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Muscarinic Receptor Activation Enables Persistent Firing in Pyramidal Neurons from Superficial Layers of Dorsal Perirhinal Cortex

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Abstract

Persistent-firing neurons in the entorhinal cortex (EC) and the lateral nucleus of the amygdala (LA) continue to discharge long after the termination of the original, spike-initiating current. An emerging theory proposes that endogenous persistent firing helps support a transient memory system. The present study demonstrated that persistent-firing neurons are also prevalent in rat perirhinal cortex (PR), which lies immediately adjacent to and is reciprocally connected with EC and LA. Several characteristics of persistent-firing neurons in PR were similar to those previously reported in LA and EC. Persistent firing in PR was enabled by the application of carbachol, a non-selective cholinergic agonist, and it was induced by injecting a supra-threshold current or by stimulating supra-threshold excitatory synaptic inputs to the neuron. Once induced, persistent firing lasted for seconds to minutes. Persistent firing could always be terminated by a sufficiently large and prolonged hyperpolarizing current; it was prevented by antagonists of muscarinic cholinergic receptors (mAChRs); and it was blocked by flufenamic acid. The latter has been suggested to inhibit a Ca^{2+} -activated non-specific cation conductance (G_{CAN}) that normally furnishes the sustained depolarization during persistent firing. In many PR neurons the discharge rate during persistent firing was a graded function of depolarizing and/or hyperpolarizing inputs. Persistent firing was not prevented by blocking fast excitatory and inhibitory synaptic transmission, demonstrating that it can be generated endogenously. We suggest that persistent-firing neurons in PR, EC, LA, and certain other brain regions may cooperate in support of a transient-memory system.

Keywords

Acetylcholine; transient memory; working memory; trace fear conditioning; TRPC channels; lateral amygdala; entorhinal cortex; postsubiculum; anterior cingulate cortex

INTRODUCTION

Transient memory is essential for behavior and cognition (Baddeley, 2007; Jonides et al., 2008; Baddeley, et al., 2009). Diverse theories exist regarding the neurophysiological basis of transient memory (Durstewitz et al., 2000; Major and Tank, 2004; Teramae and Fukai, 2005; Fransen et al., 2006; Mongillo et al., 2008). Most theories can be broadly divided into two types, which are not mutually-exclusive. The first focuses on enduring spiking within recurrent networks of neurons (Compte et al., 2000; Rodriguez and Levy, 2001;

Constantinidis and Wang, 2004; Lau and Bi, 2005). The second type, discussed below, emphasizes non-synaptic mechanisms that are intrinsic to individual neurons. Of immediate interest, in this respect, is the phenomenon of endogenous persistent firing, as first described in brain slices of the entorhinal cortex (EC; Egorov et al., 2002; Fransen et al., 2006; Tahvildari et al., 2007) and the lateral nucleus of the amygdala (LA; Egorov et al., 2006).

Persistent-firing neurons in EC and LA discharge for seconds to minutes after terminating a supra-threshold, current step (Egorov et al., 2002; Fransen et al., 2006; Reboreda et al., 2007; Tahvildari et al., 2007). The capacity for persistent firing in these neurons is enabled by activating muscarinic cholinergic receptors (mAChRs). A rapidly-emerging theory proposes that mAChR-enabled persistent firing supports the transient memory function that is required to associate stimuli that are separated in time (Egorov et al., 2002; McGaughy et al., 2005; Fransen et al., 2006; Hasselmo, 2006; Hasselmo and Stern, 2006; Kholodar-Smith et al., 2008b; Bang and Brown, 2009; Esclassan et al., 2009; Reboreda et al., 2011). Several lines of evidence indicate that mAChR activation is critical for aspects of working or transient memory (Seeger et al., 2004; McGaughy et al., 2005; Hasselmo and Stern, 2006; Bang and Brown, 2009; Esclassan et al., 2009; Yoshida and Hasselmo, 2009).

The present study asked whether neurons in perirhinal cortex (PR) also exhibit mAChR-enabled persistent firing. PR is immediately adjacent to LA and EC and is reciprocally connected to both structures (Burwell and Amaral, 1998a, b; Pitkanen et al., 2000; Pikkariainen and Pitkanen, 2001; Furtak et al., 2007b; Kerr et al., 2007). Persistent-firing neurons in both PR and EC have been hypothesized to support trace fear conditioning (Kholodar-Smith et al., 2008b; Bang and Brown, 2009; Esclassan et al., 2009), a Pavlovian paradigm in which a temporal gap (a “trace” interval) separates the offset of the conditional stimulus (CS) from the onset of the unconditional stimulus (US). The trace interval commonly lasts 10 - 30 s (McEchron et al., 1998; Moyer and Brown, 2006; Bangasser et al., 2006). Trace fear conditioning is severely impaired by damage to PR (Kholodar-Smith et al., 2008b) and by infusing PR with a nonselective mAChR antagonist (scopolamine; Bang and Brown, 2009). PR infusion with scopolamine had no effect on delay fear conditioning or context conditioning, procedures in which the CS and US are not separated by a trace interval. The present study demonstrated that mAChR-enabled persistent-firing is prevalent among pyramidal neurons from layer II/III of rat PR.

METHODS

Procedures involving animal subjects have been reviewed and approved by Institutional Animal Care and Use Committee of Yale University, conform to NIH guidelines, and were carried out with strict adherence to all university regulations. The methods were chosen to enable direct comparisons with hundreds of previous whole-cell recordings (WCRs) from rat PR neurons (Faulkner and Brown, 1999; Beggs et al., 2000; McGann et al., 2001; Moyer et al., 2002; Moyer and Brown, 2007). In these previous recordings, which were from rat PR brain slices bathed in artificial cerebral spinal fluid (aCSF), none of the cells exhibited persistent firing in response to either direct depolarization or excitatory synaptic stimulation. Based on this fact, we reasoned that even a small increase in the probability of persistent firing should be detectable in a sample of about 100 neurons.

