Long-Term Depression in Identified Stellate Neurons of Juvenile Rat Entorhinal Cortex

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Yue P-Y, Lei S. Long-term depression in identified stellate neurons of juvenile rat entorhinal cortex. J Neurophysiol 97: 727–737, 2007. First published November 29, 2006; doi:10.1152/jn.01089.2006. The entorhinal cortex (EC) serves as a gateway to the hippocampus and plays a pivotal role in memory processing in the brain. Superficial layers of the EC convey the cortical input projections to the hippocampus, whereas deep layers of the EC relay hippocampal output projections back to the superficial layers of the EC or to other cortical regions. Whereas the EC expresses long-term potentiation (LTP) and depression (LTD), the underlying cellular and molecular mechanisms have not been determined. Because the axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus granule cells of the hippocampus, we studied the mechanisms underlying the long-term plasticity in identified stellate neurons. Application of high-frequency stimulation (100 Hz for 1 s, repeated 3 times at an interval of 10 s) or forskolin (50 μM) failed to induce significant changes in synaptic strength, whereas application of pairing (presynaptic stimulation at 0.33 Hz paired with postsynaptic depolarization from −60 to −10 mV for 5 min) or low-frequency stimulation (LFS, 1 Hz for 15 min) paradigm-induced LTD. Pairing- or LFS-induced LTDs were N-methyl-d-aspartate receptor-dependent and occluded each other suggesting that they have the similar cellular mechanism. Pairing-induced LTD required the activity of calcineurin and involved AMPA receptor endocytosis that required the function of ubiquitin–proteasome system. Our study provides a cellular mechanism that might in part explain the role of the EC in memory.

INTRODUCTION

Long-term potentiation (LTP) and depression (LTD) are considered as the cellular models for learning and memory (Izquierdo 1994). The entorhinal cortex (EC) is part of a network that aids in the consolidation and recall of memories (for reviews, see Dolcos et al. 2005; Haist et al. 2001; Squire et al. 2004; Steffenach et al. 2005). The EC has been regarded as the gateway to the hippocampus because it mediates the majority of connections between the hippocampus and other cortical areas (Witter et al. 1989, 2000). Sensory inputs converge onto the superficial layers (layers I–III) of the EC (Burwell 2000), which give rise to dense projections to the hippocampus (Amaral and Witter 1995). On the other hand, neurons in the deep layers of the EC (layers IV–VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (Dolorfo and Amaral 1998a,b; Gloveli et al. 2001; Kohler 1986, 1988; van Haefen et al. 2003) and to other cortical areas (Amaral and Witter 1995; Witter et al. 1989). Similar to other synapses in the brain, the EC expresses N-methyl-d-aspartate (NMDA) receptor-dependent LTP (Alonso et al. 1990; de Curtis and Llinas 1993; Yang et al. 2004) and LTD (Bouras and Chapman 2003; Cheong et al. 2002; Kourrich and Chapman 2003; Solger et al. 2004; Yang et al. 2004; Zhou et al. 2005). However, the synapse specificity and the cellular and molecular mechanisms underlying the long-term plasticity in the EC remain to be determined.

About 70% of the neurons in layer II of the EC are stellate neurons (Klink and Alonso 1997) the axons of which form the perforant path that innervates the dentate gyrus granule cells, CA1 and CA3 pyramidal neurons, and several subtypes of hippocampal interneurons (Ruth et al. 1982, 1988; Steward and Scoville 1976). Stellate neurons themselves receive glutamatergic innervations from the pyramidal neurons in the deep layers of the EC (Witter et al. 2000). The excitability of the stellate neurons therefore is likely to play a pivotal role in controlling the functions of the hippocampus. Whereas the cellular and molecular mechanisms of long-term plasticity at hippocampal synapses are well studied, those at the EC synapses have not been determined. In the present study, we studied the long-term plasticity at synapses formed between layer II stellate neurons and the inputs of the pyramidal neurons in the deep layers of the EC in juvenile rats. Our results indicate that stellate neuron synapses display NMDA receptor-dependent LTD with no expression of LTP. We have also shown that the expression of LTD was mediated by AMPA receptor endocytosis that required the functions of calcineurin and ubiquitin–proteasome system.

