Inhibition of the slow afterhyperpolarization restores the classical spike timing-dependent plasticity rule obeyed in layer 2/3 pyramidal cells of the prefrontal cortex

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In its simplest form, the STDP protocol consists of pairing single excitatory postsynaptic potentials (EPSPs) evoked by presynaptic APs with single postsynaptic APs at low frequency (Bi and Poo 1998; Feldman 2000; Froemke and Dan 2002). However, many studies have reported that low-frequency pre- or postsynaptic stimulation did not result in the expected STDP plasticity and that the application of brief high-frequency bursts of pre- and/or postsynaptic APs was necessary to induce plasticity (Kampa et al. 2006; Markram et al. 1997b; Nevin and Sakmann 2006; Sjostrom et al. 2001, 2008; Zilberter et al. 2009). The mechanisms that make synaptic plasticity sensitive to spike timing are not yet fully understood; however, STDP seems to depend on an interplay between the dynamics of N-methyl-D-aspartate receptor (NMDAR) activation and the timing of APs, which backpropagate through the dendrites of the postsynaptic neuron (Abbott and Nelson 2000; Sjostrom et al. 2008).

The STDP rules are not rigid and vary substantially across different brain regions, cells, and synapse types (Abbott and Nelson 2000). In addition, these rules are influenced by neuromodulatory inputs through different receptors (Couey et al. 2007; Lin et al. 2003; Seol et al. 2007; Xu and Yao 2010). Recently, slow Ca2+-dependent K+-mediated afterhyperpolarization (sAHP), which is strongly inhibited by neuromodulatory inputs (McCormick et al. 1993; Nicoll 1988; Satake et al. 2006), was shown to change the temporal window for STDP in CA1 pyramidal neurons (Fuenzalida et al. 2007). In the present work, we studied STDP induction in the proximal synapses of layer 2/3 pyramidal cells of the rat medial prefrontal cortex (PFC), an area that has largely been neglected in studies of long-term plasticity, and the effects of sAHP inhibition on the STDP rules. We show that the induction of synaptic plasticity at proximal synapses in this cortical layer by a positive timing STDP protocol does not obey the classical spike-timing induction rule. Thus STDP stimulation protocols, including those in which presynaptic APs preceded postsynaptic APs, induced a presynaptically expressed tLTD. Such a tLTD was dependent on the activation of cannabinoid receptors type 1 (CB1Rs) and NMDARs. We found that the inhibition of sAHP by the selective blocker N-trityl-3-pyridinemethanamine (UCL2077) or by activation of neuromodulatory transmitter receptors by the β-adrenergic receptor agonist isoproterenol or the cholinergic agonist carbachol. Thus we demonstrate that neuromodulators can affect synaptic plasticity by inhibition of the sAHP. These findings shed light on a fundamental question in the field of memory research regarding how environmental and behavioral stimuli influence LTP, thereby contributing to the modulation of memory.

β-adrenergic receptor; cannabinoid receptor type 1; excitatory postsynaptic currents; long-term depression; long-term potentiation
METHODS

Slice Preparation

Male Wistar albino rats (14–17 postnatal days) were deeply anesthetized with chloroform and decapitated in accordance with the animal care and experimental protocol, approved by the Department of Health, Republic of Ireland. The brain was rapidly removed and immersed in ice-cold, preoxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (ACSF), and 350 μm-thick coronal slices were cut with a vibratome (Intralac, Herts, UK). The slices were maintained in ACSF for at least 1 h at 36°C and then transferred to a recording chamber and perfused with ACSF. Throughout all experiments, the ACSF of the following composition was used (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, 1.3 NaHCO3, and 20 dextrose. All salts used were obtained from Sigma-Aldrich (Dorset, UK).

Patch-Clamp Electrophysiological Recordings

Whole-cell patch-clamp recordings were made in pyramidal neurons in layers 2/3 of medial PFC. Cells were identified visually by the triangular shape of cell bodies using an infrared-transmitted illumination, fixed-stage upright microscope (model BX51WI; Olympus UK, London, UK), equipped with differential interference contrast optics and a CF 8/4 near-infrared spectroscopy video camera (Kappa Optronics GmbH, Gleichen, Germany) for contrast enhancement. Patch electrodes (3–5 MΩ) were pulled from borosilicate capillary glass. The internal solution contained (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 4 ATP-Mg, and 0.3 GTP (pH was adjusted to 7.25 with KOH). Pyramidal cells were voltage clamped −70 mV.