Brain Slice Preparation

The preparation and imaging of PR brain slices, along with the neurophysiological methods, have been described elsewhere in considerable detail (Moyer and Brown, 2007). Briefly, male Sprague-Dawley rats (13 - 31 days) were deeply anesthetized with halothane and decapitated. The brain was quickly removed and placed in ice-cold oxygenated (95% O₂/5% CO₂) sucrose-aCSF containing (in mM): 206 sucrose, 2.8 KCl, 1 CaCl₂, 1 MgCl₂, 2

MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 D-glucose for about 3 min. The brain was blocked and glued to the tray of a temperature-controlled vibratome (Vibratome 3000, Vibratome Company Inc, MO). Coronal slices (300 μm) containing perirhinal cortex were cut at ~1° C.

Slices were immediately moved to a 24-well slice incubation chamber (see Moyer and Brown, 2007) which was maintained at room temperature (22 – 28° C). The oxygenated aCSF (pH 7.4, 295 mOsmol) contained (in mM): 124 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 D-glucose. After at least 1 hr in the incubation chamber, individual slices were transferred to a submerged-type recording chamber, where they continued to be perfused with oxygenated aCSF. Recordings were performed on slices maintained at 31°C using an automatic temperature controller (Warner Instrument Co, Hamden, CT).

Electrical Stimulation and Recording

The WCR pipettes (3 – 5 MΩ) were fabricated from thin-walled glass capillaries (i.d. 1.12 mm, o.d. 1.5 mm; WPI) using a Sutter Instruments model P-97 puller. The standard pipette solution (pH 7.3; 290 mOsmol) contained (in mM): 110 K-gluconate, 10 HEPES, 20 KCl, 2.0 MgCl₂, 2.0 Na₂ATP, 0.3 Na₃GTP, and 10 phosphocreatine (di-Tris). The electrode resistance was calculated from the current that was required to produce a 5 mV hyperpolarizing voltage step. The seal resistance was calculated from the current that was required to produce a 20 mV hyperpolarizing voltage step. Recordings used an Axopatch 200B amplifier (Axon Instruments, CA) in current-clamp mode. Methods for capacitance and series-resistance compensation are described elsewhere (Moyer and Brown, 2007). Signals were filtered at 2 kHz, digitized at 44 kHz on-line using an Instrutech ITC-16 (Great Neck, NY), and acquired in real time with custom data acquisition and analysis software written using IgorPro (ver. 5.0; Wavemetrics, Lake Oswego, OR). Current steps were controlled by a Master-8 pulse stimulator (A.M.P.I., Israel) or by a programmable digital-to-analog (DAC) converter (RP2.1 Real-Time Processor, Tucker-Davis Technologies, Alachua, FL).

PR neurons were visualized using either of two upright microscopic systems. The first was a Zeiss Axioskop equipped with infrared-filtered (IR) light, differential interference contrast (DIC) optics, a Hamamatsu C2400 video camera, and a video enhancement device, as previously described (Faulkner and Brown, 1999; Beggs et al., 2000; McGann et al., 2001; Moyer et al., 2002; Moyer and Brown, 2007). The second microscope was an Olympus upright (BX51) that was similarly equipped for IR-DIC imaging. With either microscope, cells were imaged using a 60X water-immersion lens (NA 0.9).

Because of the extreme neuroanatomical and neurophysiological diversity of PR neurons (Faulkner and Brown, 1999; Beggs et al., 2000; McGann et al., 2001; Moyer et al., 2002; Furtak et al., 2007a; Moyer and Brown, 2007), recordings were restricted to upright pyramidal neurons with cell bodies in layer II/III of dorsal PR (Area 36). The rationale was that this subset of PR neurons might be most homogeneous. Pyramidal neurons constitute about half of the total number of neurons in layer II/III (Furtak et al., 2007a). Healthy pyramidal neurons were visually-selected for WCRs based on IR-DIC images, as previously described (Faulkner and Brown, 1999; Moyer and Brown, 2007). To be included in the analysis, cells had to exhibit overshooting action potentials, a resting potential of –60 mV or more negative, and an input resistance of at least 120 MΩ. None of the cells included in the analysis died or became unstable during the experiment. Cells were first recorded in aCSF and then again after adding 10 μM carbachol to the aCSF. As in previous studies of persistent firing, the membrane potentials reported here were not corrected for junction potentials (about –13 mV; Moyer and Brown, 2007).

The firing patterns that were directly elicited by just supra-threshold depolarizations were classified as described elsewhere (Faulkner and Brown, 1999; Moyer and Brown, 2007; see Results). In some experiments, a bipolar concentric stimulating electrode (o.p. 200 μm SS; i.p. 50 μm Pt/Ir, FHC, Bowdoinham, ME) was placed in layer I of PR using a 4X objective. Stimulation of layer I evoked excitatory post-synaptic potentials (EPSPs) in layer II/III pyramidal cells (Faulkner and Brown, 1999; Beggs et al., 2000; Moyer and Brown, 2007). The current was adjusted so that a single stimulation elicited a supra-threshold EPSP when the somatic membrane potential was maintained at -60 ± 1 mV. In an attempt to induce persistent firing, the input was then stimulated at 20 Hz for 2 s.

The possibility of antidromic responses was eliminated based on the sub-threshold evoked waveform (the EPSP) and the supra-threshold evoked waveform (an action potential arising from the EPSP). In contrast to orthodromic responses, antidromic responses are all-or-nothing functions of the stimulus intensity and they emerge from the baseline rather than from an EPSP. We have never observed antidromic responses using the present stimulation and recording procedures, possibly because layer II/III pyramidal cell axons tend not to project to layer I.

All drugs were bath-applied at the desired concentrations. The APV (DL-2-amino-5-phosphonovaleric acid), carbachol, atropine, pirenzepine and 4-DAMP (4-diphenyl-acetoxy-N-methyl-piperidine) stock solutions were dissolved in distilled water and then diluted to the final concentration with aCSF. The DNQX (6-cyano-7-nitroquinoxaline-2,3-dione), AFDX-116, PD102807 and flufenamic acid stock solutions were dissolved in DMSO. When stock solutions were made in DMSO, the final concentration of DMSO in the aCSF was 1%. Picrotoxin was made fresh in aCSF each time. The final drug concentrations in the bath are given in the Results section.