METHODS

Hippocampal slice preparation

Horizontal hippocampal slices (400 μm) including the EC, subiculum and hippocampus were cut using a Vibratome (Leica VT1000S) usually from 15- to 22-day-old Sprague Dawley rats as described previously (Deng and Lei 2006; Deng et al. 2006). After being deeply anesthetized with isoflurane, rats were decapitated, and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 5.0 MgCl₂, and 10 glucose, saturated with 95% O₂-5% CO₂, pH 7.4. Slices were initially incubated in the preceding solution at 35°C for 40 min for recovery and then kept at room temperature (24°C) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

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Whole cell and perforated-patch recordings

Whole cell patch-clamp recordings using an Axopatch 200B or a Multiclamp 700B (Axon Instruments, Foster City, CA) in current- or voltage-clamp mode were made from stellate neurons in layer II of the EC visually identified with infrared video microscopy (Olympus BX51WI) and differential interference contrast optics. Unless stated otherwise, recording electrodes were filled with the following (in mM): 100 K-glucuronate, 0.6 EGTA, 5 MgCl₂, 8 NaCl, 2 ATP₂Na, 0.3 GTPNa, 40 HEPEs, and 0.2 QX-314, pH 7.4. For perforated-patch recordings, recording pipettes were tip-filled with the above intracellular solution and then back-filled with freshly prepared intracellular solution containing amphotericin B (200 µg/ml, Calbiochem, San Diego, CA) (Rae et al. 1991). Patch pipettes had resistances of 4–6 MΩ when filled with the preceding solution. Stable series resistances (50–70 MΩ) were usually obtained ∼30 min after forming a gigahm seal. The extracellular solution comprised (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaHPO₄, 2.5 CaCl₂, 1.5 MgCl₂, 10 glucose, and 0.01 bicuculline methobromide, saturated with 95% O₂-5% CO₂, pH 7.4. Stellate neurons were identified by their location, shape, and electrophysiological properties. Stellate neurons are located in layer II and the border of layer II and III, and they have larger and polygonal soma with variable number of main dendrites radiating out from the cell body but are devoid of a clearly dominant dendrite (van der Linden and Lopes da Silva 1998) (Fig. 1, A and B). These neurons have unique electrophysiological properties, i.e., hyperpolarizing current pulse injection always caused the membrane potential to attain an early peak and then “sag” to a steady-state level (Alonso and Klink 1993) (Fig. 1C). The generation of sag response is due to the selective expression of hyperpolarization-activated cation channels (H-channels) in stellate neurons (Dickson et al. 2000). Because QX-314 is a potential H-channel blocker (Bischoff et al. 2003) and its inclusion in the recording pipettes would reduce the sag response, we initially tried to use intracellular solution that did not contain QX-314. However, the contamination of the action potential prevented reliable recordings of excitatory postsynaptic currents (EPSCs). We therefore included a low concentration (0.2 mM) of QX-314 in the recording pipettes. After formation of whole cell recordings, we quickly (usually in <2 min) recorded the voltage responses by injecting currents from +0.1 to −1 nA at an interval of ∼0.1 nA (Fig. 1C) in current clamp. In this condition, we can still observe nice initial sag response but prevented the contamination of action potentials on the recordings of EPSCs when QX-314 was dialyzed into the soma and dendrites later. The effect of QX-314 on sag response was likely to be negligible because action potentials were still observed in this period (Fig. 1C). We used the method developed by van der Linden and Lopes da Silva (1998) and Dorval and White (2005) by calculating the percentage of the sag according to the equation [(peak – steady-state)/peak × 100%]. Plot of the percentage of the sag responses versus the currents injected showed a linear relationship (Fig. 1D). Because Dorval and White (2005) defined stellate neurons as cells displaying >20% sag response and our cells surpassed this criterion at every current injected (Fig. 1D), we defined cells exhibiting >20% sag in response to −1 nA hyperpolarizing current injection as stellate neurons. After identifying the stellate neurons in current clamp, we switched to voltage clamp to record AMPA receptor-mediated EPSCs evoked by placing a stimulation electrode in layer IV to stimulate the inputs from the deep layers (Fig. 1A). The holding potential was at −60 mV. In this condition, the recorded responses were completely blocked by 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10 µM), indicating that they were mediated by AMPA/kainate receptors. Series resistance was rigorously monitored by the delivery of 5-mV voltage steps after each evoked current. Experiments were discontinued if the series resistance changed by >10%. Synthetic responses were evoked at 0.33 Hz by low-intensity stimulation (80- to 100-µs duration; 40- to 80-µA intensity) via a constant-current isolation unit (A360; World Precision Instrument, Sarasota, FL) connected to a patch electrode filled with oxygenated extracellular solution. Data were filtered at 2 kHz, digitized at 10 kHz, acquired on-line, and analyzed off-line with pClamp9 software (Axon Instruments). Synthetic responses were included in analysis if the rise time and decay time constants were monotonic and possessed no apparent multiple or polysynaptic waveforms. Three paradigms were used to induce long-term plasticity at the stellate neuron synapses: high-frequency stimulation (HFS, 100 Hz for 1 s, repeated 3 times at an interval of 10 s, holding potential: −60 mV for whole cell and perforated-patch recordings), pairing (presynaptic stimulation at 0.33 Hz paired with postsynaptic depolarization from −60 to −10 mV for 5 min) and low-frequency stimulation (LFS, 1 Hz for 15 min, holding potential: −60 mV). HFS was conducted with PluseMaster A300 (World Precision Instrument).

