Enhanced intrinsic excitability and EPSP-spike coupling accompany enriched environment-induced facilitation of LTP in hippocampal CA1 pyramidal neurons

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Malik R, Chattarji S. Enhanced intrinsic excitability and EPSP-spike coupling accompany enriched environment-induced facilitation of LTP in hippocampal CA1 pyramidal neurons. J Neurophysiol 107: 1366–1378, 2012. First published December 7, 2011; doi:10.1152/jn.01009.2011.—Environmental enrichment (EE) is a well-established paradigm for studying naturally occurring changes in synaptic efficacy in the hippocampus that underlie experience-induced modulation of learning and memory in rodents. Earlier research on the effects of EE on hippocampal plasticity focused on long-term potentiation (LTP). Whereas many of these studies investigated changes in synaptic weight, little is known about potential contributions of neuronal excitability to EE-induced plasticity. Here, using whole-cell recordings in hippocampal slices, we address this gap by analyzing the impact of EE on both synaptic plasticity and intrinsic excitability of hippocampal CA1 pyramidal neurons. Consistent with earlier reports, EE increased contextual fear memory and dendritic spine density on CA1 cells. Furthermore, EE facilitated LTP at Schaffer collateral inputs to CA1 pyramidal neurons. Analysis of the underlying causes for enhanced LTP shows EE to increase the frequency but not amplitude of miniature excitatory postsynaptic currents. However, presynaptic release probability, assayed using paired-pulse ratios and use-dependent block of N-methyl-D-aspartate receptor currents, was not affected. Furthermore, CA1 neurons fired more action potentials (APs) in response to somatic depolarization, as well as during the induction of LTP. EE also reduced spiking threshold and after-hyperpolarization amplitude. Strikingly, this EE-induced increase in excitability caused the same-sized excitatory postsynaptic potential to fire more APs. Together, these findings suggest that EE may enhance the plasticity of CA1 neurons, not only by strengthening synapses but also by enhancing their efficacy to fire spikes—and the two combine to act as an effective substrate for amplifying LTP.

contextual fear learning; action potential threshold; theta-burst stimulation; miniature excitatory postsynaptic currents; after-hyperpolarization

THE RODENT HIPPOCAMPUS has served as a powerful model system for understanding the physiological and molecular bases of long-term, use-dependent changes in synaptic strength and its relationship to certain forms of learning and memory (Lynch 2004; Malenka and Bear 2004; Martinez and Derrick 1996). The most extensively studied synaptic plasticity mechanism underlying memory formation in the hippocampus is long-term potentiation (LTP), in which, brief, high-frequency activation of afferents induces a persistent increase in synaptic strength (Bliss and Collingridge 1993; Bliss and Lømo 1973). Bottom-up strategies using gene-deletion techniques have greatly advanced analyses of the links among LTP, its underlying biochemical signaling mechanisms, and hippocampus-dependent memory (Chen and Tonegawa 1997; Hédou and Mansuy 2003; Huang et al. 1995; Tonegawa et al. 2003; Tsien et al. 1996). A complementary top-down approach involving experience-induced modulation of learning and memory has also contributed to our understanding of LTP and its underlying biochemical signaling mechanisms, and hippocampus-dependent learning in enriched rats (Duffy et al. 2001; Nithianantharajah et al. 2008; Rosenzweig 1966; Schrijver et al. 2004; van Praag et al. 2000; Woodcock and Richardson 2000). For instance, EE enhances synaptic connectivity in hippocampal circuits by promoting the growth of dendrites and spines (Faherty et al. 2003; Greenough and Volkmar 1973; Leggio et al. 2005; Moser et al. 1994; Rampon et al. 2000). Exposure to EE also elicits changes in biochemical signaling pathways, which play a pivotal role in experimentally induced forms of synaptic strengthening (Duffy et al. 2001; Ickes et al. 2000; Mohammed et al. 2002; Paylor et al. 1992; Williams et al. 2001).

Whereas there is broad agreement that EE enhances the molecular and structural substrates of synaptic plasticity in a manner that is expected to facilitate hippocampal LTP, the impact of EE varies between different subregions of the hippocampus. For instance, in hippocampal area CA1, electrophysiological recordings from acute slices have shown increased basal excitatory transmission following EE (Foster and Dumas 2001; Irvine and Abraham 2005), which has been interpreted as a manifestation of enhanced synaptic transmission caused by a natural, LTP-like phenomenon over the course of EE. However, there are other studies that did not find any effect of EE on basal synaptic transmission but did demonstrate enhancement of LTP in area CA1 when tested after exposure to EE (Artola et al. 2006; Duffy et al. 2001). In contrast to the CA1 area, in vitro and in vivo extracellular field
potential recordings in the dentate gyrus (DG) have shown prior exposure to EE to occlude the induction of LTP at perforant path inputs (Eckert et al. 2010; Foster et al. 1996). Furthermore, this occlusion of LTP was accompanied by an increase in the basal synaptic transmission at perforant path synapses to DG granule cells (Foster et al. 1996; Gagné et al. 1998; Green and Greenough 1986; Irvine et al. 2006). A particularly striking finding comes from in vivo extracellular recordings in freely moving rats, wherein exposure to EE caused a significant increase in the population-spike amplitude of DG granule cells (Irvine et al. 2006). Similar increases in population-spike amplitudes have been observed in vitro, even when EE failed to enhance the level of LTP induced by tetanic stimulation in the DG (Green and Greenough 1986). Importantly, it has been suggested that such a change may reflect a form of activity-induced hippocampal plasticity called excitatory postsynaptic potential (EPSP)-spike (E-S) potentiation (Irvine et al. 2006), which is thought to be the result of a stronger coupling between the EPSP and spike and is mediated by increased neuronal excitability (Andersen et al. 1980; Daoudal and Debanne 2003; Daoudal et al. 2002). However, in earlier studies that relied on extracellular field potential recordings, it was not possible to directly demonstrate EE-induced modulation of E-S coupling. This issue is also significant, because previous research has focused primarily on experience-induced changes in synaptic strength, thereby overlooking potential changes in intrinsic excitability of neurons (Abraham 2008; Frick and Johnston 2005; Frick et al. 2004; Johnston and Narayan 2008; Kim and Linden 2007; Poirazi and Mel 2001; Sjostrom et al. 2008; Zhang and Linden 2003). Whereas extracellular recordings from freely moving rats are strongly suggestive of a role for intrinsic plasticity in mediating E-S potentiation-like effects in the DG, little is known about the impact of EE on intrinsic properties of DG neurons. Even in area CA1, where clear evidence exists for EE enhancing LTP, it is not clear whether this is caused by changes in synaptic strength alone or whether intrinsic plasticity, such as strengthening of E-S coupling, also plays a role. Therefore, in the current study, we address some of these unresolved issues by using whole-cell recordings in CA1 pyramidal cells to test whether EE modulates both synaptic strength and intrinsic excitability and whether the two can combine to act as an effective substrate for amplifying LTP and its behavioral consequences.