Persistent Firing during Noise Injections

For persistent firing to serve its hypothesized mnemonic functions, it cannot be brittle in the face of random synaptic fluctuations. Here we illustrate the effects of three different bandwidths of Gaussian current noise at two different amplitudes. The noise amplitudes were defined by their standard deviations, which were chosen to be roughly comparable to the amplitudes of spontaneous and evoked postsynaptic currents in PR neurons (Faulkner and Brown, 1999; Beggs et al., 2000; Moyer et al., 2002; Moyer and Brown, 2007). White noise with a Gaussian amplitude distribution was generated using a programmable digital-to-analog (DAC) converter (RP2.1 Real-Time Processor, Tucker-Davis Technologies, Inc. Alachua, FL). The noise was band-pass filtered with a lower cutoff at 1 Hz and upper cutoffs at 20 Hz, 50 Hz, or 500 Hz. Finally, the standard deviation of the current noise was adjusted to be 0 pA, 20 pA or 50 pA (Fig. 5). The current noise was sub-threshold for directly eliciting firing. Noise was introduced 60 - 120 s before the first current step and it continued throughout the recording window. Firing was directly elicited by a 100 pA depolarizing current that lasted 2 s.

Statistical Analysis

The statistical significance of differences in group means was determined using *t* tests or *F* tests. Differences in relative frequencies were evaluated using χ^2 tests. Correlations between continuous and true dichotomous variables were computed using a point-biserial correlation (r_{pb}). The standard error is denoted SE.

RESULTS

Neurophysiology of Persistent Firing

Adjustment of the Resting Potential—In the first group of experiments, WCRs were made from 99 cells in PR brain slices that were initially bathed in aCSF and then in aCSF containing 10 μ M carbachol. Before the carbachol infusion, the mean ($\pm$ SE) resting potential was -68.0 ± 0.5 mV and the mean input resistance was 183.7 ± 5.7 M Ω . After infusing carbachol, the mean resting potential was -63.3 ± 0.5 mV and the mean input resistance was 219.5 ± 7.5 M Ω . The carbachol-produced depolarization ($t_{98} = 10.9$; $P < 0.001$) was expected from previous studies (Egorov et al., 2002; Fransen et al., 2006). The 4.7 mV depolarization never caused spontaneous firing. In an effort to create greater neurophysiological uniformity, a steady depolarizing or hyperpolarizing current was injected into each cell to adjust the resting membrane potential to -60 ± 1 mV, which was the minimum resting potential for initial inclusion in the study (see Methods). After adding carbachol, the adjustment required a depolarizing current in 66 cells, a hyperpolarizing current in 25 cells, and no current in 8 cells. Little current was required because of the large input resistance of these cells (>120 M Ω ; see Beggs et al., 2000). The carbachol-induced depolarization was clearly not the cause of persistent firing, since the same depolarization in the absence of carbachol failed to enable persistent firing.

Prevalence, rate, duration, and termination of persistent firing—In agreement with hundreds of previous recordings from PR neurons (Faulkner and Brown, 1999; Beggs et al., 2000; McGann et al., 2001; Moyer et al., 2002; Moyer and Brown, 2007), persistent firing could not be elicited while the slices were being bathed in aCSF. However, after infusion with aCSF plus 10 μ M carbachol, depolarizing current steps induced persistent firing lasting at least 10 s in 85% of the cells (84/99). Figures 1 - 7 show representative examples of persistent firing in various experimental conditions or protocols. As discussed below, different rates of persistent firing were sometimes intentionally induced in the same neuron (see Figs. 2 - 4). Among the 84 cells that exhibited at least 10 s of persistent firing, the mean ($\pm$ SE) lower rate of persistent firing was 1.84 ± 0.9 Hz. The mean upper rate of persistent firing was 2.56 ± 0.12 Hz.

The mean duration of persistent firing was not determined because repetitive firing was usually terminated by the experimenter (84/99 cells), based on practical considerations. Bearing this in mind, the observed duration distribution was as follows: 1 - 9 s ($n = 9$), 10 - 19 s ($n = 3$), 20 - 29 s ($n = 34$), 30 - 39 s ($n = 35$), 40 - 49 s ($n = 7$), 50 - 59 s ($n = 0$), 60 - 69 s ($n = 3$), 70 s or longer ($n = 2$). Persistent firing failed to occur in 7% (6/99) of the cells. In these 6 neurons, a depolarizing after-potential (DAP) was evident following the offset of the current step. Examples of DAPs are illustrated in Figs. 3 and 6. At some point in each recording, persistent firing was completely terminated by a large and prolonged hyperpolarization (see Fig. 1). Following its termination, persistent firing could always be re-induced by a second depolarization.

In a subset of these cells (57/99 neurons), the experimental protocol included recordings lasting at least 30 s after the offset of the depolarizing stimulus. Eighty-three percent of these neurons (47/57 neurons) exhibited persistent firing lasting at least 30 s. Since there are known developmental changes in persistent firing in EC (Reboreda et al., 2007), we examined the relationship between the age of the animals, which ranged from 13 to 30 days old, and the occurrence of persistent firing. The mean ($\pm$ SE) age was 21 ± 1 days. There was no significant correlation between the age of the animals and the occurrence of 30 s of persistent firing ($r_{pb(57)} = -0.16$, $P = 0.90$). In this group of 57 neurons, no attempt was made to induce different rates of persistent firing. The mean firing rate was 1.86 ± 0.12 Hz ($n = 47$). Twelve percent of the cells exhibited persistent firing that lasted less than 10 s

(7/57 neurons). Five percent of the cells (3/57 neurons) failed to exhibit persistent firing. In these three cells, a DAP was evident after the offset of the current step.

Fig. 1 shows an example of persistent firing that was allowed to continue for 60 s. The frequency-time histogram gives the number of action potentials in successive 1-s intervals. After a minute of continuous discharge, persistent firing was terminated by a hyperpolarizing current step. The longest duration of persistent firing was terminated after 3 min. Additional examples of persistent firing, lasting 30 s or longer, are illustrated in Figs. 4, 5, and 7.