Recordings of evoked field potentials

An extracellular recording pipette (2–5 MΩ) containing 2 M NaCl was positioned in layer II of the EC and a concentric bipolar stimulation electrode (Frederick Haer) was placed in layer IV to stimulate the inputs from the deep layers. Constant current pulses (0.1 ms, 100–300 µA) were delivered using a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360). Evoked field potentials were amplified 100 times, filtered at 1 Hz, and digitized at 10 kHz. The slope of evoked field potential responses was measured using the program Clampfit (Axon Instruments).

Peak-scaled nonstationary variance analysis

Peak-scaled nonstationary variance analysis was used to calculate the conductance and numbers of synaptic AMPA receptors (Benke et al. 1998; Lei and McBain 2002; Lei et al. 2003; Traynelis and...
Jaramillo 1998; Traynelis et al. 1993) before and after the induction of LTD. The recorded AMPA receptor EPSCs were initially inspected visually to exclude those responses contaminated with spontaneous synaptic activity. Only those traces showing fast rise time (20–80% rise time <0.8 ms) and smooth decay (successful fitting of a period of 87 ms from the peak by two exponential functions) were selected for analysis. The selected EPSCs were aligned and averaged. The average response was scaled to the peak and subtracted from individual responses to compute the variance. A period of 0–87 ms commencing at the EPSC peak was selected for analysis. The average EPSC response was then divided into 100 equally sized bins and the corresponding variances pooled. The binned variance was plotted against the mean current amplitude, and the single-channel current and the number of AMPA receptors were calculated by fitting the data according to the following equation: $\sigma^2 = il - P/N + \sigma_{base}$, where $\sigma^2$ is the variance, $i$ is the mean current, $N$ is the number of open channels, $l$ is the single-channel current, and $\sigma_{base}$ is the background variance. The single-channel conductance was measured by $\gamma = il(E - E_{rev})$, where $E$ is the holding potential, and $E_{rev}$ is the reversal potential that was measured to be close to 0 mV under our recording conditions.

Data analysis

Data are presented as the means ± SE. Student’s paired or unpaired $t$-test or ANOVA was used for statistical analysis as appropriate; $P$ values are reported throughout the text and significance was set as $P < 0.05$.

Chemicals

AM251, (±)-amino-4-carboxy-methyl-phenylacetic acid (MCPG), lactacystin, pep2m peptide (KRMKVAKNAQ), and (2S,2R,3R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) were products of Tocris (Ellisville, MO). Epoxomicin and forskolin were purchased from BIOMOL (Plymouth Meeting, PA). All other compounds were products of Sigma-Aldrich (St. Louis, MO).

RESULTS

Stellate neuron synapses are devoid of HFS-induced LTP but exhibit LFS- and pairing-induced LTD

Stellate neurons in layer II of the EC receive glutamatergic innervation from the pyramidal neurons in the deep layers and the axons of stellate neurons form the perforant path that innervates the dentate gyrus granule cells. We initially examined the long-term plasticity at the stellate neuron synapses. We recorded from the stellate neurons in layer II of the EC and stimulated the inputs from the deep layers by placing a stimulation electrode in layer IV (Fig. 1A). After recording sag response in current clamp to identify stellate neurons, we switched to voltage clamp to record AMPA receptor-mediated EPSCs. GABA$_A$ responses were blocked by including bicuculline (10 μM) in the extracellular solution. In this condition, HFS (100 Hz for 1 s, repeated 3 times at an interval of 10 s), a protocol used widely to induce LTP, failed to induce significant changes in AMPA EPSCs (99 ± 7% of control, $n = 8$, $P = 0.87$, Fig. 2A). We tested the efficacy of the induction protocol by recording from CA1 pyramidal neurons of the hippocampus and placing the stimulation electrode in the stratum radiatum to stimulate Schaffer collateral fibers. At this synapse type, application of the same HFS paradigm induced robust posttetanic potentiation and LTP (167 ± 19% of control, $n = 5$, $P = 0.02$, Fig. 2B), excluding the inefficiency of the induction protocol. To test the possibility that intracellular constituents required for LTP induction in stellate neurons of the EC were washed out during the period of whole cell
recordings, we utilized perforated-patch recordings to perform the experiments. Application of the HFS induction protocol still failed to induce LTP (101 ± 8% of control, n = 6, P = 0.92, Fig. 2C) in this recording configuration.

