Intrinsic Ca\(^{2+}\)-dependent theta oscillations in apical dendrites of hippocampal CA1 pyramidal cells in vitro

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Hansen AK, Nedergaard S, Andreasen M. Intrinsic Ca\(^{2+}\)-dependent theta oscillations in apical dendrites of hippocampal CA1 pyramidal cells in vitro. *J Neurophysiol* 112: 631–643, 2014. First published May 14, 2014; doi:10.1152/jn.00753.2013.—Behavior-associated theta-frequency oscillation in the hippocampal network involves a patterned activation of place cells in the CA1, which can be accounted for by a somatic-dendritic interference model predicting the existence of an intrinsic dendritic oscillator. Here we describe an intrinsic oscillatory mechanism in apical dendrites of in vitro CA1 pyramidal cells, which is induced by suprathreshold depolarization and consists of rhythmic firing of slow spikes in the theta-frequency band. The incidence of slow spiking (29%) increased to 78% and 100% in the presence of the β-adrenergic agonist isoproterenol (2 μM) or 4-aminoypyridine (2 mM), respectively. Prior depolarization facilitated the induction of slow spiking. Applied electrical field polarization revealed a distal dendritic origin of slow spikes. The oscillations were largely insensitive to tetrodotoxin, but blocked by nimodipine (10 μM), suggesting the contribution from voltage-dependent K\(^{+}\) channels. Antagonists of T-, R-, N-, and P/Q-type Ca\(^{2+}\) channels had no detectable effect. The slow spike dimension and properties capable of generating rhythmic voltage fluctuations in the theta-frequency band.

**Abstract**

Intrinsic Ca\(^{2+}\)-dependent theta oscillations in apical dendrites of hippocampal CA1 pyramidal cells in vitro have been shown to be critical for the generation of theta oscillations in the hippocampus. These oscillations are thought to be involved in the encoding of spatial information. In this study, the authors investigate the role of Ca\(^{2+}\) channels in these oscillations. They find that the oscillations are largely insensitive to tetrodotoxin, but blocked by nimodipine. This suggests that the oscillations are dependent on voltage-dependent K\(^{+}\) channels. The authors also report that prior depolarization facilitates the induction of slow spiking. Applied electrical field polarization revealed a distal dendritic origin of slow spikes. The oscillations were largely insensitive to tetrodotoxin, but blocked by nimodipine, suggesting the contribution from voltage-dependent K\(^{+}\) channels. Antagonists of T-, R-, N-, and P/Q-type Ca\(^{2+}\) channels had no detectable effect. The slow spike dimension and properties capable of generating rhythmic voltage fluctuations in the theta-frequency band.

**Intracellular oscillations**

Intrinsic Ca\(^{2+}\)-dependent theta oscillations in apical dendrites of hippocampal CA1 pyramidal cells in vitro have been shown to be critical for the generation of theta oscillations in the hippocampus. These oscillations are thought to be involved in the encoding of spatial information. In this study, the authors investigate the role of Ca\(^{2+}\) channels in these oscillations. They find that the oscillations are largely insensitive to tetrodotoxin, but blocked by nimodipine. This suggests that the oscillations are dependent on voltage-dependent K\(^{+}\) channels. The authors also report that prior depolarization facilitates the induction of slow spiking. Applied electrical field polarization revealed a distal dendritic origin of slow spikes. The oscillations were largely insensitive to tetrodotoxin, but blocked by nimodipine, suggesting the contribution from voltage-dependent K\(^{+}\) channels. Antagonists of T-, R-, N-, and P/Q-type Ca\(^{2+}\) channels had no detectable effect. The slow spike dimension and properties capable of generating rhythmic voltage fluctuations in the theta-frequency band.

**Conclusion**

The results of this study suggest that Ca\(^{2+}\) channels play a critical role in the generation of theta oscillations in the hippocampus. The authors propose a model where a somatic-dendritic interference mechanism is responsible for the induction of slow spiking. This model predicts that the oscillations are dependent on activation of voltage-dependent K\(^{+}\) channels. The authors also report that prior depolarization facilitates the induction of slow spiking. Applied electrical field polarization revealed a distal dendritic origin of slow spikes. The oscillations were largely insensitive to tetrodotoxin, but blocked by nimodipine, suggesting the contribution from voltage-dependent K\(^{+}\) channels. Antagonists of T-, R-, N-, and P/Q-type Ca\(^{2+}\) channels had no detectable effect. The slow spike dimension and properties capable of generating rhythmic voltage fluctuations in the theta-frequency band.

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**References**

dendritic interference model has also gained support from recent in vitro dual somato-dendritic recordings (Losonczy et al. 2010). The precise cellular and synaptic mechanisms underlying the intracellular dynamics observed in place cells are still uncertain (Harvey et al. 2009). One important question is whether dendritic membrane currents are involved and, if so, to what extent these are dynamically modulated by synaptic inputs. The dendritic membrane is endowed with a large number of active conductances (for review, see Johnston and Narayan 2008), some of which form the basis for oscillating and/or resonating properties (Hu et al. 2009; Kamondi et al. 1998; Narayan and Johnston 2007), and it has been predicted that an intrinsic dendritic oscillator is necessary to allow full 360° phase precession in place cells (Lengyel et al. 2003). The fact, that place cells in the dorsal hippocampus have a somewhat higher firing frequency compared with place cells in the ventral hippocampus, despite no difference in the local theta field oscillation (Maurer et al. 2005), has been taken as indirect evidence for the presence of an intrinsic dendritic oscillator (O’Keefe and Burgess 2005). The nature of this intrinsic dendritic oscillator has yet to be uncovered (for review, see Buzsáki 2002), but it seems likely to involve high-threshold Ca²⁺ currents (Kamondi et al. 1998). In the present study, we have used intradendritic recordings from hippocampal brain slices with the aim of verifying the presence of an intrinsic dendritic oscillator in CA1 pyramidal cells, and investigate its ionic mechanism and functional properties.