Recordings were made with MultiClamp 700B amplifiers (Axon Instruments, Union City, CA). Signals were filtered at 4 kHz using a Bessel filter and acquired at a sampling rate of 10 kHz using a Digidata 1322A analog-digital interface (Axon Instruments). Series resistance (typically 10–20 MΩ) and input resistance were monitored constantly and remained relatively stable during the experiments (∼30% changes).

Synaptic responses were evoked with a monopolar glass pipette electrode positioned in the same layer and 50 μm away from the recording cell (Fig. 1A). Stimuli to afferents consisted of 0.1- to 0.5 ms constant-current, square-wave pulses. Just-suprathreshold for presynaptic activation excitatory post synaptic currents (EPSCs) were evoked by increasing the extracellular stimulus level until a stable, small-evoked EPSC was elicited without failures; usually, the amplitude of extracellular stimulus was 30–100 μA. To isolate excitatory responses, all recordings were obtained in the presence of the GABAa receptor antagonist picrotoxin (100 μM).

Synaptic plasticity was induced by STDP or HFS protocols. Two STDP protocols with positive intervals were used—either a single, presynaptic spike preceding three postsynaptic spikes (50–100 Hz) by 10 ms, repeated 50 times at a frequency of 0.1 Hz, or five presynaptic spikes preceding five postsynaptic spikes, both at 50 Hz, and presynaptic APs leading postsynaptic APs by 5 ms (Fig. 1, B and C). STDP protocol with negative timing consisted of three postsynaptic spikes (50 Hz) preceding a single presynaptic spike by 10 ms, repeated 50 times at a frequency of 0.1 Hz. Postsynaptic spikes were evoked by 2 ms-long current injections (0.8–1.4 nA). The HFS protocol consisted of 10 trains of eight stimuli at 100 Hz, at an intertrain interval of 0.5 s, repeated three times (one/10 s). Both protocols were applied under current-clamp condition. Changes in EPSC amplitude, >10% of test values, were considered to indicate the induction of synaptic plasticity.

Pharmacology

\( \text{d}-(\text{−})-2\text{-Amino-5-phosphonopentanoic acid (AP5; 50 μM), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoline-7-sulfonamide disodium salt (10 μM), N}(\text{piperidin-1-yl})\text{-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 1 μM; a selective CB}1_{\text{R}}\text{ antagonist), UCL2077 (10 μM), isoproterenol (10 μM), and carbocoll (10 μM) were obtained from Sigma-Aldrich or from Tocris Bioscience. All drugs were bath applied.}

Data Analysis and Statistics

Data were analyzed using Clampfit 9.2 software. EPSC decay time (time constant of a monoeXponential decay function) was determined on traces obtained by averaging 30–50 consecutive responses. Synaptic plasticity was quantified by calculating the ratio of the average EPSC amplitude of the consecutive responses collected between 20 and 30 min after induction protocols to the average control EPSC amplitude. Paired-pulse ratios (PPRs) were measured at a 100 ms interval.
interval between EPSCs. PPRs were defined as being second EPSC amplitude/first EPSC amplitude. Values are given as mean ± SE, and error bars in figures also indicate SE unless otherwise indicated.

RESULTS

All recordings were carried out in layer 2/3 pyramidal cells in the rat medial PFC. To reduce the number of activated axons, evoked EPSCs were recorded at the minimal stimulation level that did not produce failures (see METHODS). The evoked responses had a short and constant latency that was consistent with that of monosynaptic EPSCs. This type of stimulation evoked test EPSCs with an average amplitude of 101 ± 9 pA (n = 53). As neocortical excitatory synaptic connections are usually multisynaptic (Feldmeyer et al. 2006; Markram et al. 1997a), and because the average amplitude of evoked EPSCs was approximately six times larger than that of isolated, miniature EPSCs (17.4 ± 1.7 pA; n = 13), the number of axons stimulated in the present study was likely to be small. The equivalent average EPSP recorded under current clamp conditions was 3.9 ± 0.6 mV (n = 16).