**MATERIALS AND METHODS**

**Animals used.** Male Sprague-Dawley rats were used in this study. Animals were maintained in a temperature-controlled room with a 14:1 light:10-h dark cycle with ad libitum access to food and water. All experimental protocols used in this study were approved by the Institutional Animal Ethics Committee of the National Centre for Biological Sciences (Bangalore, India).

**Housing conditions.** At postnatal day 25, rats were randomly assigned to either control (CON) or EE housing conditions. In CON housing condition, two to three rats were housed together in standard laboratory cages and were handled only for routine animal maintenance procedures (Fig. 1B). In EE housing condition, 12–13 rats were group housed in larger cages (25 × 20 × 12 in; Fig. 1C). Each day for 4 h (during the light phase of the rats), enriched rats were transferred to a playing arena (34 × 34 × 30 in), which contained novel objects (tunnels, ladders, balls, etc; Fig. 1D). The placement of these objects was changed on a daily basis, and new objects were added to the arena twice every week. Following 30–35 days of exposure to EE, animals were used for morphological, behavioral, or electrophysiological analysis. From each batch of enriched rats, six to eight rats were used for electrophysiological recordings, and the rest were used for morphological and behavioral testing. The EE animals were not exposed to the playing arena on the day of the experiment, and all recordings were carried out 1 day after the end of EE. The experimenter was aware of the housing condition for electrophysiology experiments. The morphological and behavioral analyses were done blind.

**Contextual fear conditioning.** Training was conducted in a Plexiglas rodent conditioning chamber with a metal grid floor (Coulbourn Instruments, Whitehall, PA), which was enclosed within a sound attenuating chamber (Coulbourn Instruments). The chamber was illuminated dimly by a single house light, and a ventilation fan provided a background noise of 60–65 dB. The floor and the walls of the chamber were wiped using 70% alcohol between each trial. On the day of training, each rat was placed in the conditioning chamber and given one footshock (1 s, 1 mA) 16 s later. Rats were removed from the chamber 120 s after the shock. Twenty-four hours later, each rat was returned to the chamber, and contextual fear learning was quantified manually during a 2-min period (from the videotape). Freezing was used as the index for contextual fear learning. Freezing involved absence of all body movements, except respiration-related movements (Blanchard and Blanchard 1969; Bouton and Bolles 1980; Fanselow 1980; Phillips and LeDoux 1994).

**Golgi staining and morphological analysis.** Animals were anesthetized using halothane and decapitated. The brain was removed quickly, and blocks of tissue containing the hippocampus were dissected and processed for the Golgi-Cox technique at room temperature. The brains were processed, and coronal sections were obtained as described before (Pawlak et al. 2005).

By using the Neurolucida image analysis system (MicroBrightField, Williston, VT) attached to an Olympus BX61 microscope (100×, 1.3 N.A., Olympus BX61, Olympus, Tokyo, Japan), all protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft. The primary branch to the apical dendrite (main shaft) of the area CA1 neurons was selected for spine analysis. Primary branches from both short-shaft and long-shaft neurons were included in the analysis. The branches selected for analysis originated 50–150 μm away from the soma. Starting from the origin of the branch and continuing away from the cell soma, the number of spines was counted in successive steps of 10 μm each, for a total of eight steps (i.e., extending a total length of 80 μm).

**Hippocampal slice preparation.** Rats were anesthetized using halothane and decapitated. The brain was removed quickly from the
skull and transferred to oxygenated, ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 115 NaCl, 25 glucose, 25.5 NaHCO3, 1.05 NaH2PO4, 3.3 KCl, 2 CaCl2, and 1 MgSO4. The hippocampi were dissected out of the two hemispheres, and transverse sections (400 μm) were obtained using Vibratome 1000 Plus (Vibratome, St. Louis, MO). In experiments where the slices were disinhibited during the recordings, area CA3 was surgically removed to avoid spontaneous activity. Slices were transferred to a holding chamber and were allowed to recover for 1 h at room temperature.

Individual slices were transferred to a submerged recording chamber (28 ± 2°C) and visualized using infrared differential interference contrast optics and Dage camera system attached to an Olympus BX51WI microscope. Cells were selected for recording, based on their pyramidal shape, smoothness of the membrane, and low-contrast appearance.

Whole-cell recordings. Patch pipettes (3–5 MΩ, ~2 μm tip diameter) were pulled from thick-walled borosilicate glass on a P-97 Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA). In experiments where evoked responses were recorded, a bipolar electrode (25 μm diameter Platinum/Iridium, FHC, Bowdoin, ME) or a silver-coated glass electrode filled with extracellular ACSF (~2 μm tip diameter), connected to an ISO-Flex stimulus isolator (A.M.P.I., Jerusalem, Israel), was used to stimulate the Schaffer collateral inputs. Data were recorded using an EPC 10 Plus amplifier (HEKA Elektronik, Germany), filtered at 2.9 kHz and digitized at 20 kHz. Stimulus delivery and data acquisition were performed using Patchmaster software (HEKA Elektronik). Cells were used for recording if initial resting membrane potential (V_m) ≤ −60 mV, and series resistance (R_s) was 15–25 MΩ. During the course of the experiments, the neuron’s input resistance (R_in) and R_s were monitored continuously by applying hyperpolarizing current or voltage pulse, and experiments were rejected if R_s or R_in changed by >20% of their respective initial values. All analysis of electrophysiological data was performed using custom-written programs in IGOR Pro software (WaveMetrics, Lake Oswego, OR), unless stated otherwise.