**MATERIALS AND METHODS**

Animal care and housing facilities, as well as the experimental protocol for the euthanasia of animals, were in accordance with Danish and European law and approved by the Animal Experimentation Board under the Danish Ministry of Justice. Experiments were performed on hippocampal slices prepared from male Wistar rats (4–5 wk old). The rats were anesthetized with isoflurane and decapitated, after which the brain was removed and quickly placed in a dissection medium (see below) at 4°C. The hippocampus was dissected free, and 400–μm slices were cut on a McIlwain tissue chopper. One slice was immediately transferred to the recording chamber, where it was placed on a nylon-mesh grid at the interface between warm (31–33°C) standard perfusion medium (see below) and warm humidified carbogen (95% O₂, 5% CO₂). Perfusion flow rate was 1 ml/min. The slice was allowed to rest for at least 1 h before recording. The remaining slices were placed in a storage container at room temperature.

Recordings were obtained using sharp borosilicate glass microelectrodes (1.2 mm outer diameter, Harvard Apparatus, Edenbridge, UK). For intracellular recordings, the electrode was filled with a solution of 3 M KCl and 0.1 M K⁺-acetate (tip resistance: 40–70 MΩ). For extracellular recordings, the electrode was filled with 1 M NaCl (tip resistance: 10–20 MΩ). A bipolar Teflon-insulated platinum electrode (tip diameter 50 μm, interpert distance: 25 μm) placed in stratum (str) radiatum at the border between CA3 and CA1 was used for orthodromic stimulation of the Schaffer collateral-commissural fibers. Conventional recording techniques were employed using a high-input impedance amplifier (Axoclamp 2A or MultiClamp 700B, both Molecular Devices) with bridge balance and current injection facilities. Signals were digitized on-line via a Digidata 1440A converter and transferred to a computer employing pCLAMP (version 10.0, Molecular Devices) acquisition software for off-line analysis. Signal analysis was performed using pCLAMP or Spike2 software (Cambridge Electronic Design).

To ensure that recordings were from the distal half of the apical dendrites, penetrations were made near the border between str. radiatum and str. lacunosum-moleculare. The distance from the recording site to the superficial border of str. pyramidalae was estimated using an eyepiece micrometer. A dendritic penetration was identified based on the characteristic response to current injections (Andreasen and Lambert 1995). Inclusion criteria were a stable resting membrane potential (RMP) less than or equal to −50 mV and a membrane input resistance (Rᵢ) ≥ 10 MΩ.

**Stimulation protocols.** Once an intradendritic recording was established, a series of stimulation protocols were employed to determine the current-voltage (I/V) relationship of the membrane, and the threshold and frequency of spike activity. Oscillatory behavior was evoked using a protocol consisting of a series of 4-s-long depolarizing current pulses of increasing intensity (steps: 0.05–0.2 nA) with an interpulse interval of 1–3 s. Each pulse was preceded by a brief hyperpolarizing current pulse (−0.3 nA, 50 ms), to ensure correct bridge balance. This protocol is referred to as the standard pulse (SP) test. In some experiments, the SP test protocol was combined with a fixed 6-Hz sine-wave current injection (0.2 nA) applied for the duration of the square pulse. This protocol will be referred to as the SPₛᵣₑ test.

For synaptic stimulation, we used short trains of four stimuli at 100 Hz with a train frequency of 5 Hz. When train stimulation was combined with the SP test, the duration of the current pulse was increased to 5 s. This protocol will be referred to as the SPₛᵣₑ test.

**Extracellular electric field stimulation.** To enable differential polarization of the CA1 pyramidal cells, we used electrical field application (for details, see Andreasen and Nedergaard 1996). In brief, constant current (intensity: ±0.5–2.5 mA; duration: 2 s) was passed between a pair of platinum electrodes, positioned on either side of the slice and connected to a constant-current source (Isolator-11, Axon Instruments). The slice was oriented so that the current flow was perpendicular to str. pyramidalae to ensure maximal transmembrane polarization of the CA1 pyramidal cells (Tranchina and Nicholson 1986). Parallel to the current flow, the extracellular voltage gradient across the CA1 region was close to being linear (measured at 200-μm intervals at 150-μm depth) and was, on average, 62 ± 3 mV-mm⁻¹-m⁻³ (n = 5 slices). To measure the transmembrane potential, the tip of an extracellular reference electrode was placed as close as possible to the tip of the intracellular recording electrode. The transmembrane potential was obtained by subtracting the potentials measured by the two electrodes using a custom-built differential amplifier with a gain balance. Before each penetration, we confirmed that the recorded voltage deflection imposed by a test field application was similar from the two electrodes, and any (small) deviation from zero in the differential output signal was compensated by adjusting the gain.

**Analysis.** The I/V-relationship in the region of the RMP was evaluated by plotting the changes in membrane potential (Vₑ), measured at the end of the current pulses, as a function of current intensity. Rᵢ was then calculated as the slope of the best linear fit. The slow spike threshold was taken as the Vₑ immediately before the occurrence of the first slow spike during the SP test protocol. Due to the occurrence of repetitive fast spiking, the Vₑ was measured in the interval between the fast spikes.

The amplitude of Vₑ oscillations induced by the SPₛᵣₑ test was evaluated using a sine curve fitting routine after low-pass filtering with a cutoff frequency of 25 Hz to remove fast spikes and noise. The first response induced by each current pulse was discarded as it often had a shape reminiscent of a compound spike (Andreasen and Lambert 1995). Unlike subsequent events, and thus may represent a different mechanism than regular oscillatory spikes. To permit intercellular comparison, the amplitude of the membrane oscillations at any given Vₑ was normalized with respect to the amplitude of oscillations evoked at RMP.

Responses obtained with the SPₛᵣₑ test was evaluated after low-pass filtering, similar to that used for membrane oscillations. For each 100-Hz train stimulation, the area of the resulting postsynaptic potential was measured from the time of the first stimulus to the time where...
the voltage had returned to (or was closest to) the pretrain baseline $V_m$. For each depolarizing step, the area of the postsynaptic responses and the pretrain baseline potentials were averaged. The averaged areas were then normalized with respect to the area measured at $V_m$ and the pretrain baseline potentials were averaged. The averaged areas bubbled with carbogen; pH 7.3; 0.1 mM-L-ascorbic acid was added to MgSO$_4$, 2; bubbled with carbogen. The composition of the standard perfusion medium was as follows (in mM): NaCl, 120; KCl, 2; KH$_2$PO$_4$, 1.25; HEPES acid, 6.6; NaHEPES, 2.6; NaHCO$_3$, 20; d-glucose, 10; CaCl$_2$, 2; MgSO$_4$, 2; bubbled with carbogen. The composition of the standard perfusion medium was as follows (in mM): NaCl, 124; KCl, 3.25; NaH$_2$PO$_4$, 1.25; NaHCO$_3$, 20; CaCl$_2$, 2; MgSO$_4$, 2; d-glucose, 10; bubbled with carbogen; pH 7.3; 0.1 mM-L-ascorbic acid was added to prevent oxidation of isoproterenol. In control experiments L-ascorbic acid was found to have no effect on dendritic properties ($n = 9$). In experiments where CdCl$_2$ was added, sulfate and phosphate were substituted with Cl$^-$ to avoid precipitation.

