consistent with single cell mechanisms for supporting persistent firing.

Recently, using mutant mice, Suh et al. [38] showed the specific importance of the temporoammonic (TA) pathway, the direct projection from layer III of the medial entorhinal cortex (MEC) to the hippocampal CA1 field, in spatial working memory and temporal association memory. Although place cells and spatial reference memory were normal in these mutant mice with suppressed synaptic transmission in the TA pathway, they exhibited a deficit in spatial working memory tasks such as the delayed matching-to-place (DMP) version of the water maze task (delay 30 s) and the delayed non-matching-to-place (DNMP) version of the T-maze task (delay 15 s). Mutants also had a deficit in a non-spatial temporal association task such as trace fear-conditioning with 20 s trace period. They further showed that infusion of a cholinergic receptor antagonist and a group I mGlu receptor antagonist in MEC layer III was effective in disrupting the task in control mice but no effect was seen in mutants. This study, therefore, indicated a particular importance of MEC layer III in active maintenance of memory possibly through persistent firing supported by individual cells.

Although we have reported that group I mGlu receptor activation supports persistent firing in principal cells in the MEC layer III [39], it remains unknown whether cholinergic receptor activation can support persistent firing in these cells. Therefore, in this study, we tested the induction of persistent firing in rat MEC layer III cells using *in vitro* ruptured and perforated whole cell patch recordings in the cholinergic receptor agonist carbachol. We show that persistent firing can be greatly enhanced in carbachol in a dose dependent manner. We further show that multiple stimulations can gradually terminate persistent firing.

2. Materials and methods

2.1. Ruptured whole-cell patch recording

All ruptured whole-cell patch recordings were conducted at Boston University. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Boston University. Long-Evans rats (postnatal days 21–27; Charles River, Wilmington, MA) were deeply anesthetized with ketamine/xylazine (95 mg/kg ketamine and 2.8 mg/kg xylazine) through intraperitoneal injection. After the absence of both pedal and tail pinch reflex was confirmed, ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM) 110 choline chloride, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 7 glucose, 3 pyruvic acid and 1 ascorbic acid (pH adjusted to 7.4 by saturation with 95% O_2–5% CO_2) was intracardially perfused. The brain was then removed from the cranium and placed in ice-cold modified ACSF. 350 μm-thick slices were cut horizontally using a Vibroslicer (World Precision Instruments, Sarasota, FL, USA). Slices were transferred to a holding chamber, where they were kept submerged at 30 °C for 30 min and then at room temperature at least 30 more min before recording. The holding chamber was filled with ACSF containing (in mM) 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.6 $CaCl_2$, 1.8 $MgSO_4$, 10 glucose (pH adjusted to 7.4 by saturation with 95% O_2–5% CO_2).

Slices were transferred to a submerged recording chamber and superfused with ACSF, maintaining the temperature in between 34 and 36 °C for recordings. Patch pipettes were fabricated from borosilicate glass capillaries by means of a P-87 horizontal puller (Sutter Instrument, Novato, CA, USA). Patch pipettes were filled with intracellular solution containing (in mM) 120 K-gluconate, 10 HEPES, 0.2 EGTA, 20 KCl, 2 MgCl, 7 phosphocreatine-diTris, 4 Na_2ATP, 0.3 TrisGTP and 0.1% biocytin (pH adjusted to 7.3 with KOH). When filled with this solution, the patch pipettes had a resistance of 3–5 MΩ. Slices were visualized with an upright microscope (Zeiss Axioskop 2), equipped with a 40× water-immersion objective lens, and a near-infrared charge-coupled device (CCD) camera (JAI CV-M50IR, San Jose, CA, USA). Tight seals (>1 GΩ) were formed on cell bodies and the membrane was ruptured with negative pressure. Current-clamp recordings were made with a Multi Clamp 700B amplifier (Axon Instruments, Foster City, CA, USA). Signals were lowpass filtered at 5 kHz or 10 kHz and sampled at 10 kHz or 20 kHz, respectively, using Clampex 9.0 software (Axon Instruments, Foster City, CA, USA). A liquid junction potential of 10 mV was not corrected. All whole-cell recordings were performed in the presence of synaptic blockers to suppress ionotropic glutamatergic and GABAergic synaptic transmission using kynurenic (2 mM) acid and picrotoxin (100 μM).