For current clamp recordings, the patch pipettes were filled with internal solution containing (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 2 NaCl, 4 MgATP, 0.3 NaGTP, 10 phosphocreatine (pH 7.3, KOH ~290 mOsm). For the voltage clamp internal solution, potas- sium was replaced with equimolar cesium. For all recordings, neurons were held at ~70 mV.

mEPSC recordings. CA1 neurons were voltage clamped at ~70 mV, and 2-amino-3-(5-methyl-3-oxo-1, 2-oxazol-4-yl) propanoic acid receptor (AMPA)-mediated miniature excitatory postsynaptic currents (mEPSCs) were isolated by TTX (0.5 μM) and picrotoxin (100 μM). Continuous current traces of 5-min duration (recorded at least 5 min after achieving whole-cell configuration) were analyzed using the Mini Analysis Program (Synaptosoft, Fort Lee, NJ).

Paired-pulse measurements. Paired stimuli with an interstimulus interval (ISI) of 50, 175, 100, 150, 200, and 250 ms were delivered every 20 s (10 sweeps each) to the Schaffer collateral inputs while clamping the cell at ~70 mV in the presence of picrotoxin (100 μM). Paired-pulse ratio (PPR) was defined as the ratio of peak amplitude of the second EPSC (measured for 1 ms) to the first.

MK-801 experiments. CA1 neurons were voltage clamped at +40 mV in the presence of cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and picrotoxin (100 μM), and N-methyl-d-aspartic acid receptor (NMDAR)-mediated mEPSCs were recorded by stimulating the Schaffer collaterals and measuring the NMDA receptor-evoked miniature postsynaptic currents (mEPSCs). Continuous current traces of 5 min, MK-801 (5 μM) was added to the bath, and synaptic stimulation was resumed, and 100 trials were recorded for each cell. The peak amplitudes (measured for 10 ms) of NMDAR-EPSCs were measured and normalized to the amplitude of the first trace in MK-801. The decay in NMDAR-EPSC amplitudes in the presence of MK-801 was fit to a single exponential (Manabe and Nicoll 1994; Murthy et al. 1997). Time constants (τ) obtained from the exponential fits were used for statistical comparisons.

LTP. Recordings were obtained from CA1 neurons in current clamp mode in the presence of picrotoxin (100 μM), and the cells were held at ~70 mV (±2 mV) by injecting hyperpolarizing current. The LTP protocol involved acquisition of 5-min stable, baseline EPSPs (0.05 Hz), followed by application of theta-burst stimulation (TBS), which consisted of five bursts (at 5 Hz) of four pulses (at 100 Hz) each (see Fig. 5A). Post-TBS, EPSPs were recorded for 30 min at the baseline stimulation frequency (0.05 Hz). LTP experiments were excluded from the analysis if the TBS application was not within 10–12 min after achieving whole-cell configuration. The amplitudes of EPSPs during baseline acquisition were kept within 5–10 mV for all cells. LTP was quantified using averaged and normalized initial slope values, defined as the rise in amplitude for the first 1–2 ms of EPSPs. For statistical comparisons, EPSP slope values at 25–30 min after TBS were compared with the 5-min average baseline.

To analyze burst-induced depolarization, the traces recorded during TBS were filtered at 100 Hz, and the action potentials (APs) were subtracted from the waves (see Fig. 5, B and C). The peak amplitudes for every burst of the resultant waveform were used for comparison.

Passive and active membrane properties. Current clamp recordings were obtained in the presence of picrotoxin (100 μM), amino-phosphonopentanoic acid (30 μM), and CNQX (10 μM). R_s was compensated to accurately measure the AP properties. The current-voltage relations were obtained by plotting the steady-state voltage responses to 600 ms, 10 pA current steps (~50 pA to ~50 pA). R_m was calculated from the slope of the linear fit of the voltage-current plot (Staff et al. 2000). The membrane τ was calculated from the average of the monoexponential fits of 200 ms current steps (~20, ~10, 10, and 20 pA). The sag voltage was calculated by subtracting the steady-state voltage from the peak-voltage responses to 600 ms hyperpolarizing current injections (~600 to ~300 pA) (Moyer et al. 1996). The resonance frequency was measured from the cell’s response to a sinusoidal current of constant amplitude with its frequency linearly spanning 0–20 Hz in 20 s (Narayanan and Johnston 2007). The peak after-hyperpolarization potential (AHP) was analyzed using a 100-ms depolarizing pulse that reliably elicited a train of four to five APs (Moyer et al. 1996). The after-depolarization potential (ADP) was measured from the peak amplitude during the AP repolarization phase. AP properties were measured from single APs elicited by 5-ms, 1-nA current injection. The AP threshold was measured as the voltage at which the first derivative of the voltage response (dV/dt) reached 40 mV/ms. Rheobase current was determined as the minimal depolarizing current amplitude (3 ms) required to elicit an AP, and AP amplitude was measured from the V_m to the AP peak, and the duration was measured at one-half amplitude.

To study the membrane excitability, neurons were injected with 600 ms depolarizing current pulses ranging from 50 to 500 pA. The number of APs elicited by each current injection was counted for individual traces and plotted as a function of injected current amplitude.

E-S coupling. E-S relationships were measured for a cell by stimulating the Schaffer collateral inputs at 0.05 Hz, while recording the slope of the resulting EPSP in the presence of picrotoxin (100 μM). The E-S data were plotted by binning the slope values and finding a probability to spike for each slope bin. This E-S curve was fit with a sigmoid function and the EPSP value at the 0.5-spike probability point (EC-50) determined for each cell (Daoudal et al. 2002). The EC-50 values were used for statistical comparison.

EPSP amplification. The EPSP slope data were binned in 0.3 mV/ms bins (range on x-axis: 0.2–2.3 mV/ms), and the average EPSP amplitude was calculated for individual CA1 cells. A linear fit of the amplitude vs. slope plot was obtained for individual cells, and the slope of the linear fit for individual cells was used for statistical comparison.
Statistical analysis. All values are expressed as mean ± SE. Statistical comparisons were done after using Levene’s test and single sample Kolmogorov-Smirnov (K-S) test for appropriate assumptions of variance and normality of distribution. Comparisons between two groups were done using unpaired Student’s t-test. Comparison for cumulative distributions was done using two-sample K-S test. All statistical analyses were conducted using SPSS 9.0 (IBM SPSS, Armonk, NY) or IGOR Pro (WaveMetrics).