The preceding experiments were performed in the presence of bicuculline to block GABA_A receptors. To test whether inclusion of bicuculline prevented the induction of LTD, we recorded the evoked field potentials in the absence of bicuculline from layer II of the EC by positioning the stimulation electrode in layer IV to stimulate the inputs from the deep layers. In the presence of normal Ca^{2+} concentration (2.5 mM), application of the HFS induction paradigm failed to induce LTP (105 ± 14% of control, n = 5, P = 0.72, Fig. 2D). Furthermore, increasing the extracellular Ca^{2+} concentration to 5 mM failed to induce LTP (99 ± 5% of control, n = 5, P = 0.91, Fig. 2E). Because all the preceding experiments were conducted in slices from juvenile rats, we then extended our experiments to adult rats to test whether the expression of LTD was developmentally regulated. Application of the HFS paradigm did not induce LTD in slices cut from adult rats (94 ± 6% of control, n = 7, P = 0.35, Fig. 2F). Taken together, we concluded that the stellate neuron synapses do not express HFS-induced LTD.

We then used the pairing paradigm (presynaptic stimulation at 0.33 Hz together with postsynaptic depolarization from −60 to −10 mV for 5 min) to test whether stellate neuron synapses exhibit other forms of LTD. Application of the pairing paradigm did not induce LTD but instead induced LTD (60 ± 5% of control, n = 20, P < 0.00001, 20 min after the induction protocol, Fig. 3A). Although the pairing-induced LTD in some cells developed slowly and stabilized ~15 min after the induction protocol (Fig. 3A), it was not due to the run-down of synaptic strength because postsynaptic depolarization from −60 to −10 mV for 5 min without presynaptic stimulation did not significantly change AMPA receptor-mediated EPSCs (98 ± 7% of control, n = 6, P = 0.76, Fig. 3A). To test whether LTD could be induced after preconditioning the slices with the pairing-induced LTD, we applied HFS induction protocol after the expression of the pairing-induced LTD. In five experiments, application of the pairing protocol induced the expression of LTD (56 ± 9% of control, n = 5), but subsequent application of HFS did not significantly change the synaptic strength (97 ± 2% of the value prior to HFS paradigm, n = 5, P = 0.11, Student’s paired t-test, data not shown), suggesting that there is no bidirectionality of synaptic plasticity. Furthermore, application of the pairing paradigm induced robust LTD at the Schaffer collateral-CA1 pyramidal neuron synapses of the hippocampus (213 ± 28% of control, n = 6, P = 0.009, Fig. 3B) excluding the inefficacy of the induction paradigm. We also used perforated-patch recordings to test whether intracellular molecules required for LTD induction were washed out during the processes of whole cell recordings. In this recording configuration, application of the pairing paradigm still induced LTD (57 ± 5% of control, n = 9, P < 0.0001, Fig. 3C). We also performed the experiments in high concentration of extracellular Ca^{2+} (5 mM). Application of the pairing paradigm in 5 mM Ca^{2+} still induced LTD (47 ± 8% of control, n = 7, P = 0.0005, Fig. 3D).

We next examined whether the stellate neuron synapses express chemical LTD by applying forskolin, an adenylyl cyclase activator that increases the generation of cyclic AMP and induces LTD (Huang et al. 1994; Maccaferri et al. 1998; Weisskopf et al. 1994). Application of forskolin (50 μM) did not induce LTD at the stellate neuron synapses (95 ± 6% of control, n = 7, P = 0.44, Fig. 3E), whereas application of the same concentration of forskolin induced robust LTD at the

![Diagram of EC stellate neuron and CA1 pyramidal neuron synapses](http://jn.physiology.org/figure/10.1152/jn.00875.2005/B63730_10.1152/jn.00875.2005.B63730.html)

**Fig. 3.** Stellate neuron synapses express pairing- and low-frequency stimulation (LFS)-induced long-term depression (LTD) but not forskolin-induced LTD. A: application of pairing (presynaptic stimulation at 0.33 Hz paired with postsynaptic depolarization from −60 to −10 mV for 5 min) paradigm induced LTD (n = 20), whereas postsynaptic depolarization from −60 to −10 mV for 5 min without presynaptic stimulation did not change synaptic strength (n = 6). Top: traces averaged from 10 AMPA EPSCs as indicated in the figure. The rest of the figure was arranged in the same way. B: application of the same pairing paradigm induced LTD at the Schaffer collateral-CA1 pyramidal neuron synapses (n = 6). C: pairing-induced LTD at the stellate neuron synapses recorded by perforated patch recordings (n = 9). D: pairing still induced LTD when extracellular Ca^{2+} concentration was elevated to 5 mM (n = 7). E: application of forskolin (50 μM) did not induce LTD at the stellate neuron synapses (n = 7). F: stellate neuron synapses expressed LFS (1 Hz, for 15 min)-induced LTD (n = 6).
mossy fiber-CA3 pyramidal neuron synapses of the hippocampus (188 ± 17% of control, n = 5, P = 0.006, data not shown), suggesting that the stellate neuron synapses do not express forskolin-induced chemical LTP.