Most pharmacological compounds were made up in aqueous stock solutions of 100–1,000 times the required final concentration and diluted in the perfusion medium as appropriate. Nimodipine and salbutamol, $\omega$-agonist-IVA, 4-aminopyridine (4-AP), linopirdine, nimodipine, isoproterenol, carbacholine chloride, tetraethylammonium chloride (TEA), NiCl$_2$, CdCl$_2$, $\alpha$-dendrotoxin, l-ascorbic acid and bicuculline methobromide were purchased from Sigma. ZD7288 was purchased from Tocris.

RESULTS

A total of 181 intradendritic recordings were obtained with an average distance of 233 ± 3 μm from the superficial border of str. pyramidale. The averaged RMP and $R_m$ were $-61.1 \pm 0.3$ mV ($n = 171$) and $21.9 \pm 0.4$ MΩ ($n = 171$), respectively.

Characterization of dendritic slow spikes. Intradendritic injection of incrementing suprathreshold current pulses (SP test) induced repetitive fast spiking at increasing frequencies (Fig. 1A). In 29% (12/42) of dendrites, high-intensity current injections, in addition, repetitive firing of slow spikes distinguished by a prolonged rise time and a marked after-hyperpolarization (exemplified by arrows in Fig. 1B). Dendrites were

![Fig. 1. Depolarization-induced rhythmic slow spiking in apical dendrites. A: top: standard current injection protocol [standard pulse (SP) test]. Each depolarizing current pulse is preceded by a short (50 ms) hyperpolarizing current pulse (−0.3 nA). Middle: intradendritic response to SP test stimulation in standard perfusion medium. Bottom: intradendritic responses from another dendrite in the presence of isoproterenol (2 μM). Dotted lines in this and the following figures mark the baseline potential indicated to the right. B: responses marked by * and ** in A shown on an expanded time scale. Note the difference in rhythmic firing of slow spikes (indicated by arrow). C: the incidence of dendrites displaying slow spiking in response to the SP test stimulation in control conditions ($n = 42$), in the presence of isoproterenol (2–4 μM, $n = 104$) and in the presence of 4-aminopyridine (4-AP) (2 mM, $n = 10$).](http://jn.physiology.org/doi/10.1152/jn.00753.2013)
categorized as being slow spiking only if slow spikes appeared during the first run of the SP test. We found that application of the adrenergic β-receptor agonist isoproterenol (2 μM) enhanced the occurrence of slow spikes (Fig. 1B) and increased the incidence of slow spiking dendrites to 78% (81/104; Fig. 1C), which was significantly higher than in control conditions (P < 0.01, χ² test). In seven recordings exhibiting slow spiking both before and after isoproterenol (2–4 μM), we found no significant difference in spiking frequency (control: 5.2 ± 0.5 Hz, isoproterenol: 4.8 ± 0.7 Hz; P = 0.4). However, the mean amplitude of the spikes increased in the presence of isoproterenol (control: 14.8 ± 3.1 mV, isoproterenol: 22.1 ± 3.4 mV, P < 0.05). The selective adrenergic β₁-receptor agonist salbutamol (2–10 μM, n = 2) did not change the slow spiking properties of the apical dendrites, suggesting that the effect of isoproterenol depends on activation of β₁-receptors. In the presence of carbacholine (1–2 μM), unstable slow spiking was observed in only two out of five recordings, indicating that muscarinic receptors are less efficient in promoting slow spiking than adrenergic β₁-receptors.

As exemplified in Figs 1B and 4A, the frequency of the slow spikes stabilized during the course of the depolarizing pulse, and the latency from pulse onset to development of regular slow spiking decreased with increasing depolarization. The outline of the slow spikes was quite uniform, consisting of a slow ramplike depolarization, during which high-frequency fast spikes were usually present, followed by a fast repolarization which continued into a distinct after-hyperpolarization. Once a regular rhythm was established, there was a smooth transition between the rising phase of the slow spike and the after-hyperpolarization of the preceding spike, resulting in a sinusoidal-like oscillation of the Vₘ (Figs. 1B and 4A). A single compound spike (Andreasen and Lambert 1995) was often observed to precede the initiation of slow spikes. Compound spikes were not classified as slow spikes because they never occurred repetitively, and their composition deviated from that of the slow spikes, suggesting a different induction mechanism. Compound spikes are variable, complex-shaped Ca²⁺-dependent spikes, which are triggered during the repolarization phase of a fast Na⁺-dependent spike (Andreasen and Lambert 1995; Golding et al. 1999), thus explaining why compound spikes typically appeared at more negative Vₘ than the slow spikes. In contrast, the slow spikes were simple and uniform in shape, and they showed no temporal correlation to Na⁺-dependent spikes. Furthermore, the occurrence of compound spikes was not predictive for the ability to induce slow spiking, a notion which further supports that these two types of responses are distinct.

In 34.1% of dendrites in which the initial SP test was inefficacious, repeating the SP test resulted in slow spiking (Fig. 2). As such dendrites were categorized as non-slowspiking, by our criteria, this finding show that the true proportion of slow-spiking dendrites is larger than our estimate. Furthermore, in a high proportion (55.6%) of the dendrites in which slow spiking was induced by the initial SP test, repeating the SP test lowered the threshold for slow spiking.