Chemicals. Most of the chemicals and toxins were obtained from Sigma (St. Louis, MO), unless mentioned otherwise. TTX was obtained from Alomone Labs (Jerusalem, Israel). MK-801 maleate was obtained from Tocris Bioscience (Bristol, UK).

RESULTS

EE improves contextual fear learning and enhances spine density on hippocampal CA1 neurons. To confirm the efficacy of our experimental protocol for EE, we relied on behavioral and cellular measures that have been established by earlier studies. At the behavioral level, we used previous findings on hippocampus-dependent contextual fear learning being enhanced by EE (Duffy et al. 2001; Woodcock and Richardson 2000). At the cellular level, we took note of earlier reports on EE, leading to an increase in dendritic spine density in hippocampal area CA1 neurons (Faherty et al. 2003; Greenough and Volkmar 1973; Leggio et al. 2005; Moser et al. 1994; Rampon et al. 2000).

Many lines of evidence support the involvement of hippocampus-dependent spatial learning in contextual fear acquisition (Lee and Kesner 2004; Maren et al. 1998). Therefore, we compared contextual fear conditioning between rats subjected to EE and rats that were housed for the same period of time in control cages (see MATERIALS AND METHODS). Memory for contextual fear conditioning, measured in the same context 24 h after training, was enhanced significantly (95% increase, P < 0.01) in the number of spines/10 μm, measured along an 80-μm dendritic segment in EE neurons (CON: 11.1 ± 0.6, n = 14 cells, n = 4 rats; EE: 13.5 ± 0.4, n = 16 cells, n = 4 rats; Fig. 2C). A more detailed segmental analysis in steps of 10 μm showed significantly higher spine density in all 10-μm segments along the entire 80-μm length of the apical dendrite in EE neurons (Fig. 2D). Thus consistent with previous reports, the EE paradigm used in the present study also caused structural plasticity in the excitatory neurons in hippocampal area CA1.

EE increases the frequency but not the amplitude of mEPSCs in CA1 pyramidal neurons. Does the higher spine density after EE have a physiological correlate that is manifested as an increase in excitatory synaptic transmission? To test this, we used whole-cell, voltage-clamp recordings to compare the frequency and amplitude of spontaneous mEPSCs in CA1 pyramidal cells from control and enriched rats (Fig. 3, A1 and A2). These mEPSCs were blocked completely by CNQX, confirming that they were AMPAR-dependent synaptic currents (data not shown). EE neurons exhibited a significant increase (57%, P < 0.01) in mEPSC frequency (CON: 0.28 ± 0.02 events/s, n = 14; EE: 0.44 ± 0.03 events/s, n = 13; Fig. 3B1), whereas mEPSC amplitude was not affected (CON, 22.4 ± 1.5 pA, n = 14; EE: 21.4 ± 0.53 pA, n = 13; Fig. 3B2). The increase in mEPSC frequency was also reflected in a leftward shift of the cumulative probability plot of interevent interval for EE neurons relative to controls (Fig. 3B1). Furthermore, the decay time of mEPSCs was significantly longer (17% increase, P < 0.01) in EE neurons (CON: 5.88 ± 0.21 ms, n = 14; EE: 6.9 ± 0.33 ms, n = 13; Fig. 3B3).

![Graphs showing EE effects on spine density and contextual fear learning](image-url)
EE has no effect on measures of presynaptic release probability. Whereas the enhanced mEPSC frequency is consistent with an increase in the number of spines after exposure to EE, the increase in mEPSC frequency may also be indicative of enhanced presynaptic release probability (Turrigiano and Nelson 2004). Therefore, we analyzed the potential impact of EE on presynaptic release probability using two different assays (Murthy et al. 1997). First, we measured PPR of EPSCs across a range of ISIs at Schaffer collateral inputs using whole-cell, voltage-clamp recordings from CA1 neurons (Fig. 3C). We found no difference in PPR of evoked EPSCs in EE cells compared with controls (PPR at 50 ms interpulse interval, CON: 2.5 ± 0.2, n = 12; EE: 2.5 ± 0.3, n = 11; Fig. 3C). This lack of effect on PPR reflects an absence of change in presynaptic release probability after EE. This was probed further using a second assay that involved repeated stimulation of Schaffer collateral inputs to CA1 cells in the presence of the NMDAR open-channel blocker MK-801. This led to a progressive decay of NMDAR-EPSCs (Fig. 3D1 and D2), the τ of which is inversely related to the probability of release (Manabe and Nicoll 1994; Rosenmund et al. 1993). The decay kinetics were fit by a single exponential, and the τ of decay between the two groups was not found to be significantly different (CON: 59 ± 10.6 ms, n = 10; EE: 63.6 ± 10.1 ms, n = 10; Fig. 3D2). Together, these data showed that EE had no effect on the release probability at Schaffer collateral inputs to CA1 neurons, suggesting that the increase in mEPSC frequency is likely to be an electrophysiological correlate of the higher number of synapses on CA1 neurons in EE rats (Prange and Murphy 1999; Turrigiano and Nelson 2004).