Firing patterns during the depolarizing current step—In direct response to a depolarizing current step, different PR neurons can exhibit one of six distinguishable firing patterns (Faulkner and Brown, 1999; Moyer and Brown, 2007). The frequency distribution of firing patterns varies as a joint function of the morphological cell type and the cortical layer. All of the cells recorded in the present study exhibited either a regular-spiking (RS) or a late-spiking (LS) firing pattern, as expected based on previous studies of layer II/III pyramidal neurons (Faulkner and Brown, 1999; Beggs et al., 2000; Moyer and Brown, 2007). Thirty-eight cells were classified as RS neurons. Of these, 29 showed persistent firing (76%). Sixty-one cells were classified as LS neurons, 55 of which showed persistent firing (90%). There was no significant relationship between the firing pattern elicited by the current step and the occurrence of persistent firing ($\chi^2_{(1)} = 2.0$, $P = 0.16$). These results do not necessarily generalize to other cell morphologies or firing patterns in PR. In addition to the regular-spiking and late-spiking neurons recorded in the present study, other firing patterns include fast-spiking, burst-spiking, single-spiking, and irregular spiking (Faulkner and Brown, 1999; Moyer and Brown, 2007).

Graded nature of persistent firing—The graded or analog nature of persistent firing was explored using three methods that were borrowed from studies of persistent firing in EC (Egorov et al., 2002; Fransen et al., 2006; Tahvildari et al., 2007) and LA (Egorov et al., 2006). The first approach asked whether the rate of persistent firing depends on the amplitude of the spike-eliciting current step. This procedure was applied to 9 of the 47 cells in which recordings lasted at least 30 s after the offset of the current step. An example of the effect of the current amplitude on the rate of persistent firing is illustrated on the left-hand side of Fig. 2 (before the hyperpolarizing current step). The histograms show the numbers of spikes in successive 1-s time bins.

As expected, increasing the current-step amplitude increased the firing rate during the current step. The mean firing rates ($n = 9$) during the smallest, intermediate, and largest current steps, respectively, were 2.95 ± 0.22 Hz, 5.35 ± 0.18 Hz, and 6.40 ± 0.13 Hz ($F_{(2, 38)} = 130.4$, $P < 0.001$). When tested on a single-cell basis, the effect of the current-step amplitude on the persistent-firing rate was statistically-significant (F tests, $P < 0.05$) in 7 of the 9 cells that were tested. In the remaining 2 cells, the smallest tested current that caused persistent firing resulted in almost the maximum rate of persistent firing.

The second method examined the effect of a 5 s hyperpolarizing current step on the rate of persistent firing ($n = 25$). In every case, firing ceased during the current step. In all 25 cells, the hyperpolarization either terminated persistent firing or reduced the rate of persistent firing. As shown in the right-hand part of Fig. 2, the effect of a hyperpolarizing current step of fixed amplitude (110 pA) depended on the persistent-firing rate prior to the hyperpolarization. When the persistent-firing rate was lowest (Fig. 2A), the hyperpolarizing current step terminated firing. When the persistent-firing rate was somewhat higher, the same hyperpolarizing current step, applied to the same cell, failed to terminate firing, but it did reduce the mean rate of persistent firing (Fig. 2B).

In the example illustrated in Fig. 2B, the mean rate of persistent firing in response to an intermediate current step was 5.35 ± 0.18 Hz before the hyperpolarization and 3.57 ± 0.37 Hz after the hyperpolarization. This 33 percent reduction in the firing rate was statistically significant ($t_{(6)} = 6.97$, $P < 0.001$), based on firing rates in the 7 time bins (1 s each) before and after the hyperpolarization. In the same cell, illustrated in Fig. 2C, the mean firing rate during a larger current step was 6.40 ± 0.13 Hz before the hyperpolarization and 5.14 ± 0.40 Hz after the hyperpolarization. This 20% reduction was also statistically-significant based on the same time bins, $t_{(6)} = 2.714$, $P < 0.05$).

When tested on a single-neuron basis, these reductions in firing rate were statistically significant in 22 of the 25 cells (t tests, $P < 0.05$). In the remaining 3 neurons, the hyperpolarization was also accompanied by a reduction in the firing rate, but the firing-rate change was not statistically significant. In the examples shown in Fig. 2, the reductions in the persistent-firing rates may not have been long-lasting. Following the reinstatement of firing, the discharge rates were gradually increasing during the recorded interval. Fig. 4, discussed below, illustrates a longer-duration reduction in the firing rate. As noted earlier, persistent firing could always be terminated by a sufficiently large and prolonged hyperpolarization.

The third procedure was to inject one or more depolarizing currents after the onset of persistent firing ($n = 13$). An example is shown in Fig 3, where the frequency-time histogram gives the number of action potentials in successive 1-s time bins. The first current step was supra-threshold for eliciting a train of action potentials (at 4 - 5 spikes per 1-s time bin) and a DAP, but it was sub-threshold for inducing persistent firing. The second current step directly elicited firing at 6 spikes per time bin, and it induced persistent firing at 1 or 2 spikes per time bin. The qualitative difference between the effect of the first and second current steps suggests that some residuum of the first depolarization must have persisted during the 16 s interval between the offset of the first current step and the onset of the second. Note that repetitive depolarizations were not necessary to trigger persistent firing. A sufficiently-large initial depolarization always elicited persistent firing in cells that are capable of this discharge pattern.

The mean persistent-firing rates after the second and third current steps were significantly different from each other ($t_{(16)} = 7.78$, $P < 0.001$), based on a comparison of the 17 time bins before and after the second current step. There was no significant difference between the mean firing rates following the third and fourth current steps, which directly elicited firing at 7 spikes per 1-s bin, followed by persistent firing at 2 - 3 spikes per bin. Further statistical analysis compared the effects of the two smallest current steps that elicited persistent firing. Eight of the 13 cells showed a statistically significant increase in firing rate (t tests, $P < 0.05$) following the larger of the two current steps. In the group as a whole, the mean persistent-firing rate following the smallest depolarization that elicited persistent firing (2.18 ± 0.25 Hz) was significantly lower than the mean firing rate following the next-largest depolarization (2.54 ± 0.26 Hz; $t_{(12)} = 2.99$, $P < 0.05$).