Because of this dynamic feature, we measured the threshold for slow spiking in a standardized fashion by taking the Vₘ just before the first slow spike evoked during the initial SP test, thereby avoiding any influence from preconditioning. Measured in this way, the averaged threshold in the presence of isoproterenol was ~34.6 ± 0.7 mV (n = 81, Fig. 3A). This value was not significantly different from that measured in the absence of isoproterenol (-34.5 ± 2.7 mV, n = 12, P = 0.93, one-way ANOVA). Hence, the increased incidence of slow spiking observed in isoproterenol was not related to a decrease in threshold, nor could it be related to a change in the dendritic RMP (control: -61.2 ± 0.7 mV, isoproterenol: -61.0 ± 0.4 mV, P = 0.75, one-way ANOVA) or Rᵢ (control: 21.1 ± 0.7 MΩ, isoproterenol: 22.0 ± 0.6 MΩ, P = 0.37, one-way ANOVA). However, we observed that isoproterenol altered the I/V relationship at depolarized voltages. In control conditions, the membrane exhibited outward rectification close to and below the threshold for slow spiking. In isoproterenol, the rectification was greatly diminished, resulting in significantly larger depolarizations in response to currents ≥ 1 nA, thus allowing the Vₘ to cross the threshold (Fig. 3B, P < 0.05, two-way ANOVA). In the absence of isoproterenol, approximately twice the amount of current was needed to reach the threshold.
Significant deviation from control (slow spikes. Note that 4-AP has increased the slope of the average depolarization required to reach threshold for induction of rhythmic 26), in isoproterenol (above mV/mV, with depolarization in an almost linear fashion (slope: 1.38 R noise level, reliable spike identification failed at Vm values in the presence of isoproterenol (Fig. 4). The mean threshold (mV) is indicated by the horizontal bars. 10). The mean threshold (SE) is indicated by the horizontal bars. 10). Dashed line marks the average depolarization required to reach threshold for induction of rhythmic slow spikes. Note that 4-AP has increased the slope of the I/V curve, and that both 4-AP and isoproterenol block the outward rectification of the membrane. *Significant deviation from control (P < 0.05; two-way ANOVA).

average threshold, thus reducing the probability of slow spike induction.

To investigate the voltage dependency of the dendritic slow spikes, we used a pattern recognition template (pCLAMP) to automatically identify slow spikes induced by the SP test. After visual confirmation of the reliability of the recognition template, the corresponding values of amplitude, frequency, and Vm was obtained for multiple events in 16 dendrites recorded in the presence of isoproterenol (Fig. 4A). Due to the increased noise level, reliable spike identification failed at Vm values above −27 mV. The amplitude of the slow spikes decreased with depolarization in an almost linear fashion (slope: 1.38 mV/mV, n = 2,048 spikes, R² = 0.9714, Fig. 4B). The frequency of slow spikes, measured toward the end of each current pulse, and taken as an average from all Vm values, was 6.22 ± 2.1 Hz (mean ± SD, n = 1,880). Although there was a tendency for an increase in mean frequency at potentials positive to −45 mV (Fig. 4C), linear regression analysis showed no significant correlation between Vm and frequency (slope: 0.125 Hz/mV, R² = 0.0596). An interesting notion from Fig. 4C is the apparent increase in frequency at potentials below −45 mV (6.97 ± 1.3 Hz, n = 37). The reason for this jump is not clear, but it should be pointed out that, at these potentials, slow spikes are primarily evoked following multiple pulse injections, and the abrupt increase in frequency could perhaps reflect a facilitatory effect of repetitive depolarization (see above).

As fast spiking was typically absent during the repolarization of the slow spikes, a general consequence of their fusion into a rhythmic oscillation was that fast spiking changed from regular into a pattern of short bursts occurring at theta-frequency (Fig. 4A).

Slow dendritic spikes involves activation of L-type Ca²⁺-channels (L-VDCC). In the following experiments, we investigated the pharmacology of the slow spikes. Isoproterenol (2 μM) was routinely added to the perfusion medium to ensure a high incidence of events. First, the effect of blocking voltage-gated Na⁺ channels with TTX (1 μM) was examined. In 56% (5/9) of recordings, regular slow-spike activity was induced following the introduction of TTX (Fig. 5). In one recording, the firing rhythm became irregular, and, in the remaining 33% (3/9), slow spiking was absent. With TTX present, slow spikes had broader base caused by prolonged rise and decay times and thus a slower frequency (Fig. 5).

We next investigated the role of voltage-gated Ca²⁺-channels (VDCC). The L-VDCC antagonist nimodipine (10 μM), applied during the recording, completely blocked slow spiking in all four cells tested (Fig. 5A). In another 14 experiments, the slices were preincubated with nimodipine (10 μM) for at least 30 min before the recording. In these circumstances, slow spikes could not be evoked in 93% (13/14) of recordings. In one recording, randomly occurring atypical slow spikes was observed (not shown). We also investigated the effect of drop application (see MATERIALS AND METHODS) of the P/Q-VDCC antagonist ω-agatoxin-IVA (2 μM, n = 7) or the N-VDCC antagonist ω-conotoxin-GVIA (40 μM, n = 7). Although these toxins reduced synaptic excitation postynaptic potentials by 56 ± 18.4% (ω-agatoxin-IVA, n = 3) and 75.3 ± 4.6% (ω-conotoxin-GVIA, n = 3, not shown), they had no significant effect on the slow spikes. We did observe that ω-conotoxin-GVIA reduced the mean amplitude of the slow spikes by 26.9 ± 10.5%, but this was not significant (P = 0.08). No effect was obtained with the T/R-VDCC antagonist Ni²⁺ (100 μM, n = 4) or the selective R-VDCC antagonist SNX-482 (2 μM, n = 3).

Rhythmic slow spiking involves activation of potassium channels. The dendritic membrane expresses several K⁺ channels (Chen and Johnston 2004), some of which are known to be involved in controlling the initiation and repolarization of dendritic Ca²⁺ spikes (Golding et al. 1999; Hoffman et al. 1997). In particular, the transient outward A-type K⁺ current (Iₜₒ) is important for controlling the activation of dendritic VDCC (Frick et al. 2003; Hoffman et al. 1997; Magee and Carruth 1999). We, therefore, investigated the effect of the Iₜₒ blocker 4-AP (2 mM). Similar to isoproterenol, 4-AP increased the incidence of slow spiking (100%, n = 10/10, Fig. 1C) without any significant change in the threshold (−38.4 ± 2.2 mV, P = 0.29, one-way ANOVA, Fig. 3A). Furthermore, 4-AP increased the general slope of the I/V curve and also diminished the outward rectification at depolarized potentials, resulting in larger depolarizations in response to current injections (Fig. 3B).
We next investigated the possible influence of outward currents on the shape of the slow spikes. At low concentrations (100 μM), 4-AP increased the amplitude of the slow spikes (Fig. 5B). In six experiments, the average increase was 47.4% (control: 19.4 ± 3.6 mV, 4-AP: 28.6 ± 5.4 mV, Fig. 5C), which was significant (P < 0.01). In addition, there was a 13% reduction in frequency (control: 5.3 ± 0.6 Hz, 4-AP: 4.6 ± 0.6 Hz, Fig. 5C), which was borderline significant (P < 0.05).