EE enhances LTP at Schaffer collateral inputs to CA1 pyramidal neurons. The morphological and electrophysiological results presented thus far point to an overall enhancement of excitatory synaptic transmission by EE. In addition, at the behavioral level, the same EE improves hippocampus-dependent memory. A large body of evidence has identified a pivotal role of EE in improving learning and memory, and the results presented here provide a mechanistic basis for this effect.
role for hippocampal synaptic plasticity mechanisms, such as LTP, in mediating learning and memory (Bliss and Collingridge 1993; Malenka and Bear 2004). Indeed, several earlier studies have reported EE-induced enhancement of LTP in the CA1 area of the hippocampus (Artola et al. 2006; Duffy et al. 2001). A majority of these earlier studies, however, used extracellular field potential recordings to examine the effects of EE on LTP. We, therefore, analyzed the impact of EE at the level of single CA1 pyramidal neurons in hippocampal slices. To this end, we used a LTP induction protocol, TBS, which resembles the physiologically relevant in vivo firing patterns in the theta frequency range (4–8 Hz) seen during memory acquisition and retrieval in rodents (Bland 1986; Larson and Lynch 1986; Larson et al. 1986; Nguyen and Kandel 1997). In hippocampal slices obtained from control rats, TBS applied to Schaffer collateral inputs to CA1 pyramidal neurons led to robust LTP (percent increase in EPSP slope relative to pre-TBS baseline, CON: 101.9 ± 19.4, n = 11; Fig. 4A1). Strikingly, the same TBS protocol induced significantly greater LTP in CA1 neurons from enriched rats (percent increase in EPSP slope relative to pre-TBS baseline, EE: 191.7 ± 43.1, n = 11, 90% increase compared with LTP in control slices, P < 0.05; Fig. 4). Thus EE facilitates the ability of excitatory glutamatergic synapses in area CA1 to undergo LTP, and this, in turn, is consistent with the enhanced hippocampal memory.

**EE enhances AP firing during LTP induction.** What may be the underlying mechanisms that lead to enhanced LTP in CA1 neurons after EE? Physiological and morphological changes in the excitatory synapses of CA1 neurons provide an ideal substrate for enhanced LTP but may not be the only determinants of the magnitude of LTP induced. The level of postsynaptic depolarization reached during the delivery of LTP-induc-
ing stimuli is known to play an important role in its efficacy to elicit potentiation (Urban and Barrionuevo 1996). Therefore, we first carried out a detailed analysis of the levels of membrane depolarization achieved through the four stimulus pulses, given 10 ms apart (i.e., intraburst frequency of 100 Hz), which constituted each of the five bursts delivered at an interburst interval of 200 ms (i.e., 5 Hz frequency; Fig. 5A; see MATERIALS AND METHODS). The peak amplitude of each of the five depolarizing envelopes elicited by the five bursts was quantified for each cell and averaged for the control and EE groups (Fig. 5B, C and D). This analysis showed that the mean amplitude of peak depolarization underlying each individual burst of the TBS was not different between the two groups for any of the five bursts (mean burst depolarization, CON: 14.4 ± 2.4 mV, n = 11; EE: 15.7 ± 2.3 mV, n = 11; Fig. 5D). Therefore, we shifted our focus to the number of APs fired by EE and control cells within each of the five bursts of the TBS induction protocol used to elicit LTP (Pike et al. 1999; Thomas et al. 1998) (Fig. 5E). This analysis showed that the number of APs fired during the first of the five bursts was significantly higher (80% increase, P < 0.01) in EE cells compared with control cells (CON, 2.1 ± 0.25, n = 11; EE, 3.8 ± 0.24, n = 11; Fig. 5E). During the remaining four bursts, the enriched cells continued to fire more APs, although the difference was no longer statistically significant (Fig. 5E). The total number of APs fired through all five bursts was significantly higher in CA1 cells from enriched rats (CON, 3.36 ± 0.9, n = 11; EE, 7.2 ± 1.3, n = 11; Fig. 5E). Importantly, the number of APs fired during TBS correlated positively with the magnitude of potentiation in the EPSP slope for all EE and control cells (r = 0.7, P < 0.05; Fig. 5F). The number of APs fired and the magnitude of LTP in EE cells spanned a greater range compared with their control counterparts (Fig. 5F). Taken together, these results highlight differences in the efficacy of firing APs during the induction of LTP, which, in turn, correlates with the level of synaptic potentiation achieved.

**EE enhances intrinsic excitability of CA1 pyramidal cells.** Since our results suggested that increased spiking of CA1 cells might have contributed to the effects of EE on LTP, we examined if and how key parameters related to neuronal excitability were modulated by exposure to EE. We first examined whether APs, evoked by somatic injection of increasing steps of depolarizing currents, differed between EE and control cells (Fig. 6A). CA1 neurons from enriched rats fired a significantly higher number of APs relative to controls for several values of current injected (number of APs for 200 pA current injection, CON: 9.7 ± 0.9, n = 16; EE: 12.4 ± 0.73, n = 16, P < 0.05; Fig. 6B). Furthermore, the instantaneous frequencies for the first interspike intervals (ISIs) were significantly higher in EE neurons (instantaneous frequency for 200 pA current injection; CON: 41.6 ± 5.7 Hz, n = 16; EE: 66.26 ± 6.43 Hz, n = 16, P < 0.01; Fig. 6C). Thus consistent
with our observations on EE neurons exhibiting enhanced spiking during TBS-induced LTP, somatic injections of depolarizing currents also led to enhanced AP firing.

Next, we examined the basis of this EE-induced enhancement in firing by comparing the threshold to fire APs in EE vs. control cells. The voltage threshold at which somatic depolarization evoked an AP was reduced significantly in CA1 neurons from EE rats (CON: $-51.5 \pm 1.1$ mV, $n = 16$; EE: $-55.5 \pm 1.3$ mV, $n = 16$, $P < 0.05$; Fig. 6D1). Correlating with a decrease in the voltage threshold, a significant reduction was also observed in the current threshold or the rheobase of EE neurons (25% decrease; CON: $0.7 \pm 0.03$ nA, $n = 16$; EE: $0.56 \pm 0.03$ nA, $n = 16$, $P < 0.01$; Table 1). Next, we focused on another facet of neuronal excitability—the AHP—following a depolarizing somatic current injection, which reliably elicited a train of four to five APs (Fig. 6E1). Area CA1 pyramidal neurons from EE rats exhibited a significant reduction (42% decrease, $P < 0.01$) in AHP amplitude (CON: $2.7 \pm 0.2$ mV, $n = 16$; EE: $1.9 \pm 0.5$ mV, $n = 16$, $P < 0.001$; Fig. 6D2). Analysis of other active membrane properties (Table 1) did not show any impact of EE on the ADP, AP amplitude, and half-width or the effects of EE on the ADP, AP amplitude, and half-width or the voltage response. $*P < 0.01$; $†P < 0.05$, Student’s t-test.