Because application of LFS using field potential recordings induced LTD in the superficial layers of EC (Cheong et al. 2002; Kourrich and Chapman 2003; Solger et al. 2004), we then tested whether stellate neuron synapses express this type of LTD. Application of LFS (1 Hz for 15 min) produced robust LTD (59 ± 11% of control, n = 6, P = 0.01, Fig. 3F), suggesting that stellate neuron synapses express LFS-induced LTD.

Stellate neuron LTDs are mediated by an increase in postsynaptic Ca\(^{2+}\) and are NMDA receptor-dependent

Among all the paradigms used to induce long-term synaptic plasticity, we observed the pairing- and LFS-induced LTD. We next examined whether increases in intracellular Ca\(^{2+}\) were required for these two forms of LTD. Inclusion of bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid (BAPTA; 30 mM) in the recording pipettes blocked pairing-induced LTD (95 ± 4% of control, n = 7, P = 0.22, Fig. 4A), indicating that an increase in intracellular Ca\(^{2+}\) is required for pairing-induced LTD. Similarly, inclusion of BAPTA (30 mM) in the recording pipettes blocked LFS-induced LTD (96 ± 2% of control, n = 6, P = 0.16, Fig. 4B). These results suggest that both pairing- and LFS-induced LTDs require an increase in postsynaptic Ca\(^{2+}\).

We then tested whether NMDA receptors were required for pairing- or LFS-induced LTDs. Application of DL-APV (100 μM) blocked both pairing-induced LTD (99 ± 9% of control, n = 11, P = 0.96, Fig. 4C) and LFS-induced LTD (98 ± 3% of control, n = 5, P = 0.55, Fig. 4D), suggesting that NMDA receptors are required for both pairing- and LFS-induced LTDs consistent with previous results (Cheong et al. 2002; Kourrich and Chapman 2003; Solger et al. 2004).

If NMDA receptors are required for both the pairing- and LFS-induced LTDs, it is possible that these two forms of LTD share the same mechanism. If so, expression of one form of LTD would occlude the expression of the other and vice versa. We applied the induction protocol for the first form of LTD three times to saturate its expression before applying the induction protocol for the second form of LTD. Application of the pairing paradigm the second time reduced AMPA EPSCs to 61 ± 7% of control (n = 6); this was not significantly different from the EPSCs after the third application of the induction protocol (53 ± 10% of control, n = 6, P = 0.28, Fig. 4E), suggesting that the expression of LTD was saturated by the pairing paradigm. Subsequent application of LFS protocol (1 Hz for 15 min) did not induce LTD (96 ± 17% of the value before application of 1-Hz protocol, n = 6, P = 0.76, Fig. 4E). Similarly, application of LFS protocol (1 Hz for 15 min) twice reduced AMPA EPSCs to 39 ± 8% of control (n = 5); this was not significantly different from the EPSCs after the third application of the induction protocol (31 ± 5% of control, n = 5, P = 0.38, Fig. 4F), suggesting that LFS-induced LTD was saturated. In this condition, application of the pairing paradigm failed to induce LTD (95 ± 9% of the value prior to the application of pairing protocol, n = 5, P = 0.44, Fig. 4F).

These results suggest that pairing- and LFS-induced LTDs share the similar mechanism at stellate neuron synapses.

Stellate neuron synapses express lower NMDA/AMPA EPSC ratio

Activation of NMDA receptors can produce either LTP or LTD. It is generally believed that high and fast increases in intracellular Ca\(^{2+}\) are supposed to induce LTP, whereas low and slow increases in intracellular Ca\(^{2+}\) generate LTD. The density of NMDA receptors at individual synapses is likely to
be a determinant that controls the amount and velocity of Ca$^{2+}$ influx when NMDA receptors are activated. Because application of the pairing-paradigm induced LTP at CA1 pyramidal neuron synapses but LTD at stellate neuron synapses, we reasoned that the distinct effects might be due to differences in NMDA receptor density at those two synapses, i.e., the density of NMDA receptors at the stellate neuron synapses is lower than that at the CA1 pyramidal neuron synapses. We tested this possibility by comparing NMDA/AMPA EPSC ratio at these two synapse types. We initially recorded AMPA EPSCs at −60 mV and then recorded the EPSCs mediated by both NMDA and AMPA receptors at +40 mV. NMDA receptor-mediated current was measured 50 ms after the stimulus artifact at +40 mV (Lei and McBain 2004). NMDA/AMPA EPSC ratio at the stellate neuron synapses (0.81 ± 0.13, n = 6) was lower than that measured at CA1 pyramidal neuron synapses of the hippocampus (1.37 ± 0.10, n = 7, P = 0.005, Fig. 5A), suggesting that the low density of NMDA receptors at the stellate neuron synapses might be the reason that application of the pairing paradigm at this synapse type induced LTD instead of LTP.