In summary, graded persistent firing is common among pyramidal neurons from superficial layers of dorsal PR. The graded nature of persistent firing eliminates a whole class of candidate mechanisms that would result in strictly bi-stable states (Brody et al., 2003; Fransen et al., 2006).

Persistent firing elicited by synaptic stimulation—Persistent firing was readily elicited by stimulating excitatory synaptic inputs to the recorded neuron (4/4 cells tested). As in the previous experiments, the slices were bathed in aCSF containing $10 \mu\text{M}$ carbachol. A typical example is illustrated in Fig. 4, where the time histogram again gives the number

of action potentials in successive 1-s time bins. During the synaptic stimulation, the firing rate ranged from 10 – 17 spikes/bin. The subsequent rate of persistent firing peaked at 5 spikes/bin and then gradually declined to 3 – 4 spikes/bin. Following a hyperpolarizing current step, the rate stabilized at 3 spikes/bin. This decrease in the firing rate was statistically significant ($t_{(17)} = 9.22$, $p < 0.001$), based on the 18 time bins (1 s each) before and after the current step.

Among the 4 neurons that were tested, the rate of synaptically-elicited persistent firing ranged from 1.6 to 4.1 Hz. The mean ($\pm$ SE) firing rate was 2.61 ± 0.26 Hz. After terminating persistent firing, with a sufficiently large and prolonged hyperpolarizing current step, it could be re-elicited by a second synaptic stimulation and/or by a second depolarizing current step (4/4 cells). Repetitive supra-threshold synaptic stimulation does not cause persistent firing in layer II/III pyramidal neurons bathed in aCSF (Beggs et al., 2000; also see Moyer and Brown, 2007).

Persistent firing during random current noise—The hypothesis that persistent firing mediates aspects of transient memory requires that the underlying mechanism be robust to random noise and distracters (Brody et al., 2003; Fransen et al., 2006). An additional set of experiments ($n = 8$ cells) examined persistent firing in the presence of Gaussian current noise of three different amplitudes and in three different frequency bands (see Methods). Representative results are illustrated in Fig. 5. The three rows (A – C) are recordings from three different neurons that were subjected to noise in three different frequency bands: 1 – 20 Hz (top row); 1 – 50 Hz (middle row), and 1 – 500 Hz (bottom row). The three columns illustrate the effects of increasing the amount of current noise, measured by its standard deviation (SD), which was either 0 pA (left column, no-noise control condition), 20 pA (middle column), or 50 pA (right column). Persistent firing was not blocked in any of the noise conditions (8/8 cells).

Neuropharmacology of Persistent Firing

Antagonists of muscarinic cholinergic receptors—Several mAChR antagonists demonstrated that the pharmacological action of carbachol on persistent firing was mediated by mAChRs. Atropine (1 μ M), an antagonist of all mAChR-receptor subtypes, blocked both persistent firing and DAPs ($n = 5$; Fig. 6A). More selective antagonists of M1 – M4 receptors were also explored. Pirenzepine (0.3 μ M; $n = 3$; Fig. 6B), a M1 receptor antagonist, blocked persistent firing (3/3 neurons), as did 4-DAMP (1 μ M; $n = 5$; Fig. 6C), a M3 receptor antagonist (5/5 neurons). Both antagonists also blocked DAPs.

Perfusion with the M2 and M4 receptor antagonists, AFDX-116 and PD102807, respectively, produced more variable effects on persistent firing and DAPs. Following infusion with AFDX-116 (10 μ M; $n = 7$), none of the tested cells showed persistent firing, but DAPs remained in 3 of the 7 neurons (see Figure 6D). Following the infusion with PD102807 (0.25 μ M; $n = 5$), only 1 of the 4 tested cells continued to show persistent firing. In this cell, the firing rate was low and it self-terminated several seconds after the offset of the current step (Fig. 6E). A DAP remained in 3 cells that did not exhibit persistent firing. Thus, at the tested concentrations, antagonists for M1 and M3 receptors were comparable to atropine in blocking persistent firing and DAPs, whereas the M2 and M4 antagonists were not comparable to atropine.

Antagonist of a non-specific cation conductance—According the standard hypothesis (Egorov et al., 2002; Fransen et al., 2006), the sustained current supporting endogenous persistent firing in EC and LA is a Ca^{2+} -dependent, non-specific, cation conductance (G_{CAN}) that is activated by the combination of agonist binding to mAChRs and

a large elevation in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Flufenamic acid reliably blocks persistent firing in LA and EC, presumably by inhibiting G_{CAN} (Egorov et al., 2002; Fransen et al., 2006). Perfusion with flufenamic acid (100 μM) reliably eliminated persistent firing (5/5 neurons; Fig. 6F). Recently, flufenamic acid was also shown to block mAChR-enabled persistent firing in the postsubiculum (PoS; Yoshida and Hasselmo, 2009) and in the anterior cingulate cortex (ACC; Zhang and Seguela, 2010). Although the specificity of flufenamic acid for G_{CAN} can be questioned (Wang et al., 2006), the results demonstrate another parallel to findings in other brain regions.

Antagonists of ionotropic glutamate and GABA receptors—An additional 23 neurons were studied under conditions in which fast synaptic transmission through ionotropic receptors was blocked. As in the earlier-described group of 57 cells, recordings in this group of 23 neurons lasted at least 30 s after the offset of the initial depolarizing current. The ages of the animals ranged from 13 to 31 days old. The mean age ($\pm$ SE) was 21 ± 1 days. The point-biserial correlation between the age of the animal and the occurrence of 30 s of persistent firing was not statistically significant ($r_{pb(21)} = 0.13$, $P = 0.55$). Thus the ages of the animals were quite similar in both groups and in neither group was there a significant correlation between age and the occurrence of 30 s of persistent firing.