Increasing the concentration to 2 mM gave an additional increase in amplitude (4-AP: 33.1 ± 6.2 mV) and a further reduction in frequency (4-AP: 3.3 ± 0.5 Hz); however, neither values was significantly different from what was obtained by the low concentration (amplitude: P > 0.20, frequency: P = 0.09, Fig. 5C). The nonselective K⁺-channel blocker TEA (10 mM) also increased the amplitude of the slow spikes with a concomitant reduction in frequency (Fig. 5B). On average, the amplitude was increased by 54.7% (control: 24.7 ± 1.9 mV, TEA: 38.1 ± 3.9 mV, n = 6), and the frequency was reduced by 38.2% (control: 5.5 ± 0.3 Hz, TEA: 3.4 ± 0.4 Hz, Fig. 5C), both of which were significant (P < 0.01). In addition, TEA gave a very pronounced broadening of the spikes, associated with a slowing of their repolarization phase. In some instances, repolarization was observed to occur in a stepwise manner with TEA present (Fig. 5B).

We attempted to clarify which specific types of K⁺ channels contributed to the above effects of 4-AP and TEA by employing more selective antagonists. The following selective K⁺ channels antagonists (see DISCUSSION), α-dendrotoxin (1–10 μM, n = 10), stromatoxin (1–2 μM, n = 5), and iberiotoxin (0.1–0.2 μM, n = 5), were all without effect on slow spiking (not shown). As TEA have been reported to block the M-type K⁺ channels (IM) (Koyama and Appel 2006), we also investigated the effect of the selective M-current antagonist linopirdine. However, even at high concentrations (30 μM, n = 6), linopirdine had no effect on either the ability to induce the slow spikes, or on their properties (not shown). As L-VDCC often are colocalized with small-conductance Ca²⁺-activated K⁺ (SK) channels (Bowden et al. 2001), we also tested the selective SK-channel antagonist, apamin. Apamin (0.5 μM, n = 2) was, however, inefficacious (not shown).

The hyperpolarization-activated nonselective cation (h) channel, which is found at highest density in the distal apical dendrites (Magee 1998), has previously been implicated in the regulation of dendritic oscillatory behavior in the theta frequency band (Hu et al. 2002, 2009; Ulrich 2002). In the present study, the selective Ih antagonist ZD7288 (20 μM) did cause a slight, but significant, decrease in the frequency of slow spik-
Rhythmic slow spiking can arise in the dendritic regions. In an attempt to locate the subcellular origin of the intrinsic rhythmic slow spiking, we applied an electric field across the CA1 region parallel to the somato-dendritic axis of the pyramidal cells. This procedure induces a differential polarization of the transmembrane potential, such that the distal apical dendrites are polarized in one direction, and the remaining parts of the cells are polarized in the opposite direction (Andreasen and Nedergaard 1996). In three out of four experiments, we found that F− (soma-hyperpolarizing field), applied...
during a subthreshold current injection, immediately provoked rhythmic firing of slow spikes, whereas F+ did not (Fig. 6). These effects were obtained using 0.5–2.5 mA applied current, which corresponds to a voltage gradient in the tissue of 31–155 mV/mm. This is comparable to the F− intensity required for generating dendritic Na+ and Ca2+-dependent spikes from resting potential (60–375 mV/mm; Andreasen and Nedergaard 1996). In the remaining cell tested, electrical field stimulation of both polarities induced slow spiking (not shown). Fast spiking was generally blocked during F−, except for occasional spikes evoked at the peaks of the slow spikes. In contrast, during F+, the fast spikes were still present but had reduced amplitudes. Both findings are consistent with a somatic origin of these fast spikes.

**Dendritic amplification of oscillatory inputs.** Evidence suggests that L-VDCC are well suited to act as amplifying current for oscillatory Vm changes (Hutcheon and Yarom 2000). We investigated whether such role of the L-VDCC could be of functional significance in the apical dendrites. To emulate dendritic oscillatory activity observed during in vivo field theta-activity (Kamondi et al. 1998), we used a 6-Hz sine-wave paradigm in combination with depolarizing current pulses of increasing intensity (SPsin test, Fig. 7A). The experiments were performed in the presence of isoproterenol (2 μM) and TTX (1 μM), to ensure sufficient depolarization of the dendritic membrane and to avoid contamination by fast spiking, respectively. Control experiments showed that the amplitude of the dendritic voltage oscillations imposed by the sine-wave pulse increased in the potential range from −50 to −25 mV, with a peak amplitude obtained at an average Vm of −29.4 ± 1.3 mV (Fig. 7, A and B, n = 6). The maximal voltage-dependent amplification normalized with respect to the amplitude at RMP was, on average, 3.7 ± 0.3. Nonselective blockade of VDCC by adding 0.5 mM Cd2+ and 0.3 mM Ni2+ to the perfusion medium significantly reduced the maximal amplification to 1.6 ± 0.1 (P < 0.05, n = 4, Fig. 7B). A similar effect was obtained with nimodipine (10 μM, maximal amplification: 1.8 ± 0.3, P < 0.01, n = 4, Fig. 7C). There was no significant difference between the effects obtained with Cd2+/Ni2+ and nimodipine (P = 0.61). These results, therefore, indicate that L-VDCC can act to amplify membrane resonance at suprathreshold potentials.

To test if the amplification mechanism could significantly influence synaptic computation, we activated the CA3-CA1 synapses by short bursts of four stimuli at 100 Hz with an interburst interval of 200 ms (SPsyn test, see MATERIALS AND METHODS, Fig. 8A). In preliminary experiments, we found that slow spikes were truncated by fast inhibitory postsynaptic potentials, and it is thus expected that any amplifying property of the dendritic membrane also would be dampened by activation of inhibitory synapses. To avoid this, we included the GABA_A-receptor antagonist bicuculline (10 μM) in addition to isoproterenol (2 μM). As expected, the size of the postsynaptic burst responses decreased in response to dendritic depolarization (Fig. 8A). Normalized data revealed a nonlinear voltage dependency of the summarized excitatory postsynaptic potential area (Fig. 8B). Thus the area-Vm plot showed that the curve region positive to −35 mV had a smaller slope (0.007 unit/mV, R^2: 0.8292) than the region positive to −35 mV (slope: 0.03 unit/mV, R^2: 0.998). An equivalent curve obtained in the presence of nimodipine (10 μM) displayed an almost linear voltage dependency over the whole voltage range tested (Fig. 8B, slope: 0.021 unit/mV, R^2: 0.972). The overall difference between the values obtained in control and in nimodipine were statistically significant (P < 0.01, two-way ANOVA).