Table 1. Summary of passive and active membrane properties of CA1 neurons from control and enriched rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CON</th>
<th>EE</th>
<th>$P$ value</th>
</tr>
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<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>$-64.8 \pm 0.8$ nA, $n = 16$</td>
<td>$-66.4 \pm 0.5$ nA, $n = 16$</td>
<td>0.1</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>$100.6 \pm 3.8$ mΩ, $n = 16$</td>
<td>$105.3 \pm 4.4$ mΩ, $n = 16$</td>
<td>0.5</td>
</tr>
<tr>
<td>Membrane time constant (ms)</td>
<td>$27.9 \pm 1$ ms, $n = 16$</td>
<td>$31.2 \pm 1.7$ ms, $n = 16$</td>
<td>0.1</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>$2.7 \pm 0.2$ mV, $n = 16$</td>
<td>$1.9 \pm 0.2$ mV, $n = 16$</td>
<td>0.009†</td>
</tr>
<tr>
<td>ADP (mV)</td>
<td>$16.2 \pm 0.4$ mV, $n = 16$</td>
<td>$15.8 \pm 0.5$ mV, $n = 16$</td>
<td>0.1</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>$120.1 \pm 1.1$ mV, $n = 16$</td>
<td>$118.8 \pm 1.2$ mV, $n = 16$</td>
<td>0.45</td>
</tr>
<tr>
<td>AP half-width (ms)</td>
<td>$1.6 \pm 0.02$ ms, $n = 16$</td>
<td>$1.6 \pm 0.04$ ms, $n = 16$</td>
<td>0.6</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>$-51.5 \pm 1.1$ mV, $n = 16$</td>
<td>$-55.5 \pm 1.3$ mV, $n = 16$</td>
<td>0.02†</td>
</tr>
<tr>
<td>Max. dV/dt (KV/s)</td>
<td>$0.42 \pm 0.001$ s, $n = 16$</td>
<td>$0.42 \pm 0.001$ s, $n = 16$</td>
<td>0.9</td>
</tr>
<tr>
<td>Current threshold (nA)</td>
<td>$0.7 \pm 0.03$ nA, $n = 16$</td>
<td>$0.56 \pm 0.03$ nA, $n = 16$</td>
<td>0.008*</td>
</tr>
<tr>
<td>Sag voltage (mV)</td>
<td>$13.3 \pm 0.3$ mV, $n = 16$</td>
<td>$12.7 \pm 0.5$ mV, $n = 16$</td>
<td>0.6</td>
</tr>
<tr>
<td>Resonance frequency (Hz)</td>
<td>$1.7 \pm 0.2$ Hz, $n = 16$</td>
<td>$2 \pm 0.2$ Hz, $n = 16$</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The CA1 neurons in the environmental enrichment (EE) group have decreased- after-hyperpolarization potential (AHP) amplitude and have a reduced action potential (AP) threshold. CON, control (routine animal maintenance procedures); ADP, after-depolarization potential; Max. dV/dt, maximum first derivative of the voltage response. $*P < 0.01$; $†P < 0.05$, Student’s t-test.
why CA1 neurons from EE rats are prone to firing more APs when they are activated.

**EE strengthens E-S coupling in CA1 pyramidal cells.** In our earlier analysis of the possible reasons underlying enhanced LTP in EE neurons, two observations were prominent. First, in hippocampal slices from EE rats, CA1 neurons fired a higher number of APs during the activation of synaptic afferents with TBS (Fig. 5). This, in turn, was consistent with the increased, intrinsic excitability assessed through somatic depolarization (Figs. 6, A–E). Second, these measures of enhanced spiking and excitability caused by EE stood in striking contrast to the absence of any effect on the subthreshold membrane depolarization seen during TBS delivery (Fig. 5D). Furthermore, this lack of effect was also consistent with the finding that EE did not affect the amplitude of mEPSCs. This suggested that the likelihood of firing APs was greater in EE neurons during LTP induction, despite no apparent increase in the amplitude of postsynaptic depolarization caused by TBS. How does TBS-induced activation of synaptic inputs, which leads to comparable levels of postsynaptic depolarization in EE and control cells, nonetheless lead to enhanced AP firing in EE neurons? A possible mechanism is suggested by earlier studies that report an enrichment-induced increase in population-spike amplitudes in DG granule cells (Green and Greenough 1986; Irvine et al. 2006). These findings have also suggested that this effect is reminiscent of tetanus-induced E-S potentiation, a form of hippocampal plasticity, wherein a stronger coupling between the EPSP and spike is manifested as greater population-spike amplitude, even in the absence of any potentiation of the synaptic response (Andersen et al. 1980; Chavez-Noriega et al. 1989, 1990; Daoudal and Debanne 2003; Daoudal et al. 2002). These earlier reports on EE-induced E-S potentiation-like effects relied on extracellular field potential recordings in the hippocampus. In this study, we used whole-cell recordings that provide a sensitive test of this idea at the single-cell level by quantifying the strength of the E-S coupling, an index of the likelihood of firing an AP for a given synaptic depolarization. The E-S relationships were compared between CA1 cells taken from EE and normal rats under control conditions, not after inducing LTP. To this end, the Schaffer collateral inputs were stimulated while recording the slope of the resulting EPSP. The E-S data were plotted by binning the slope values and finding a probability to spike for each slope bin. This E-S curve was then fit with a sigmoid function and the EC-50 determined for each cell. This analysis shows a leftward shift in the E-S curve for EE neurons (Fig. 6F). The EPSP slope values at 0.5-spike probability (EC-50) were significantly lower (22% decrease) for EE neurons (CON, 3.26 ± 0.16 mV/ms, n = 10; EE, 2.68 ± 0.27 mV/ms, n = 8, P < 0.05; Fig. 6F). Furthermore, the amplitude and slope of the EPSPs recorded during the E-S coupling experiments were also analyzed to test whether EE affected the EPSP amplification (amplitude/slope ratios). We found no significant difference in EPSP amplification between the two groups (CON, 6.98 ± 0.3, n = 10; EE, 6.29 ± 0.7, n = 8, P = 0.4; data not shown) (Campanac and Debanne 2008). Thus exposure to EE strengthens the E-S coupling such that the same-sized EPSP is likely to cause the firing of more APs in CA1 neurons from enriched rats.