Both Pre-leading-Post and Post-leading-Pre Spike Pairings Induced Mainly LTD

The classical rule of STDP is that a repetitive pairing of pre- and postsynaptic APs induces a form of synaptic plasticity that depends on the temporal order of the pre-/postpairing. Pre-leading-post spike pairing induces LTD, whereas post-leading-pre pairing induces tLTD (Bi and Poo 1998; Feldman 2000; Froemke and Dan 2002; Kampa et al. 2006; Markram et al. 1997b; Sjostrom and Nelson 2002). Two different protocols with pre-leading-post spike pairings were used in the present study to induce synaptic plasticity. The first protocol involved a pairing of a single presynaptic AP with a train of three postsynaptic action spikes at 50 Hz, the presynaptic AP leading postsynaptic APs by 10 ms. This protocol was applied, consisting of pairing a train of five EPSPs with five postsynaptic spikes, both at 50 Hz and presynaptic APs leading postsynaptic APs by 5 ms (five EPSPs plus five APs protocol), as used by Sjostrom et al. (2001) in the visual cortex. Both of these protocols have been shown previously to induce a strong tLTD in the somatosensory and visual cortex (Nevian and Sakmann 2006; Sjostrom and Nelson 2002). However, in the present study, both of these protocols induced tLTD. Across all cells, these protocols induced a small tLTD (0.91 ± 0.05 of test values, t-test = 2.08; P < 0.05; n = 24; Fig. 2, A and B).

The protocols with a post-leading-pre spike pairing typically induced tLTD (Rodriguez-Moreno and Paulsen 2008; Sjostrom et al. 2001, 2003). A reversed three APs plus one EPSP protocol was applied, consisting of pairing a train of three postsynaptic action spikes at 50 Hz and a single presynaptic AP, with the last postsynaptic AP leading the presynaptic AP by 10 ms. Across all cells, a tLTD of 0.87 ± 0.05 was induced (t-test = 3.35; P < 0.01; n = 10; Fig. 2, C and D). It is also worth noting that the STDP-induced tLTD, independently of the temporal order of pre- and postsynaptic spikes, had a slow rise time, with maximum amplitude attained 15–20 min post-induction (Fig. 2, B and D).

The induction of synaptic plasticity at excitatory synapses in layer 2/3 of rat PFC therefore did not obey the classical spike-timing induction rules: tLTD was induced by spike pairings with both negative and positive timing. The disturbances in STDP rules were revealed recently at distal synapses in cortical pyramidal neurons (Froemke et al. 2005; Letzkus et al. 2006; Sjostrom and Hausser 2006). However, in the present study, the activation of the distal synapses was unlikely, because the stimulation electrode was placed in layer 2/3 in close proximity (~50 μm) to the recorded neurons. The time course of synaptic responses is correlated with the dendritic location of the active synaptic contacts, with a faster rise time indicating a more proximal location of contacts (Gonzalez-Burgos et al. 2009; Markram et al. 1997a; Sjostrom and Hausser 2006). In reported experiments, evoked EPSCs exhibited a fast time course (10–90% rise time = 1.41 ± 0.13 ms; n = 19), consistent with the proximal location of synapses. In contrast, stimulation from layer 1 and therefore, activation of distal synapses resulted in EPSCs with a significantly slower 10–90% rise time (3.5 ± 0.8 ms; n = 6). In addition, no correlation was noted between the 10–90% rise time and the changes in the synaptic strength [r = 0.18; n = 19; P = not significant (n.s.)].

HFS Protocol Induced LTP

Although the STDP protocol was applied just 8–12 min after membrane rupture, the possibility remains that some diffusible cytoplasmic factors, needed to trigger LTP, were washed out at that time (Malinow and Tsien 1990). To exclude this possibility, an additional set of experiments using a HFS induction protocol was performed. The HFS protocol was applied with a similar delay following membrane rupture and consisted of 10 trains of eight stimuli at 100 Hz at an intertrain interval of 0.5 s, repeated three times every 10 s. Across all cells (n = 16), the HFS protocol induced LTP of 1.22 ± 0.07 (t-test = 3.08; P < 0.01). Moreover, LTP induced by HFS had a rapid onset, reaching its peak amplitude within 5 min (Fig. 2E).

The Inhibition of the sAHP Currents Enabled the STDP Rule

The sAHP has been shown to regulate synaptic excitation (Lancaster et al. 2001), to increase the threshold for LTP induction (Le Ray et al. 2004; Sah and Bekkers 1996), and to change the temporal window for STDP (Fuenzalida et al. 2007). The sAHP has also been found to reduce the amplitude of the NMDA EPSP component (Fernandez de Sevilla et al. 2007) and consequently, to reduce calcium influx through NMDA channels. Thus a suppression of the sAHP during a given protocol would be expected to result in a larger depolarization, a larger calcium influx, and a lower threshold for induction of LTP. Therefore, the effect of the inhibition of the sAHP on synaptic plasticity in PFC pyramidal cells was investigated in the present study.