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**Fig. 6.** Effect of differential polarization on slow spiking. The dendritic transmembrane potential change during 4-s depolarizing current pulses (intensity just below threshold for induction of slow spikes) in combination with external electrical field application (bars). With a field polarity (F−) that depolarizes the distal apical dendrite region, rhythmic slow spiking is evoked (top), whereas the reversed polarity (F+) has no such effect (bottom). Applied current was 2.5 mA in both cases. Recordings were performed in the presence of isoproterenol (2 μM).

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**Fig. 7.** L-type voltage-dependent Ca2+ channel (VDCC) amplify voltage signals at theta frequency. A: top: current injection protocol (SPsin test) consisting of 6-Hz sine waves of fixed intensity (0.2 nA) in combination with depolarizing current pulses of increasing intensity (step: 0.2 nA). Bottom: the dendritic response to the SPsin test in the presence of isoproterenol (2 μM) and TTX (1 μM). B: the wave amplitude as function of Vm before (control), during perfusion of 0.5 mM Cd2+ and 0.3 mM Ni2+, and following washout. Same cell as in A. C: histogram of the average peak amplitude (±SE) measured in control medium (n = 6), in Cd2+ and Ni2+ (n = 4), and in nimodipine (10 μM, n = 4). All data were normalized with respect to the amplitudes measured at RMP. *Significant deviation from control (P < 0.05).
DISCUSSION

Intrinsic dendritic theta oscillations in CA1 pyramidal neurons. The intrinsic dendritic V_m oscillations described in the present study closely resemble those observed in in vivo recordings from the apical dendrites of CA1 pyramidal neurons (Kamondi et al. 1998). In both cases, induction required suprathreshold depolarization of the dendritic membrane, and the postsynaptic response consisted of rhythmic firing of slow ramplike spikes at frequencies within the theta-band. In the present study, the average frequency (6.2 ± 2.1 Hz) lies within the region (6–9 Hz) that is strongly correlated with locomotion, spatial navigation and explorative behavior (Ahmed and Mehta 2009; Düzel et al. 2010; Skaggs et al. 1996). The frequency showed no significant voltage dependency, which is in contrast to the pronounced voltage dependency observed by Kamondi et al. (1998). The authors did not specify the precise voltage-range examined, but inspection of their illustrations suggests a similar range as tested in the present study. The basis for these contrasting results is uncertain, but could be due to the different preparations (in vivo vs. in vitro), the age and strain of the rats used.

A dendritic origin of rhythmic slow spiking has been inferred but not firmly established. The high threshold found in the present study and in the study by Kamondi et al. (1998) could indicate that slow spikes originate at a distance from the recording electrode, e.g., soma. However, we found that external field application evoked slow dendritic spiking during selective depolarization of the distal apical dendrites in all four cells tested (Fig. 7). In contrast, reversing the polarization of the cells only induced dendritic slow spiking in one out of four cases. These findings are, therefore, in strong support of a distal dendritic origin, but do not exclude that other areas of the pyramidal cell can support rhythmic slow spiking. Additional support for this concept has been provided by recordings from isolated apical dendrites, showing depolarization-induced rhythmic firing of slow spikelike events (Benardo et al. 1982).

Intrinsic oscillatory behavior appears to be a ubiquitous property as it was readily induced in 100% of dendrites exposed to 4-AP.

Enhancement of slow spikes by adrenergic β1-receptor stimulation. The increased proportion of oscillating dendrites following adrenergic β1-receptor stimulation with isoproterenol was not attributed to any detectable change in basic dendritic membrane properties (RMP or R_in) or in the threshold of the slow spike. However, isoproterenol blocked outward rectification at depolarized potentials (Fig. 3), which can explain its facilitatory effect. Enhancement of slow spike induction, along with an inhibition of outward rectification at steady-state depolarization, was also observed with 4-AP, indicating a common target. This is likely the slow Ca^{2+}-activated K^+ current, I_AHP, which is inhibited by both isoproterenol and 4-AP (Andreasen 2002; Madison and Nicoll 1986), and which has previously been shown to participate in controlling the excitability of the apical dendrites (Sah and Bekkers 1996). Activation of muscarinic receptors is known to have similar effect on the I_AHP (Cole and Nicoll 1983), but it also inhibits several VDCC, including the L-VDCC (Gähwiler and Brown 1987; Shapiro et al. 1999), consistent with the lack of facilitatory effect of carbacholine on the slow spikes. Isoproterenol and 4-AP also antagonizes the I_A; however, because I_A is a transient current, being suitable for shaping the spike (see below), it will likely have very little influence on steady-state outward rectification, thus making it less probable that the I_A has a major role in controlling the slow spike induction.

The importance of L-VDCC. It is well established that isolated dendritic Ca^{2+} spikes can be induced by transient depolarizations provided by locally generated fast Na^+-dependent spikes or backpropagating somatic action potentials (for review, see Higley and Sabatini 2008). The Na^+ and Ca^{2+} spikes are, therefore, temporally linked and form a complex-shaped potential, a compound spike, which is abolished by TTX (Andreasen and Lambert 1995). In contrast, slow spikes induced by long-lasting depolarization of the dendrite appear to

![Figure 8](http://jn.physiology.org/)

**Fig. 8.** L-VDCC amplify theta-like synaptic potentials. *A,* top: stimulation protocol (SP_{syn} test) consisting of rhythmic train stimulation (4 stimuli, 100 Hz) of Schaffer collateral-commissural fibers with a train frequency of 5 Hz. Train stimulation was combined with the SP test. Bottom: three voltage traces showing the dendritic response to the SP_{syn} test (depolarizing current intensity: 0, 0.9, and 1.6 nA) in the presence of isoproterenol (2 μM) and bicuculline (10 μM). *B:* mean area (±SE) of the postsynaptic response as a function of V_m in control conditions (n = 4) and in the presence of nimodipine (10 μM, n = 5). The raw data were divided into 5-mV bins and normalized with respect to the response evoked at −57.5 ± 2.5 mV.
be independent of Na\(^+\) currents, as they largely persisted in the presence of TTX.