**Pairing-induced LTD at the stellate neuron synapses is independent of L-type Ca$^{2+}$ channels, metabotropic glutamate receptors, or cannabinoids receptors**

Because the pairing-induced LTD has not been reported previously in the EC, we next studied the mechanisms underlying this form of LTD. Pairing-induced LTD was not blocked by application of nimodipine (10 μM, 50 ± 3% of control, n = 5, P < 0.0001, Fig. 5B), suggesting that L-type Ca$^{2+}$ channels are not required. To ensure that the used nimodipine was effective, we induced LTD at the mossy fiber-CA3 pyramidal neuron synapses in the presence of nimodipine because membrane depolarization (from −60 to −10 mV for 5 min) induces L-type Ca$^{2+}$ channel-dependent LTD at this synapse type (Lei et al. 2003). Application of nimodipine (10 μM) blocked depolarization-induced LTD at the mossy fiber-CA3 pyramidal neuron synapses (93 ± 8% of control, n = 6, P = 0.4), whereas depolarization still induced LTD in the absence of nimodipine at this synapse type (56 ± 3% of control, n = 6, P < 0.001, data not shown). Because metabotropic glutamate receptor (mGluR)-mediated LTD has been observed in the perirhinal cortex (McCaffrey et al. 1999), we tested whether pairing-induced LTD was mediated via activation of mGluRs. Application of the broad spectrum, nonselective group I/II mGluR antagonist MCPG (1 mM) failed to block pairing-induced LTD (47 ± 6% of control, n = 5, P = 0.001, Fig. 5C), suggesting that the function of mGluRs is not required for pairing-induced LTD at the stellate neuron synapses. We also performed a positive control experiment to ensure that MCPG was effective. Because mossy fiber terminals of the hippocampus express group II mGluRs and application of DCG-IV activates these receptors and inhibits glutamate release (Lei et al. 2003), we recorded AMPA EPSCs from the CA3 pyramidal neurons and stimulated mossy fibers by placing the stimulation electrode in the stratum lucidum. Application of DCG-IV (1 μM) inhibited AMPA EPSCs to 76 ± 3% of control (n = 6) in the presence of MCPG (1 mM), whereas application of the same concentration of DCG-IV alone inhibited AMPA EPSCs to 18 ± 2% of control (n = 6, P = 0.0001, Student’s unpaired t-test), suggesting that MCPG was effective at blocking mGluRs. Together, these results indicate that group I/II mGluRs are not required for the induction of LTD at the stellate neuron synapses.

In many cells, postsynaptic depolarization can potentially release endogenous cannabinoids, which translocate to the presynaptic terminals to transiently inhibit transmitter release (for review, see Alger 2002), and cannabinoids are involved in LTP (Carlson et al. 2002; Misner and Sullivan 1999) as well as LTD (Chevaleyre and Castillo 2003; Gerdeman et al. 2002). We therefore tested whether pairing-induced LTD requires the function of cannabinoids. In slices pretreated and perfused with the CB1 receptor antagonist AM-251 (10 μM), pairing still induced robust LTD (62 ± 10% of control, n = 6, P = 0.01, Fig. 5D). To ensure that AM-251 was effective at blocking CB1 receptors, we recorded GABA$\text{A}$ receptor-mediated inhibitory postsynaptic currents (IPSCs) from CA1 pyramidal neurons of the hippocampus by placing the stimulation electrode in the stratum radiatum. We replaced the intracellular K$^+$-glutonate with the same concentration of CsCl and the extracellular solution contained DNIQX (10 μM) to block AMPA responses. The cells were held at −60 mV. In this condition,
application of WIN 55212-2 (2 μM), an agonist for cannabinoid receptors, inhibited IPSCs to 26 ± 4% of control (n = 5; P < 0.001). This effect was blocked by application of AM-251 (2 μM, 98 ± 6% of control, n = 6, P = 0.8, data not shown), suggesting that AM-251 was effective at blocking CB1 receptors, consistent with previous results (Hajos and Freund 2002). Together, these results indicate that pairing-induced LTD is unlikely to be mediated by cannabinoids.

**Postsynaptic expression of pairing-induced LTD at stellate neuron synapses**

We then examined whether the expression of pairing-induced LTD at stellate neuron synapses was pre- or postsynaptic in origin using the following two approaches. First, we calculated the coefficient of variation (CV = SD/mean) before and after the induction of LTD (Fig. 6A). The values of CV were not changed after LTD induction (control: 0.141 ± 0.017, LTD: 0.146 ± 0.018, n = 20, P = 0.39, Fig. 6A). Second, we calculated the paired-pulse ratio (PPR) before and after LTD induction. PPR was not significantly changed after the induction of LTD (control: 1.09 ± 0.05, LTD: 1.11 ± 0.07, n = 10, P = 0.76, Fig. 6B). These data suggest that the expression of LTD is postsynaptic.