To inhibit the sAHP, we used a selective blocker, UCL2077 (10 μM) (Shah et al. 2006). First, we confirmed that application of this compound significantly reduced the amplitude of the sAHP current by ~70% (9.5 ± 0.5 pA in control, 3.1 ± 0.2 pA in UCL2077, t-test = 12.0; P < 0.001; n = 5; Fig. 3A). The effect of sAHP inhibition on plasticity was dramatic; the STDP protocol with positive timing induced a robust tLTP. Across all cells (n = 14), a significant tLTP of 1.22 ± 0.09 was observed (t-test = 2.46; P < 0.05; Fig. 3, B and C). We also tested whether sAHP inhibition would affect plasticity when induced with a post-leading-pre spike pairing (three post-APs + one pre-EPSP protocol). We found that a tLTD of 0.85 ± 0.06 (t-test = 2.45; P < 0.05; n = 7)
was induced (Fig. 3D). Figure 3E shows a histogram of the overall result of the suppression of the sAHP, with the restoration of the classical STDP rule in layer 2/3 pyramidal cell synapses.

**Neuromodulators that Inhibit sAHP Enabled the STDP Rule**

Several neurotransmitter systems [muscarnic, β-adrenergic, serotonergic, H3-histaminergic, and metabotropic glutamate (mGlu)] have been shown to inhibit the sAHP, acting via distinct, second messenger systems, for example, activation of β-adrenoceptors acting via adenyl cyclase and muscarinic receptors M1/M3 via a protein phosphatase (Dasari and Gulledge 2011; Gulledge et al. 2009; Krause et al. 2002; Krause and Pedarzani 2000; McCormick et al. 1993; Nicoll 1988; Rouse et al. 2000; Satake et al. 2008).

In the present study, the effects of the β-adrenergic receptor agonist isoproterenol (10 μM) and the cholinergic agonist carbachol (10 μM) were examined. We initially confirmed that application of both compounds significantly reduced the amplitude of the sAHP current and also observed that application of carbachol produced a slow afterdepolarization current instead of a sAHP current (Fig. 4, A and B). Previously, slow afterdepolarization induction was suggested to occur by muscarinic receptor activation and to be mediated by a nonselective cation current through transient receptor potential channels.

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Fig. 2. Induction of long-term depression (LTD) and long-term potentiation (LTP) by STDP and high-frequency stimulation (HFS) protocols. A and B: LTD was induced after both STDP (1+3) and STDP (5+5) positive-timing protocols in layer 2/3 of the prefrontal cortex (PFC). A: a representative example of the STDP experiment showing LTD. Each point denotes excitatory postsynaptic current (EPSC) amplitude for each sweep of the experiment. Dashed line shows mean EPSC at the baseline period. Series resistance (R) and input resistance were monitored constantly and remained relatively stable during the experiments. B: the graph shows the pooled and normalized EPSC amplitudes for all cells (n = 24). C and D: LTD was induced after a STDP negative-timing protocol. C: a representative example of the STDP experiment showing LTD. D: the graph shows the pooled and normalized EPSC amplitudes for all cells. E: LTP was induced after HFS protocol in layer 2/3 of the PFC. The graph shows the pooled and normalized EPSC amplitudes for all cells.
Because β-adrenergic agonists can enhance the excitatory synaptic responses in cortical pyramidal cells (Ji et al., 2008), the effect of a short bath application of isoproterenol (10 min, 10 μM) on EPSC was tested. Isoproterenol increased the amplitude of EPSCs, but this effect was reversed completely in 15–20 min after the drug was washed out (n = 4).

The effects of carbachol and isoproterenol were investigated on STDP-induced plasticity. Carbachol was bath applied during the whole experiment, whereas isoproterenol was applied for 3 min prior to the STDP protocol and during the STDP protocol (10 min in total). Application of the STDP protocol with positive timing in the presence of isoproterenol resulted in tLTP of 1.18 ± 0.06, n = 14 cells (t-test = 3.0; P = 0.01; Fig. 4, C and D). The same tendency was observed in the presence of carbachol (1.16 ± 0.08; t-test = 2.1; P = 0.06; n = 12; Fig. 4, E and F). Thus the suppression of sAHP changed the synaptic plasticity outcome induced by the STDP protocol with positive timing from tLTD to tLTP (Fig. 4H).