As before, persistent firing was studied in brain slices bathed in aCSF plus carbachol (10 μM). The position of the extracellular stimulating electrodes and the amount of current were adjusted to produce a reliable excitatory postsynaptic potential (EPSP; Fig. 7A). Next, fast synaptic transmission was blocked by perfusing the slices with 50 μM APV (an NMDA receptor antagonist), 10 μM DNQX (an AMPA receptor antagonist), and 100 μM picrotoxin (a GABA_A receptor antagonist). The cell featured in Fig. 7 demonstrates persistent firing following the block of fast synaptic transmission. The top trace in Fig. 7A shows a large EPSP before the transmission block. The middle trace demonstrates that the drug combination completely blocked the EPSP. Fig. 7B illustrates persistent firing in this same cell during the block of fast synaptic transmission. The bottom trace in Figure 7A shows the restoration of the EPSP after the neurotransmitter antagonists were washed from the bath.

During the blockade of fast synaptic transmission, 43% percent of the cells (10/23) exhibited persistent firing that lasted at least 30 s. In these 10 neurons, persistent firing was clearly endogenously-generated. In absolute terms, the proportion of cells showing 30 s of persistent firing in this group of 23 cells was smaller than in the previously-described sample of 57 cells. However, the difference in proportions did not approach statistical significance ($\chi^2_{(1)} = 2.0$, $P = .157$). As in the first set of experiments, persistent firing was always completely terminated by a hyperpolarizing current step. The mean rate of persistent firing under these conditions was 2.13 ± 0.21 , which is similar to the earlier-noted rate of persistent firing in the absence of synaptic blockade.

DISCUSSION

In agreement with several previous studies of the firing patterns of rat PR neurons (Faulkner and Brown, 1999; Beggs et al., 2000; McGann et al., 2001; Moyer et al., 2002; Moyer and Brown, 2007), persistent firing was never observed while the brain slices were being bathed in aCSF ($n = 99$). The dramatic new finding is that adding 10 μM carbachol to the bath qualitatively transformed most (85%) of the neurons by enabling persistent firing. Figures 1 - 6 give representative examples of persistent firing in these cells under various experimental conditions. In a second set of experiments, persistent firing was recorded in slices bathed in aCSF after complete blockade of fast synaptic transmission ($n = 23$). The results demonstrate that persistent firing can be generated-endogenously. Figure 7 shows a representative example of persistent firing during the complete blockade of fast synaptic

transmission. All of the recordings ($n = 122$) were from RS or LS pyramidal neurons with cell bodies in layer II/III of dorsal PR. This well-defined group furnishes a convenient and relatively homogeneous source of persistent-firing PR neurons for future cellular and molecular studies.

Some open questions include whether persistent firing occurs in ventral PR (Area 35), in other layers of dorsal or ventral PR, or in any of the numerous non-pyramidal cell types (Faulkner and Brown, 1999; Furtak et al., 2007a; Moyer and Brown, 2007). One might predict that persistent firing would not occur in single-spiking neurons, since they only fire once in response to a strong and prolonged depolarization. Similarly, persistent firing might not occur in the large, burst-firing pyramidal neurons in layer V, because bursting in these cells is normally accompanied by a large hyperpolarizing after-potential (Faulkner and Brown, 1999; Moyer and Brown, 2007). The small, fast-spiking PR neurons (Faulkner and Brown, 1999; Moyer and Brown, 2007), which are probably inhibitory local-circuit neurons, also may not exhibit persistent firing, which has thus far only been reported in large neurons. Persistent firing may be limited to the larger PR neurons, which are either pyramidal neurons or spiny stellates (Faulkner and Brown, 1999; Furtak et al., 2007a; Moyer and Brown, 2007).

Comparisons with Persistent-Firing Neurons in Entorhinal Cortex and Amygdala

In six respects, persistent firing in PR was qualitatively similar to persistent firing in EC (Egorov et al., 2002; Fransen et al., 2006; Tahvildari et al., 2007) and LA (Egorov et al., 2006). First, persistent firing in PR lasted tens of seconds to minutes (Figs. 1 – 7). Notably, these durations include the range of trace intervals that are commonly used in studies of trace fear conditioning (McEchron et al., 1998; Moyer and Brown, 2006; Kholodar-Smith et al., 2008b; Bang and Brown, 2009; Esclassan et al., 2009). Second, persistent firing was enabled by mAChR activation. More precisely, persistent firing was enabled by carbachol, a nonspecific cholinergic agonist, and it was reliably blocked by atropine and more selective muscarinic antagonists (Fig. 6). Third, persistent firing was reliably prevented by flufenamic acid (Fig. 6). Fourth, the discharge rate during persistent firing was relatively low (Figs. 1 – 7). Fifth, the persistent-firing rate was often a graded function of depolarizing and hyperpolarizing inputs to the cell (Figs. 2 – 4). Finally, persistent firing occurred after blockade of fast synaptic transmission (Fig. 7), demonstrating that it can be endogenously-generated. Five of these characteristics of persistent firing have also been reported in neurons from PoS (Yoshida and Hasselmo, 2009) and ACC (Zhang and Sequela, 2010). Still unknown is whether these neurons can exhibit graded persistent firing.

Quantitative Effects of Recording Methods on Persistent Firing Characteristics

Variations in recording methods may cause quantitative differences in persistent-firing characteristics. Persistent firing in LA and EC has usually been evaluated using sharp electrodes or perforated-patch recordings. The rationale was that the ruptured-patch technique, used in the present study, might cause “rundown” resulting from the washout of diffusible intracellular messengers. Indeed, a recent comparison between perforated-patch and ruptured-patch recordings in layer V of EC did report differences (Reboreda et al., 2007). Specifically, cells recorded in ruptured-patch configuration showed a lower probability of exhibiting graded persistent firing. In addition, the retention of graded activity greatly diminished with the passage of time.

The present study used ruptured patches to allow direct comparisons with hundreds of previously-recorded PR neurons. In spite of possible washout effects, the results demonstrated the existence and the prevalence of persistent-firing neurons in PR. The use of sharp electrodes or perforated-patch electrodes might have resulted in a larger proportion of

persistent-firing neurons in the targeted population, a higher rate or duration of persistent firing, and/or a greater range of graded-persistent firing rates.