The dendritic slow spikes are blocked by nimodipine, indicating that they are mediated by L-VDCC (Catterall 2000), in agreement with their high threshold of induction. Several other types of VDCC are present in the apical dendrites (Frick et al. 2003; Magee et al. 1998; Magee and Johnston 1995), but we found no evidence of any significant contribution from either T-, R-, N- or P/Q-type Ca\(^{2+}\) channels. This is in contrast to backpropagation-activated Ca\(^{2+}\) currents, which are reported to depend on different VDCCs, primarily the T- and R-type (Magee and Carruth 1999).

Self-sustained membrane oscillation generally arises as a result of a resonance current interacting with an amplifying current, and the L-VDCC fulfill the requirements to act as an amplifying current (Hutcheon and Yarom 2000). Indeed, L-VDCC have been implicated in oscillatory activity in a number of cell types (Guzman et al. 2009; Lipscombe et al. 2004; Nedergaard et al. 1993). Furthermore, the spatial requirements are also fulfilled, as L-VDCC (Ca\(_{1.2}\) and Ca\(_{1.3}\)) are distributed throughout the dendritic regions of CA1 pyramidal neurons (Leitch et al. 2009; Tippen et al. 2008).

Hippocampal theta burst oscillations induced in vitro by carbacholine or metabotropic glutamate receptors (mGluR) activation are sensitive to low concentrations of Ni\(^{2+}\), and evidence supports that the R-type VDCC (presumably dendritic) is involved in this activity (Gillies et al. 2002; Tai et al. 2006). This is clearly at variance with the present results, which, therefore, raises the interesting possibility that dendritic oscillatory events can be governed by different subtypes of Ca\(^{2+}\) channels, depending on a regulatory influence from incoming signals.

The importance of voltage-gated K\(^{+}\)-channels. In addition to the L-VDCC, we found evidence that voltage-dependent K\(^{+}\) channels significantly contributed to slow spiking. Both 4-AP and TEA increased the spike amplitude, indicating that K\(^{+}\) currents oppose the depolarizing phase of the slow spikes. In addition, TEA caused a pronounced increase in the duration of the slow spikes, suggesting that the repolarization of the spike is not due to inactivation of the Ca\(^{2+}\) channels, but depends on an active outward current. TEA antagonizes the delayed rectifying K\(^{+}\) current, the I\(_{m}\), and the BK-type Ca\(^{2+}\)-activated K\(^{+}\) current, I\(_{C}\) (for review, see Storm 1990). Neither the I\(_{m}\) nor the I\(_{C}\) seems to be involved in the repolarization of the slow spikes, as the selective antagonists of these currents, linopirdine and iberiotoxin, were without effect. Therefore, results, therefore, suggest that a delayed rectifier current makes a major contribution to slow spike repolarization.

The lack of effect of strophanthidin to show a significant decrease in the membrane to resonate in the theta frequency band at potentials close to firing threshold (Hu et al. 2002, 2009; Narayanan and Johnston 2007), and 2) BK channels in accumulating the depolarization of dendritic compound spikes and to suppress repetitive firing (Golding et al. 1999). The present effect of TEA seems to bear some resemblance to previous studies on suprathreshold somatic oscillations in pyramidal cells from CA1 (Leung and Yim 1991) and cortex (Amitai 1994). It is interesting to note that the delayed repolarization of the slow spikes observed in TEA often occurred in a stepwise fashion, which is indicative for the existence of separate initiation sites for the Ca\(^{2+}\) current (Reuveni et al. 1993). Hence, it is possible that the TEA-sensitive K\(^{+}\) channels both control the activation of local individual microdomains of L-VDCC, and ensure electric segregation of these microdomains (Leitch et al. 2009). In addition, the oscillation frequency was substantially reduced by both 4-AP and TEA, suggesting that a hyperpolarization provided by voltage-dependent K\(^{+}\) currents is a key step in the cycle, being significant for keeping the oscillations within the theta band.

4-AP is known to antagonize the voltage-dependent D-type K\(^{+}\) current, I\(_{D}\), and the I\(_{A}\) at micromolar and millimolar concentrations, respectively (for review, see Storm 1990), and the aforementioned effect on I\(_{SAMP}\) also requires doses in the millimolar range (Andreasen 2002). Our observation that 100 \(\mu M\) 4-AP had near maximal effect on the spike shape and frequency could, therefore, point to a primary role of I\(_{D}\). Indeed, I\(_{D}\) has been shown to control the activation of dendritic compound spikes (Golding et al. 1999). However, unlike compound spikes, the slow spiking recorded here was insensitive to \(\alpha\)-dendrotoxin, which antagonizes I\(_{D}\) (Storm 1988) by targeting K\(_{1.1}\), K\(_{1.2}\) and K\(_{1.6}\) (Coetzee et al. 1999; Harvey 2001), thus making an involvement of I\(_{D}\) unlikely. The I\(_{A}\) is known to play a major role in controlling the excitability of the dendritic membrane (Hoffman et al. 1997; Magee et al. 1998), and our data showing additional effects of 4-AP at higher dose seem consistent with a partial involvement of the I\(_{A}\) in controlling the frequency and amplitude of the slow spikes. This could also explain the increase in amplitude observed with isoproterenol, as adrenergic \(\beta\)-receptor activation leads to a downregulation of I\(_{A}\) (Hoffman and Johnston 1999). A precondition for this interpretation is that a significant amount of I\(_{A}\) is still available for activation at the spike threshold, despite a near-complete inactivation of dendritic I\(_{A}\) at voltages above \(-40 \text{ mV}\) (cf., Hoffman et al. 1997). To account for the effects seen with low dose of 4-AP, other K\(^{+}\) channel types should be considered. Members of the Kv3 group are sensitive to TEA and low concentrations of 4-AP (Gutman et al. 2005) and are implicated in action potential repolarization (for review, see Bean 2007; Johnston et al. 2010). However, Kv3 channels have been found in only 17% of pyramidal cells (Martina et al. 1998), making it difficult to explain the present data. Another slowly inactivating K\(^{+}\) channel with properties similar to Kv3 is Kv1.5, which is also sensitive to slow concentrations of 4-AP, but not to TEA (Gutman et al. 2005). The K\(_{1.5}\) channels are present in both soma and dendrites of CA1 pyramidal cells (Mallet-Savatic et al. 1995), and our results, therefore, seem coherent with Kv1.5 being a target for 4-AP.