**Stellate neuron LTD involves a reduction in postsynaptic AMPA receptor number**

LTD at the stellate neuron synapses could result from a decrease in single-channel conductance (γ) or open probability of AMPA receptors or a reduction in the number of available postsynaptic AMPA receptors. To differentiate these possibilities, we used peak-scaled nonstationary variance analysis (Benke et al. 1998; Lei and McBain 2002; Lei et al. 2003; Traynelis and Jaramillo 1998; Traynelis et al. 1993). The synaptic current amplitude varies not only as a result of random channel gating but also with variations in the transmitter release, the temporal and spatial transmitter concentration profile and the numbers of channels in the postsynaptic membranes (when synaptic currents arise from different release sites). Because variations in amplitude will contaminate the calculation of variance attributable to channel gating, the individual events have to be scaled such that their peak amplitude equals the average peak amplitude. Therefore peak-scaled variance analysis yields estimates of the single-channel conductance from 7 neurons before and after the induction of LTD. Individual experiments and mean data are indicated (□ and •, respectively). D: summarized data for the number of the AMPA receptors before and after LTD induction. Note that the number of AMPA receptors was significantly reduced after the induction of LTD (n = 7; P = 0.0007).

**FIG. 6. Postsynaptic expression of pairing-induced LTD.** A: lack of change in CV (SD/mean) before and after LTD induction (n = 20). Top: consecutive 15 EPSCs before (left) and after (right) LTD induction. Empty circles: individual experiments; black circles, mean data. B: paired-pulse ratio (PPR) evoked by 2 stimuli at an interval of 40 ms was not altered after the induction of LTD (n = 10). Top left: EPSCs evoked by 2 stimuli before (thin) and after (thick) LTD induction. Top right: EPSCs before and after LTD induction were scaled to show the lack of difference in PPR. Empty circles: individual experiments; black circles, mean data.
with the mechanisms observed at other synapses (Linden 2001).

**Stellate neuron LTD involves AMPA receptor internalization**

Postsynaptic AMPA receptor translocation is an important mechanism in the expression of NMDA receptor-dependent LTD (Carroll et al. 1999; Lüscher et al. 1999; Lüthi et al. 1999; Man et al. 2000; Matsuda et al. 2000; Sheng and Kim 2002; Song and Huganir 2002; Wang and Linden 2000). NMDA receptor-dependent LTD expression involves a pool of AMPA receptors regulated by NSF-GluR2 interactions at Schaffer collateral-CA1 pyramidal neuron (Lüscher et al. 1999; Lee et al. 2002; Lüthi et al. 1999; Noel et al. 1999) and cerebellar (Steinberg et al. 2004) synapses. NMDA receptor-induced endocytosis of AMPA receptors is dependent on both Ca$^{2+}$ and the activity of protein phosphatase (Beattie et al. 2000; Ehlers 2000; Morishita et al. 2005). We next examined whether expression of LTD at stellate neuron synapses similarly involved a translocation of AMPA receptors using the broad-spectrum NSF inhibitory peptide, commonly referred to as pep2m (Lüscher et al. 1999; Lüthi et al. 1999; Lee et al. 2002; Shi et al. 2001). Infusion of pep2m (0.5 mM) into the cells via the recording pipette led to a reduction in AMPA receptor EPSCs (51 ± 8% of control, n = 7, P = 0.0009, Fig. 8A), suggesting that translocation of AMPA receptors at stellate neuron synapses is dependent on NSF-GluR2 interactions. In the continuous presence of pep2m, application of the pairing paradigm failed to induce LTD (86 ± 7% of the value before pairing, n = 7, P = 0.1, Fig. 8A), suggesting that LTD at stellate neuron synapses involves the endocytosis of a pool of AMPA receptors that require the function of NSF.

**Stellate neuron LTD is ubiquitination-dependent**

The endocytosis of AMPA receptors has recently been shown to require the activity of the ubiquitin-proteasome system (Bingol and Schuman 2004; Burbea et al. 2002; Collledge et al. 2003; Turrigiano 2002). Ubiquitination is conducted in a stepwise manner by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, which recognize target proteins and catalyze the covalent attachment of ubiquitin to these target substrates. Ubiquitinated membrane proteins are substrates for endocytosis and eventually degraded by proteasome. Inhibition of the proteasome activity has been shown to block NMDA receptor-mediated LTD in hippocampal CA1 pyramidal neuron synapses (Collledge et al. 2003). We next tested whether the function of proteasome was required for stellate neuron LTD using two proteasome inhibitors of different structures: lactacystin and epoxomicin. Dialysis of lactacystin (10 μM) into the cells via the recording pipettes blocked pairing-induced LTD (94 ± 7% of control, n = 6, P = 0.46, Fig. 8B), whereas inclusion of the vehicle (0.05% DMSO) in the recording pipettes failed to block pairing-induced LTD (54 ± 2% of control, n = 5, P < 0.0001, Fig. 8B). Pretreatment of slices with epoxomicin (100 mM), a cell-permeable proteasome inhibitor, also blocked pairing-induced LTD at the stellate neuron synapses (90 ± 6% of control, n = 6, P = 0.63, Fig. 8C). Together, these results indicate that the function of proteasome is required for the endocytosis of AMPA receptors and LTD at the stellate neuron synapses.