**DISCUSSION**

In this study, we characterized the impact of EE on hippocampal plasticity and its functional consequences using a combination of electrophysiological, morphological, and behavioral analyses. Repeated exposure to EE for 1 mo gave rise to naturally occurring plasticity manifested as an increase in both the structural and physiological substrates of excitatory synaptic transmission. Importantly, EE also enhanced intrinsic excitability and the coupling between synaptic drive and AP firing. This naturally occurring synaptic and intrinsic plasticity, in turn, served as an ideal cellular substrate for supporting further synaptic plasticity that is manifested as enhanced LTP. The combined impact of these cellular and synaptic changes is consistent with the significant improvement in a form of contextual learning that depends on the hippocampus. Thus the changes in synaptic transmission and neuronal excitability that occurred in vivo in the intact animal during the course of the EE appear to facilitate artificially induced LTP in hippocampal slices ex vivo. Indeed, these two cellular mechanisms act in concert to improve new learning after EE, as shown here and in previous studies.

**Strengthening of the structural and physiological basis of excitatory synaptic transmission.** Previous studies have identified growth of dendrites and spines as hallmarks of structural plasticity induced by EE (Faherty et al. 2003; Greenough and Volkmar 1973; Leggio et al. 2005; Moser et al. 1994; Rampon et al. 2000). The EE protocol used in the present study also increased spine density on the primary branches of apical dendrites of CA1 pyramidal neurons. This EE-induced increase in spine density in the stratum radiatum of area CA1, which receives Schaffer collateral inputs, is consistent with earlier reports of increased excitatory synaptic transmission at the same afferents, assessed using extracellular field recordings of input-output relationships (Irvine and Abraham 2005). We probed the basis of this enhancement in greater detail using whole-cell recordings from CA1 pyramidal cells. We observed no effects on the amplitude of spontaneous mEPSCs, suggesting a lack of any significant impact of EE on the strength of individual functional synapses (Turrigiano and Nelson 2004). However, we found the frequency of mEPSCs to be higher in EE rats. Along with an increase in mEPSC frequency, we report a significant increase in mEPSC decay time course after exposure to EE. This prolongation of the decay of mEPSCs may be caused by the previously reported increase in basal responsiveness of CA1 neurons to exogenous application of AMPA (Foster et al. 1996; Gagné et al. 1998). A change in subunit composition in the AMPAR can also contribute to this increase in the decay time course. A more detailed electrophysiological characterization of the kinetics and rectification properties of AMPAR will be required to investigate these possibilities (Gagné et al. 1998; Naka et al. 2005). In light of the increase in mEPSC frequency, we also assessed the impact of EE on PPRs and use-dependent block of NMDAR currents at Schaffer collateral inputs to area CA1. We found that EE affected neither of these measures of presynaptic release (Murthy et al. 1997). Taken together, our results suggest that EE enhances basal excitatory transmission by increasing the number of synapses on area CA1 pyramidal neurons. The findings reported here are in agreement with a previous study (Foster and Dumas 2001), which carried out quantal analysis at CA3–
CA1 synapses to characterize the relative contributions of presynaptic and postsynaptic changes to the increase in synaptic strength caused by EE. Further support for postsynaptic mechanisms comes from a more recent report showing that exposure to EE causes similar postsynaptic changes in the developing hippocampus (He et al. 2010). It is interesting to note that presynaptic changes have also been seen after exposure to EE but in older animals (Artola et al. 2006). Whereas the nature of pre- and postsynaptic changes may vary with the age of experimental animals or features of the enrichment paradigm used, the strengthening of both of the structural and physiological bases of excitatory synaptic transmission reflects a robust form of naturally occurring potentiation of synaptic transmission that is developed during exposure to EE.

**Augmentation of LTP.** Natural strengthening of synaptic transmission could have diverse, functional consequences on electrically induced synaptic plasticity, tested after EE exposure. For instance, it could use up some of the available capacity of CA1 neurons to support further synaptic plasticity, thereby occluding subsequent induction of electrically induced LTP. Alternatively, the stronger synapses could serve as an effective substrate to facilitate further LTP. We find that despite EE leading to a naturally occurring increase in the number of spines and frequency of mEPSCs, it did not impair the ability of CA3–CA1 synapses to undergo further LTP after EE. Not only were these synapses able to support LTP, but also, they did so with magnitudes greater than those seen in control animals. This is in clear contrast to previous reports about the absence of an effect or occlusion of electrically induced LTP at the perforant path inputs to DG neurons after exposure to EE (Eckert et al. 2010; Feng et al. 2001; Green and Greenough 1986).

In this connection, it is also worth noting that earlier studies used LTP induction protocols involving high-frequency tetanic stimuli, which are significantly stronger than the TBS used here. Stronger induction protocols are likely to elicit LTP, which push synaptic strengths closer to saturating levels, thereby leaving less room to evaluate the full impact of an EE-induced increase in LTP. In contrast, the TBS paradigm resembles naturally occurring hippocampal theta rhythm seen in rodents during exploratory behavior (Bland 1986). Earlier studies have also established the efficacy of TBS as an optimal paradigm for triggering LTP, which uses fewer electrical pulses and hence is more physiologically relevant compared with high-frequency stimulation protocols that involve much higher levels of sustained afferent activity (Chen et al. 2006; Larson et al. 1986).

**Postsynaptic activity during LTP induction and changes in intrinsic excitability of CA1 pyramidal cells.** To probe how EE may enhance LTP, we first focused on the levels of postsynaptic activity achieved by CA1 neurons during the application of the TBS induction protocol. To this end, we compared two measures of postsynaptic activity—the number of APs fired and the underlying subthreshold depolarization—both of which have been shown to be correlated with the magnitude of LTP achieved (Linden 1999; Pike et al. 1999; Thomas et al. 1998; Urban and Barrionuevo 1996). Although exposure to EE had no effect on the mean amplitude of each of the depolarizing envelopes elicited by the five bursts of TBS, the total number of APs fired during these bursts was significantly higher in CA1 cells from EE rats. Notably, the enhanced levels of LTP in EE cells exhibited a positive correlation with the number of APs fired during TBS. These observations led us to focus on the factors that may contribute to the greater efficacy in firing APs during the induction of LTP in cells from EE animals. We found three measures of neuronal excitability to be enhanced following EE. First, somatic injections of depolarizing currents led CA1 neurons from EE rats to fire more APs. Second, there was a reduction in the threshold for firing APs in EE cells. This reduction in AP threshold was unlikely to be caused by a change in number or properties of voltage-gated sodium channels, because we did not observe a change in peak dV/dt values. Finally, EE caused a decrease in AHP amplitude in CA1 neurons, which is in agreement with a similar finding on reduction of AHP in aged rats exposed to EE (Kumar and Foster 2007). These changes are likely to act together to enhance the capacity of EE cells to fire more spikes during TBS and thereby, support larger LTP.