A second methodological difference is that the recordings in other structures were done in slices maintained at a slightly-higher bath temperature (Egorov et al., 2002; Egorov et al., 2006; Tahvildari et al., 2007; Yoshida and Hasselmo, 2009), which could have resulted in a higher persistent-firing rate. Our concern was that the combination of higher temperatures and ruptured-patch WCRs would not be favorable for stable, long-term experiments.

Involvement of Perirhinal Cortex, Entorhinal Cortex, and Amygdala in Trace Fear Conditioning

Recent behavioral analyses have identified two important similarities between PR and EC function in terms of Pavlovian fear conditioning. First, lesions of both structures severely impair trace fear conditioning without affecting delay fear conditioning (Kholodar-Smith et al., 2008b; Esclassan et al., 2009). In the delay conditioning procedures, the CS and US overlapped temporally and co-terminated, thereby obviating the requirement for transient memory (see Bangasser et al., 2006). Second, injections of mAChR antagonists into both PR and EC impaired trace but not delay fear conditioning (Bang and Brown, 2009; Esclassan et al., 2009). Additionally, the PR infusion produced no detectable effect on context conditioning (Bang and Brown, 2009), in sharp contrast to the profound effect that PR damage has on context conditioning (Bucci et al., 2000; Lindquist et al., 2004; Kolodar-Smith et al., 2008a,b). The selectivity of the mAChR antagonist for trace conditioning shows that the infusion was not simply inactivating PR function.

Based on prevailing views (Maren and Quirk, 2004; Pare et al., 2004; Fanselow and Poulos, 2005; Kim and Jung, 2006; Phelps and LeDoux, 2005; Helmstetter et al., 2008; Bach et al., 2011), one might guess that the amygdala is critically-involved in all forms of Pavlovian fear conditioning. Surprisingly, however, almost nothing is known about the role of the amygdala in trace fear conditioning. One recent study (Raybuck and Lattal, 2011) concluded that the insertion a trace interval between the CS and the US causes cue conditioning to become amygdala-independent. More specifically, muscimol infusion into the amygdala of C57BL mice had no effect on trace conditioning to an auditory cue. However, as expected from previous studies, the infusion severely impaired delay conditioning to the same cue as well as conditioning to the training context. The authors report a double dissociation, as muscimol infusion into the dorsal hippocampus was found to impair trace but not delay conditioning. To the best of our knowledge, the only other experiment on the role of the amygdala in trace conditioning was one that was recently completed in our lab. This study found that scopolamine infusion into the rat amygdala selectively impairs trace fear conditioning without affecting delay conditioning or context conditioning (Kent et al., 2011). Additional research is clearly needed to resolve the role of the amygdala in trace conditioning. The role of the hippocampus in trace conditioning is further considered below.

Persistent Firing in Other Aspects or Mechanisms of Transient Memory

The concept that persistent firing in PR supports transient stimulus representations has implications that extend well beyond trace fear conditioning. Delayed object recognition tasks, which also require transient stimulus memory, are well-known to depend on PR function (Baxter and Murray, 2001; Parsana and Brown, 2010). Furthermore, extracellular recordings from rat PR have revealed single units that maintain stimulus-specific activity during the delay period of an odor-guided version of the delayed match-to-sample task (Young et al., 1997). Recently, mAChR-enabled persistent firing in PoS was proposed to support the head-direction system in the absence of key navigational stimuli (Yoshida and

Hasselmo, 2009). From another theoretical perspective (Mongillo et al., 2008), persistent firing might be imagined to help maintain transient synaptic plasticity in recurrent circuits.

Hypothesized Conductance Mechanisms underlying Persistent Firing

The original hypothesis for persistent firing in EC, LA, and PoS (Egorov et al. 2002; Fransen et al., 2006; Yoshida and Hasselmo, 2009) proposed that Ca^{2+} influx through voltage-gated Ca^{2+} channels activates two, opposing, sustained currents. The first is a depolarizing, Ca^{2+} -dependent, nonselective cation current (I_{CAN}). The second is a hyperpolarizing, Ca^{2+} -dependent K^+ current ($\text{IK}(\text{Ca})$). Accordingly, the occurrence of persistent firing depends on the timing and the absolute and relative sizes of I_{CAN} and $\text{IK}(\text{Ca})$. Theoretically, a change in the magnitude of either of these currents could enable persistent firing. The graded nature of persistent firing was previously theorized to emerge from the dynamics of Ca^{2+} -dependent biochemical mechanisms that regulate I_{CAN} channel function (Fransen et al., 2006).

Preliminary evidence suggests that I_{CAN} may be based on transient receptor potential (TRP) channels, in particular the TRPC subfamily (Yoshida et al., 2008; Blair et al., 2009; Yan et al., 2009; Zhang et al., 2011; Reboresda et al., 2011). Importantly, some TRPC channels are gated by the combination of elevated $[\text{Ca}^{2+}]_i$ and agonist binding to extracellular receptors, including mAChRs (Ramsey et al., 2006; Venkatachalam and Montell, 2007; Blair et al., 2009; Reboresda et al., 2011). Recent evidence shows that, in layer III of EC, activation of group I metabotropic glutamate receptors (mGluRs) can also enable persistent firing, an effect that has also been attributed to TRPC channels (Yoshida et al., 2008). Interestingly, mGluR-enabled persistent firing does not depend on mAChR activation. Nothing is known about the distribution of TRPC channels and mAChRs in PR. An obvious next step, for understanding the conductance mechanisms in PR neurons, will be to determine whether or how persistent firing in PR depends on Ca^{2+} entry, elevated $[\text{Ca}^{2+}]_i$, and an increase in an inward current. Alternatively, mAChR-enabled persistent firing in PR could be supported by a decrease in a Ca^{2+} -dependent K^+ current, although this has not been reported in the other brain structures reviewed here.