Stock solutions of carbachol (10 mM, in water) was prepared and diluted more than a thousand times in the ACSF. Kynurenic acid and picrotoxin were directly dissolved in the ACSF. Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Ellisville, MO, USA).

2.2. Perforated whole-cell patch recording

All perforated patch recordings were performed at McGill University. All experimental procedures were approved by the McGill University Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care. Long-Evans rats from young (P12) to adult (6–7 weeks old) ages were anesthetized with ketamine:xylazine cocktail (60:5 mg/kg), and intracardially perfused with ice-cold modified ACSF containing (in mM) 110 choline chloride, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 0.5 $CaCl_2$, 2.5 KCl, 7 glucose, 3 pyruvic acid, and 1.3 ascorbic acid. Horizontal slices (350 μm) were obtained using a VT1000 tissue slicer (Leica) with the same modified ACSF. Slices were transferred to a holding chamber, where they were kept submerged for ~1 h at room temperature (22 °C) before recording. The holding chamber was filled with ACSF consisting (in mM) 125 NaCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $MgCl_2$, 1.6 $CaCl_2$, 2.5 KCl, 10 glucose, 3 pyruvic acid, and 1.3 ascorbic acid. ACSF was constantly bubbled with carbogen (95% O_2–5% CO_2).

Slices were transferred to a submerged recording chamber and superfused with ACSF. Slices were visualized with an upright microscope Axioskop (Zeiss, Oberkochen, Germany) equipped with a ×63 water immersion objective and differential contrast optics. A near-infrared charged-coupled device (CCD) camera (Sony XC-75) was used to visualize the neurons.

Layer III medial entorhinal neurons selected for recording using layer II and lamina dissecans as references and were filled with biocytin for later identification. The cells were recorded using the current-clamp technique at 33 ± 1 °C with an Axopatch 1D amplifier and Clampex 8.0 recording software (Axon Instruments, Foster City, CA). Whole cell recordings were performed using the perforated-patch technique. Perforated patch was obtained using amphotericin-B (175–200 μg/ml) [40]. Intracellular pipette solution was identical to that in the ruptured whole-cell patch method above. Drugs and chemicals were purchased from Sigma (St. Louis, MO).

Patch pipettes (5–7 MΩ) were pulled using a Sutter P-97 horizontal puller (Sutter Instrument, Novato, CA). Tight seals (>5 GΩ) were obtained by applying constant negative pressure. Electrical access to the cell was obtained by waiting ~30 min for amphotericin-B to attain a stable access resistance (perforated-patch configuration). Bridge correction was performed using the built-in circuit of the amplifier. Sampling rate was 20 kHz and the low-pass filter was set at 5 kHz. All recordings were performed in the presence of the same synaptic blockers as in the ruptured whole-cell patch recording (kynurenic (2 mM) acid and picrotoxin (100 μM)).

2.3. Data analysis

Clampfit 9.0 (Axon Instruments, Foster City, CA, USA) and Matlab (MathWorks, Natick, MA, USA) were used for data analysis. The latency of persistent firing was measured as the time from the offset of the stimulation and the first spike of persistent firing. The frequency of persistent firing was measured as an average firing frequency of the neuron during the period between 10 and 20 s after the termination of the current injection. The membrane potential difference after stimulation was measured as the difference between an average membrane potential during the same period (10–20 s) and the baseline membrane potential which was measured as an average of the membrane potential during the period between 1 and 5 s before current injection.

Significance levels were evaluated using paired and unpaired *T*-tests for comparisons between two groups. Comparisons of more than two groups were performed by either a one-way or repeated measures ANOVA followed by Tukey *post hoc* tests. Significance level <0.05 (ns: not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) was used. Data are expressed as means ± SEM.

2.4. Identification of anatomical location

Locations of the cells were confirmed by biocytin staining or photos taken after recordings with the pipette still attached to the cell through the low