The increase in spiking rates in EE cells may also have interesting, functional consequences for the propagation of APs from the soma back along the dendrites. A recent study reported a reduction in currents mediated by the A-type K⁺ channels in oblique dendrites of CA1 neurons from EE rats (Makara et al. 2009). This would increase excitability of dendritic branches and facilitate the conduction of back-propagating APs (bAP), which are known to play an important role in associative, synaptic LTP (Magee and Johnston 1997). Thus the enhanced LTP seen in the present study could be mediated by the higher number of APs generated at the CA1 soma and the consequent increase in the number of bAPs and their conduction along CA1 dendrites (Chen et al. 2006). This possibility awaits further investigation.

**Stronger E-S coupling.** Enhanced AP firing in response to somatic depolarization, along with a reduction in the AP threshold, explains the increase in the number of APs fired during the application of TBS in hippocampal slices from EE rats. This finding, however, does not explain why EE cells fire more APs, despite having the same levels of postsynaptic depolarization triggered by TBS (Fig. 5). This gap was bridged by the finding that EE also strengthens baseline E-S coupling (without induction of LTP); i.e., the same-sized EPSP evoked by activation of Schaffer collateral inputs is likely to fire more APs in CA1 neurons from EE rats (Fig. 6). Stronger E-S coupling is also known to contribute to hippocampal E-S potentiation (Daoudal and Debanne 2003; Daoudal et al. 2002). E-S potentiation in the CA1 area was first observed after induction of electrically induced LTP as enhancement of population-spike amplitudes over and above what is expected from the potentiation of the field EPSP alone or even in the absence of any potentiation of the EPSP (Andersen et al. 1980; Chavez-Noriega et al. 1989, 1990). With the use of both in vitro and in vivo extracellular field potential recordings, EE has also been shown to induce E-S potentiation-like effects at perforant path inputs to the DG (Green and Greenough 1986; Irvine et al. 2006). Interestingly, exposure to EE also occludes the induction of LTP at the same inputs to DG. In contrast, we find that EE enhances both LTP and E-S coupling in CA1 pyramidal cells. Indeed, a stronger coupling between the EPSP and spike appears to create optimal conditions for enhancing synaptic potentiation, not impeding it, in area CA1.

Our results on EE leading to enhanced LTP, along with increases in intrinsic excitability and E-S coupling, are also
consistent with earlier reports about molecular signaling mechanisms activated by EE. In particular, EE is known to upregulate the levels of cAMP response element-binding (CREB) and PKC (Ickes et al. 2000; Mohammed et al. 2002; Paylor et al. 1992; Williams et al. 2001). Interestingly, not only are PKC and CREB key mediators of synaptic plasticity, but also, they regulate neuronal excitability (Astman et al. 1998; Lopez de Armentia et al. 2007). Hence, future studies would be required to investigate whether EE-induced activation of these signaling mechanisms provides a common molecular substrate for enhancing intrinsic excitability and LTP and the synergy between them.

**Functional implications for hippocampal learning and memory.** The range of changes in CA1 pyramidal neurons described here is ideally positioned to support enhanced synaptic plasticity and its functional consequences at the behavioral level. Accumulating evidence has established a critical role for LTP in area CA1 in forms of spatial memory that depend on the hippocampus. In the present study, rats were given a relatively brief period of exposure to the spatial context before receiving the unconditioned stimulus (i.e., footshock). In other words, in this task, we deliberately restricted the time available to the animals to form a robust representation of the context in which they were subjected to the aversive conditioning. Strikingly, prior exposure to EE enabled the rats to overcome this challenge and exhibit significantly stronger recall compared with control animals. This result is in agreement with an earlier report (Woodcock and Richardson 2000) showing that differences between control and EE rats in contextual conditioning were evident with a 16-s pre-eshock period but not a longer pre-eshock period. These findings suggest that EE-induced facilitation of LTP and neuronal excitability acts in concert to enhance the capacity of CA1 pyramidal cells in a manner that enables the animals to perform better in a more difficult task requiring better discriminative ability (Fanselow 1986, 2000).

Our findings also point to potential differences in the effects of EE on synaptic plasticity in different subregions of the hippocampus. As reported earlier, exposure to EE caused E-S potentiation but occluded LTP at excitatory synaptic inputs to DG granule cells (Green and Greenough 1986; Irvine et al. 2006). In the CA1 area, in contrast, EE enhances both LTP and E-S coupling at the CA3–CA1 synapses. Rapid progress in cell type-restricted gene ablation techniques has greatly advanced our understanding of the distinct roles played by synaptic plasticity mechanisms in the DG and CA1 area in different facets of spatial learning and memory (Nakazawa et al. 2004). For instance, NMDARs in CA1 pyramidal cells are known to play a pivotal role in the acquisition of spatial reference memory (Nakazawa et al. 2004; Tsien et al. 1996). On the other hand, mutant mice lacking NMDARs in the DG are reported to have an impaired ability to distinguish two similar contexts but perform normally in contextual fear conditioning (Lee and Kesner 2004; McHugh et al. 2007; Nakazawa et al. 2004). These findings on region-specific differences elicited by EE highlight the need to further explore their functional implications for specific mnemonic functions performed by specific microcircuits within the hippocampus.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

Author contributions: R.M. and S.C. conception and design of research; R.M. performed experiments; R.M. analyzed data; R.M. and S.C. interpreted results of experiments; R.M. prepared figures; R.M. and S.C. drafted manuscript; R.M. and S.C. edited and revised manuscript; R.M. and S.C. approved final version of manuscript.

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