Recently, it was shown that β-adrenergic activation selectively promotes associative LTP, independently of the timing relationship between pre- and postsynaptic stimulation, because the neuromodulator primes the induction of plasticity by affecting the steps that are downstream from the activation of NMDARs (Seol et al. 2007). Additional tests were performed to determine whether the synaptic plasticity outcome for a negative-timing STDP protocol remained the same when sAHP was inhibited by isoproterenol. In contrast to the data presented by Seol et al. (2007), we found that the post-leading-pre protocol induced LTD of 0.84 ± 0.05 (t-test = 3.1; P < 0.05; n = 9; Fig. 4, G and H). We conclude that the effects of isoproterenol on plasticity were dependent on spike timing and like those of UCL2077, presumably due to inhibition of the sAHP.

**Site of Expression of the Synaptic Plasticity in the PFC**

Changes in the PPR were analyzed to investigate the expression mechanisms of the synaptic plasticity in the PFC. Overall, in the control conditions, the PPR was increased from 0.97 ± 0.05 to 1.16 ± 0.06 (paired sample t-test = 5.28; P < 0.001; n = 18), following the STDP protocols with positive timing (Fig. 5A). These results suggest a decrease in probability of release in cells with tLTP. No change in the PPR occurred in cells with LTP after the HFS protocol (the mean PPR for test EPSCs = 1.11 ± 0.13, after HFS = 1.10 ± 0.09, paired sample t-test = 0.06; P = n.s.; n = 10; Fig. 5B). The lack of change in the PPR suggests that synaptic modifications after HFS protocol in these cells did not affect the presynaptic site and took place at a postsynaptic membrane.

**Pharmacology of Synaptic Plasticity Induced in PFC by STDP and HFS Protocols**

To investigate whether STDP- and HFS-induced plasticity in layer 2/3 of the PFC is NMDAR dependent, experiments were performed.
carried out in the presence of the NMDAR antagonist AP5 (50 μM). An almost complete blockage of plasticity was seen in both sets of experiments. Across all cells, the mean amplitude of EPSCs was 0.99 ± 0.03 of test values after the STDP protocol with positive timing (n = 10; Fig. 6, A and E) and 0.97 ± 0.05 after the HFS protocol (n = 10; Fig. 6, B and F).

It was of note that the inhibition of NMDARs did not affect the time course of the EPSCs (τ decay in control = 6.1 ± 0.3 ms, n =
46, vs. 5.7 ± 0.3 ms in AP5, n = 20, t-test = 0.76; P = n.s.), which indicates a very low activation of postsynaptic NMDARs by single-pulse stimulation at the holding potential of −70 mV. A small but significant increase of the PPR occurred in the presence AP5 (control: 0.98 ± 0.04, n = 46, vs. AP5: 1.17 ± 0.07, n = 20, t-test = 2.35; P < 0.05), which suggests a decrease in the presynaptic release probability (Zucker and Regehr 2002) due to inhibition of NMDARs. This is in agreement with previous studies, which showed that there is a tonic activity of presynaptic NMDARs that facilitates spontaneous and evoked neurotransmitter release (Bender et al. 2006; Berretta and Jones 1996; Brasier and Feldman 2008; Corlew et al. 2007; Li and Han 2007; Li et al. 2008; Sjostrom et al. 2003).

Previous evidence has been presented for the involvement of endocannabinoids (eCBs) in retrograde signaling and in the mediation of both Hebbian and anti-Hebbian forms of LTD in different cortical regions (Bender et al. 2006; see review Heftes and Castillo 2009; Sjostrom et al. 2003; Tzounopoulos et al. 2007). Based on these previous findings, the involvement of CB1Rs in the induction of tLTD by the STDP protocol was investigated. A set of experiments was performed in the presence of 1 μM AM251. Application of AM251 inhibited the induction of tLTD by the STDP protocol (one EPSP plus three APs) and unmasked tLTP (Fig. 6, C and E). The STDP protocol induced tLTP of 1.13 ± 0.07 (n = 12 cells). The PPR in the overall population of tested pyramidal cells did not change after the induction protocol (before: 1.13 ± 0.10, after: 1.12 ± 0.08, paired t-test = 0.16; P = n.s.; n = 12), suggesting that the postsynaptic site was predominantly involved in the plasticity processes.