It is well known that HCN (h) channels are concentrated in dendrites and the I\(_{h}\) possesses resonance properties (Hu et al. 2009; Magee 1998; Narayanan and Johnston 2007). Our finding that the selective I\(_{h}\) antagonist ZD7288 reduced the firing frequency of slow spikes was, however, unexpected, because this current will be inactivated at suprathreshold V\(_{m}\) values and is, therefore, unlikely to contribute to slow spiking. The results with ZD7288 might be explained by a nonspecific action, as the selectivity of ZD7288 has been challenged in reports showing antagonistic effects on Na\(^{+}\) and low-threshold Ca\(^{2+}\) currents (Felix et al. 2003; Wu et al. 2012).
L-VDCC are often localized in close proximity to small-conductance SK channels (Bowden et al. 2001), and together these two channels form the basis for membrane oscillations in dopaminergic neurons (Nedergaard et al. 1993). The lack of effect of apamin indicates, however, that SK channels are not involved in controlling suprathreshold slow spiking in CA1 pyramidal neurons.

Depolarization-induced facilitation of dendritic oscillatory behavior. The threshold for slow spiking was lowered during repetition of the pulse protocol (Fig. 2), indicating that prolonged depolarization has a facilitatory effect on the oscillatory behavior of the dendritic membrane. A similar phenomenon has been reported in neocortical layer 5 pyramidal cells (Amiatal 1994). There are several accounts from different cell types of depolarization-induced facilitation of Ca\(^{2+}\) currents, in particular those involving L-VDCC (for review, see Dolphin 1996). In CA1 pyramidal neurons, Kavalali and Plummer (1996) demonstrated that prior depolarization leads to a transient lowering of the threshold for activation of L-VDCC. Such effect usually requires strong depolarizations to \(\pm 0\) mV (Dolphin 1996; Kavalali and Plummer 1996; Kourennyi and Barnes 2000). However, less depolarization can be effective if applied for a longer period (Bourinet et al. 1994; Hirano et al. 1999). In our experiments, depolarizations to less than or equal to \(-20\) mV were sustained for 4 s, which might be sufficient to facilitate activation of L-VDCC. Indeed, repetitive 1-s-long depolarizations in the subthreshold range are reported to be effective in spinal cord motoneurons (Alaburdja et al. 2002). The high threshold for induction of dendritic slow spikes indicates a primary role of Ca,1.2 channels, which have higher activation threshold compared with Ca,1.3 (Lipscombe et al. 2004), although it cannot be excluded that electrotonically distant “hot spots” of Ca,1.3 channels are involved. Prior depolarization is known to cause a hyperpolarizing shift and an increased steepness of the activation curve of neuronal Ca,1.2 channels (Bourinet et al. 1994), consistent with the depolarization-induced decrease in threshold observed in the present study.

Functional considerations. The soma-dendritic interference model proposed to account for phase-precession in place cells (see Introduction) includes a dendritic oscillator (Harvey et al. 2009; Lengyel et al. 2003; Losonczy et al. 2010), which has been suggested to represent an intrinsic mechanism that can be dynamically modulated by excitatory synaptic inputs (Lengyel et al. 2003). The intrinsic dendritic oscillator described here seems to have the capacity of being modulated by both an unspecific excitatory influence, e.g., prior depolarization, as well as a specific synaptic input, e.g., neuromodulation. The impact of prior depolarization on the threshold suggests that the oscillatory behavior of the dendrites can be up- and down-regulated according to the level of synaptic input. For instance, during theta activity, there is an increased excitatory synaptic input to the distal dendrites (Kamondi et al. 1998), which may be the first signal for tuning this area toward oscillatory behavior. The neuromodulatory influence seems to be restricted to certain neurotransmitters, as isoproterenol, but not carbacholine, was effective. This opens for the possibility that the dendritic oscillatory behavior of CA1 pyramidal neurons can be controlled from extrahippocampal areas like locus coeruleus. Interestingly, the noradrenergic system has been shown to enhance hippocampal theta activity in a behavior-dependent manner (Kocsis et al. 2007; Walling et al. 2011). The present study indicates that noradrenaline through a down-regulation of \(I_{\text{AHF}}\) and \(I_A\) can promote and enhance the intrinsic oscillatory behavior of the apical dendrites. Future studies should investigate the effect of other transmitter systems, in particular mGluR, which are known to enhance the excitability of CA1 pyramidal neurons (for review, see Pin and Duvoisin 1995) and are found throughout the dendritic arborization (Luján et al. 1997). Furthermore, type I mGluR can generate hippocampal theta frequency oscillations, which engages the apical dendrites of CA1 pyramidal neurons in a Ca\(^{2+}\)-dependent manner (Gillies et al. 2002).

Theta rhythm is suggested to mark the “on-line” state of the hippocampus, providing a timing signal to pyramidal cell firing (Hasselmo 2005). In this respect, it is of interest to note that, during intrinsic dendritic oscillations, somatic firing is converted into a theta burst rhythm, which is time-locked to the oscillations in a way that is similar to the pattern exhibited by place cells within their place field (Harvey et al. 2009).

From the present study, it appears that intrinsic oscillatory and amplifying properties of the apical dendritic only have functional relevance at suprathreshold \(V_m\) values. Sustained dendritic depolarizations of 8–12 mV are observed during theta activity (Harvey et al. 2009; Kamondi et al. 1998), but this is far from the average of 26 mV required to reach threshold for induction of slow spikes. Hence, it seems possible that expression of intrinsic oscillation is an extreme event, which only occurs under very strong excitation. However, some aspects could suggest a broader function. First, prior depolarization will lower the threshold for slow spiking and, hence, the demand for excitation. Second, synaptic conduc-
tance increases progressively with distance from the soma (Magee and Cook 2000), and the input impedance becomes larger in small dendrites. As a consequence of this “synaptic scaling,” the amplitude of postsynaptic potentials at very distal synapses is large and sufficient to induce dendritic Ca\(^{2+}\) spikes (Golding et al. 1999; Rabinowitch et al. 2008; Schiller et al. 1997). Third, properly timed stimulation of the Schaffer collateral-lateral-commisural and perforant pathways can act synergistically, leading to an enhanced postsynaptic response (Takahashi and Magee 2009).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