**Calcineurin is required for stellate neuron LTD**

In hippocampal CA1 pyramidal neurons, NMDA receptor-mediated modest increase in postsynaptic Ca$^{2+}$ preferentially activates phosphatase 2B, calcineurin, to induce AMPA receptor endocytosis (Beattie et al. 2000; Morishita et al. 2005) and LTD (Mulkey et al. 1993, 1994). We next tested whether calcineurin was required for pairing-induced LTD at the stellate neuron synapses. We pretreated the slices with the calcineurin inhibitor, FK506 (50 μM), which was also continuously bath applied during electrophysiological recordings. In this condition, application of the pairing paradigm failed to induce LTD (94 ± 3% of control, n = 6, P = 0.12, Fig. 8D), whereas pretreatment with and bath application of rapamycin (50 μM), a compound with a structure similar to FK506 but lacking any calcineurin inhibitory activity (Kunz and Hall 1993), had no effect on pairing-induced LTD (55 ± 2% of control, n = 6, P < 0.0001, Fig. 8D). These results indicate that the calcium-dependent phosphatase, calcineurin, is required for LTD at the stellate neuron synapses of the EC.
DISCUSSION

Our study is the first one to investigate the cellular and molecular mechanisms underlying LTD in the EC. Our results demonstrate that the EC stellate neuron synapses express NMDA receptor-dependent LTD. The expression of stellate neuron LTD is postsynaptic and requires an increase in intracellular Ca\(^{2+}\) and the activity of Ca\(^{2+}\)-dependent phosphatase 2B, calcineurin. Stellate neuron LTD involves a reduction in the number of postsynaptic AMPA receptors. The endocytosis of AMPA receptors requires the function of ubiquitin-proteasome system.

Whereas LFS-induced NMDA receptor-dependent LTD was detected in the superficial layers of the EC using extracellular field recordings (Cheong et al. 2002; Kourrih and Chapman 2003; Solger et al. 2004; Yang et al. 2004), the following questions still remain to be answered. At which synapse(s) in the superficial layers of the EC does LTD occur? How does NMDA receptor activation lead to a reduction in synaptic strength in the superficial layers of the EC? Are there any other forms of synaptic plasticity in this brain region? Our study addressed these questions.

Because ~70% of the cells in layer II of the superficial layers are stellate neurons (Klink and Alonso 1997) and the axons of these neurons form the major components of the perforant path that provides the major inputs to the hippocampus, we chose to study the long-term plasticity at the stellate neuron synapses. We initially identified the stellate neurons by their location, morphology, and electrophysiological properties. We then combined whole cell, perforated-patch, and field recordings and studied the cellular mechanisms of long-term plasticity at the stellate neuron synapses. Our results suggest that the stellate neuron synapses are devoid of HFS- or forskolin-induced LTD. Whereas the reason for the incompetence of stellate neuron synapses to express LTD is not absolutely clear, our results suggest that it is due to the low expression of NMDA receptors at this synapse type because the ratio of NMDA/AMPA EPSCs is lower at this synapse type. Because the ratio of NMDA/AMPA EPSCs is lower may not elevate the intracellular Ca\(^{2+}\) by proteasome resulting in a reduction in synaptic strength.

The superficial layers (II and III) of the EC relay most of the inputs from cortical associational areas to the hippocampus, whereas the deep layers (V and VI) receive inputs from the cingular cortex, are the main target of hippocampal output, and send extensive projections back to the superficial layers of the EC and to the neocortex (Witter et al. 2000). Individual neurons in the deep layers (layer V) respond to consecutive stimuli with persistent firing (Egorov et al. 2002). The persistent activity from deep layers of the EC is likely to generate continuous depolarization of the neurons such as stellate neurons in the superficial layers. Similar to the pairing protocol, persistent depolarization of stellate neurons is likely to activate NMDA receptors and induce LTD. Because the axons of the stellate neurons are the major component of the perforant path to control the function of the hippocampus, stellate neuron LTD is likely to produce a long-term inhibition of the hippocampus. Therefore the physiological significance of the pairing- or LFS-induced LTD is to provide a feedback modulation of the hippocampal-parahippocampal functions. Because the EC in the parahippocampal region is crucially involved in the acquisition, consolidation, and retrieval of long-term memory traces for which working memory operations are essential, the feedback modulation from the stellate neuron synapses is likely to influence the memory processes.

GRANTS

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