AM251 did not alter LTP induced by the HFS protocol (Fig. 6, D and F). In all 10 cells, the HFS protocol induced LTP of 1.19 ± 0.06 compared with 1.22 ± 0.07 in the control. LTP developed as rapidly in AM251 as in the control. No changes in PPR (before 1.02 ± 0.08 vs. after 0.96 ± 0.09, t-test = 0.47; P = n.s.) were detected following induction of LTP in AM251, showing that LTP was expressed postsynaptically, as in the controls.

**DISCUSSION**

The main finding of the present study is that STDP protocols with a positive timing (i.e., presynaptic preceding postsynaptic stimulation of APs) predominantly induced a presynaptically expressed tLTD rather than the expected postsynaptically expressed tLTP in layer 2/3 of the PFC. Thus the induction of synaptic plasticity in these synapses does not obey the classical spike-timing rules. Importantly, we show that the inhibition of sAHP enables the STDP rule to be restored in these synapses. Thus a STDP protocol with positive timing induces tLTP, whereas a protocol with negative timing still produces tLTD, following suppression of the sAHP.

**Fig. 5.** Site of expression of STDP- and HFS-induced plasticity. A: paired-pulse ratio (PPR) increased after LTD induction, indicating a decrease in probability of release after the STDP protocol. A1: representative example of recordings before (top trace) and after STDP protocol (bottom trace). A2: pooled data from 18 STDP experiments resulted in LTD. B: PPR did not change in cells with LTD induced by the HFS protocol, indicating a postsynaptic site of LTD expression. B1: representative example of recordings before (top trace) and after HFS protocol (bottom trace). B2: pooled data from 10 HFS experiments resulted in LTD. n.s., not significant.

**Induction and Expression Mechanisms of Plasticity in Layer 2/3 Pyramidal Cell Synapses in Rat PFC**

The central feature of STDP is that the timing of the backpropagated AP, relative to the EPSP, determines the sign and magnitude of the synaptic plasticity. Typically, if the presynaptic cell fires an AP a few milliseconds before the postsynaptic cell, a tLTP is produced. In contrast, the opposite temporal order results in a tLTD (Sjostrom et al. 2008). A commonly observed exemption from this strict classical STDP rule is that low-frequency pre- or poststimulation does not result in the expected STDP plasticity, and brief high-frequency bursts of pre- and/or postsynaptic APs have to be...
applied to induce plasticity (Kampa et al. 2006; Markram et al. 1997b; Nevian and Sakmann 2006; Sjostrom et al. 2001, 2008; Zilberter et al. 2009). These bursts of APs are required to evoke a critical level of maintained postsynaptic depolarization, especially to activate postsynaptic NMDARs, as single back-propagating brief duration APs do not provide sufficient depolarization to activate postsynaptic NMDARs (Lisman et al. 2005).

Previous studies of STDP have shown that bursts of APs induce LTP much more successfully than a low-frequency pairing in the somatosensory and visual cortex (Kampa et al. 2006; Markram et al. 1997b; Nevian and Sakmann 2006; Sjostrom et al. 2001, 2008). For this reason, we used burst STDP protocols in the present study. However, both of these pre-leading-post protocols at \( t/\text{HFS} \) induced tLTD in the PFC. The induction of this type of tLTD...
by positive timing clearly demonstrates that input to layer 2/3 cells of the PFC does not obey the classical STDP timing rules.