Memory Buffer System and its Remote Control

The fact that PR is reciprocally and strongly connected to LA and EC raises the possibility that persistent-firing neurons in combinations of these three structures may cooperate in the support of a regional memory buffer system. The previously-described neuropharmacological experiments on trace conditioning certainly support this possibility. All three of these structures also have direct and indirect reciprocal connections with the hippocampus (HC), which presumably furnishes an important part of the input to this memory buffer (Eichenbaum et al., 1994; McEchron, et al., 1998; Quinn, et al., 2002; Chowdhury et al., 2005; Bangasser et al. 2006; Yoon and Otto, 2007; Woodruff-Pak and Disterhoft, 2008). In rats, the ventral HC is especially important for trace fear conditioning (Yoon and Otto, 2007; Czerniawski et al., 2009).

The role of the hippocampus in trace conditioning depends on both the nature of the conditional response and the trace interval. Trace fear conditioning in mice has been reported to become hippocampus-independent when the trace interval is reduced to less than 10 s (Misane et al., 2005). This finding seems puzzling because the hippocampus is well-known to be essential for trace eyeblink conditioning (Solomon et al., 1986; Moyer et al., 1990; Woodruff-Pak and Disterhoft, 2008), where the trace intervals are always of the order of tenths of a second. The reason that such short intervals have been explored is that conditioning simply does not occur with longer intervals. Whether trace fear and eyeblink conditioning engage the same temporal bridging mechanisms is unknown. Conceivably,

persistent firing might not be required for bridging very short intervals, where some other mechanism might be sufficient. At the moment, there is no information as to whether PR lesions impair trace eyeblink conditioning.

Since persistent firing is enabled by mAChR activation, an obvious next question concerns the neurophysiological control of acetylcholine release. PR, EC, and LA each receive cholinergic input from neurons in the basal forebrain (Alonso and Amaral, 1995; Vaucher and Hamel, 1995; Carlsen et al., 2004; Mesulam, 2004; Winters and Bussey, 2005; Egorov et al., 2006). Nothing is known about the ultimate neurophysiological control of firing in these cholinergic neurons, which receive both cortical and subcortical inputs. The present line of reasoning predicts that cholinergic neurons should become active in tasks that require transient memory. The initial trigger for ACh release could be stimulus novelty or a more complex executive function of prefrontal cortex.

General Conclusions and Implications

The wide-spread occurrence of mAChR-enabled persistent firing invites a major reexamination of the neurophysiological foundations of transient memory. Persistent firing is a viable, non-synaptic, mechanism for supporting aspects of transient memory. Notably, persistent firing is just one of several types of activity-dependent, non-synaptic modifications that have been proposed to maintain short-term changes in cellular and circuit function (Magee and Johnston, 2005; Bucher et al., 2006; Johnston and Narayanan, 2008; Mozzachiodi and Byrne, 2010). Rapidly-accumulating evidence suggests that non-synaptic modifications may play a much larger role in transient memory than previously imagined.

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Figure 1.

Induction and termination of persistent firing. Top: the upper and lower items are voltage responses and current steps, respectively. The depolarizing current step was supra-threshold for directly eliciting repetitive firing and for inducing persistent firing. Persistent firing was terminated after a minute by a hyperpolarizing current step. Bottom: the histogram shows the number of spikes in successive 1-s time bins during and after the current step. In this figure and all others, the displayed record of persistent firing includes all of the digitally-recorded data.

Figure 2.

Graded nature of persistent firing. The left-hand side of the traces shows persistent firing rates produced in the same neuron by current steps with three different amplitudes (A-C). Depending on the rate of persistent firing, a subsequent hyperpolarizing current step either terminated firing (A) or reduced the rate of persistent firing (B and C). In every case, firing terminated during the hyperpolarization. The histogram shows the number of spikes in successive 1-s time bins.

Figure 3.

Graded-persistent firing and temporal integration. The amplitude of the first 2- s current step was adjusted to be supra-threshold for firing the cell, but sub-threshold for evoking persistent firing. Note that it generated a depolarizing after potential (DAP). The second application of the same current step induced persistent firing. The third current step increased the rate of persistent firing, while the fourth step produced no further effect. The time histogram shows the number of spikes in successive 1-s time bins.

Figure 4.

Persistent firing induced by synaptic stimulation. The top trace shows the onset of persistent firing and the bottom trace illustrates the first 70 s, during which data were stored. The histogram gives the number of spikes in successive 1-s time bins. Persistent firing was completely blocked during the prolonged hyperpolarizing current injection. Repetitive firing resumed after the offset of the hyperpolarizing current step, but a lower rate than before the hyperpolarization.

Figure 5.

Persistent firing in the presence of noise. The amplitude of the added noise, measured by its standard deviation (SD), was 0 pA (left column), 20 pA (middle column), or 50 pA (right column). Noise was filtered at 1 – 20 Hz (A), 1 – 50 kHz (B) or 1 – 500 Hz (C). None of the noise conditions blocked persistent firing.

Figure 6.

Neuropharmacology of persistent firing. A. Atropine (1 μ M), a general mAChR antagonist, blocked the DAP and persistent firing. B and C. Pirenzepine (0.3 μ M), an M1 receptor antagonist, and 4-DAMP (1 μ M), an M3 receptor antagonist, also blocked the DAP and persistent firing. In the illustrated cells and in all others, M1 and M3 receptor antagonists acted like atropine. D. AFDX-116 (10 μ M), an M2 receptor antagonist, blocked persistent firing, but a DAP remained. E. PD102807 (0.25 μ M), an M4 receptor antagonist, allowed brief persistent firing in the illustrated cell but not in others. F. Flufenamic acid (100 μ M) blocked persistent firing, leaving a DAP.

Figure 7.

Endogenous persistent firing. A. Fast synaptic transmission was reversibly-blocked by two glutamatergic receptor antagonists (50 μ M APV and 10 μ M DNQX) and a GABAergic receptor antagonist (100 μ M picrotoxin). The illustrated traces are averages of 5 successive evoked excitatory postsynaptic potentials (EPSPs). The top trace is the evoked PSP before applying the receptor antagonists. The middle trace shows the complete EPSP block during the drug application. The bottom trace shows the EPSP recovery after the antagonists were washed from the bath. B. Persistent firing elicited by a depolarizing current step during the period in which the EPSP was blocked.