Because of this unusual outcome of synaptic plasticity in PFC induced by a standard STDP protocol, we investigated the mechanism underlying this synaptic plasticity. First, we showed that the main components of plasticity-induced STDP protocols are NMDAR dependent. These results are in agreement with published data that LTP and LTD induced by STDP are NMDAR dependent (Caporale and Dan 2008). The unusual tLTD induced in the present study by a STDP protocol with positive timing had a slow onset time, attaining a peak amplitude after ~20 min, and was dependent on NMDARs and CB,Rs. This form of tLTD is very similar to that evoked by the negative timing STDP protocol shown in previous studies (Bender et al. 2006; Nevin and Sakmann 2006; Rodriguez-Moreno and Paulsen 2008; Sjostrom et al. 2003). The presynaptic tLTD does not depend on the activation of postsynaptic NMDARs; for example, presynaptic tLTD at layer 5 neocortical synapses requires presynaptic coincidence detection of eCB release and presynaptic NMDAR activation (Dugué and Sjostrom 2006; Sjostrom et al. 2003). Release of eCB might be triggered by PLC activation through activation of postsynaptic mGlu receptors (mGluRs) and Ca\(^{2+}\) influx through voltage-dependent calcium channels (Caporale and Dan 2008; Heifets and Castillo 2009). The tLTD thereby resembles known mGluR-dependent, postsynaptic, NMDAR-independent forms of LTD (Anwyl 1999). Presynaptic NMDARs were most probably involved in tLTD induction in our experiments. The activity of presynaptic NMDAR was demonstrated in our experiments with the NMDAR antagonist AP5. The PPR was significantly larger in the presence of AP5, suggesting that presynaptic NMDARs are tonically active. In contrast, the decay time course of EPSCs did not change after application of AP5, indicating a low impact of the postsynaptic NMDAR at resting membrane potential.

The tLTD may mask weak postsynaptic LTP induced by STDP with positive timing in the PFC. When a selective CB,R antagonist AM251 was applied, the same STDP protocol induced LTP. Domination of tLTD in experiments with positive timing over LTP in layer 2/3 of the PFC might be explained by a higher eCB concentration in these layers of the PFC area than other areas. It has been shown that CB,R immunoreactivity is more intense in the frontal and cingulate (prelimbic) cortex than in other cortical areas (Herkenham et al. 1991) and is also more intense in upper layers of the PFC than in deeper layers (Eggan et al. 2010; Lafourcade et al. 2007). Thus a stronger eCB impact on synaptic plasticity in layer 2/3 of the PFC than in other cortical areas and layers could be expected. The domination of tLTD could also be due to the impact of presynaptic NMDARs on tLTD being larger than that of postsynaptic NMDARs in the young animals used in the present study, but this effect declines with development, as presynaptic NMDARs are reduced (Corlew et al. 2007).

The Role of sAHP in the Regulation of Synaptic Plasticity and Functional Significance

Although the present work has identified the critical role of sAHP in the regulation of LTD in the PFC, the exact mechanism remains to be investigated further. sAHP current requires a rise in cytosolic calcium for activation and is voltage insensitive. Although many attempts have been made to identify the native channels that underlie the sAHP in neurons, their molecular identity remains a mystery (Sah and Faber 2002). In the absence of a good molecular correlate for the current and immunocytochemistry, the sAHP channel localization is as yet unknown. For example, in hippocampal pyramidal neurons, in which they have been studied extensively, their location remains controversial: on the soma, functionally colocalized with the L-type channel (Lima and Marrion 2007), or on the dendritic tree (Andreasen and Lambert 1995; Bekkers 2000; Sah and Bekkers 1996). Some studies demonstrated that sAHP is involved in the shaping of EPSPs, suggesting the dendritic location of the sAHP channels (Fernandez de Sevilla et al. 2007; Lancaster et al. 2001). The sAHP current was shown to reduce calcium influx through NMDAR channels, both directly, via the AHP hyperpolarization, and indirectly, by shunting EPSPs and reducing their depolarizing drive (Sah and Bekkers 1996); sAHP was shown to be involved in regulation of the threshold for LTP induction (Le Ray et al. 2004; Sah and Bekkers 1996) and the temporal window for STDP (Fuenzalida et al. 2007). Based on our finding that inhibition of sAHP restored the STDP rule in layer 2/3 pyramidal cells, we propose that in control conditions, the postsynaptic spikes activate a sAHP current, which can then shunt EPSPs and consequently, reduce the postsynaptic NMDAR activity. Moreover, the sAHP may downregulate the cellular excitability and AP backpropagation (Gullledge et al. 2005; Sah 1996). Both of these factors strongly decrease postsynaptic tLTP, which is masked completely in PFC synapses by the presynaptic tLTD. The suppression of sAHP would increase the NMDAR current and boost AP backpropagation, which in turn, favors LTP induction by STDP protocols with positive timing. Because the amplitudes of somatically recorded sAHP currents in PFC pyramidal cells are relatively low, and a negative timing protocol still produced tLTD after sAHP suppression, we suggest that sAHP channels are located closely to the site of LTP induction. In such a location, the sAHP channels may most effectively shunt the postsynaptic NMDARs during the STDP protocols.

It is worth noting that the small conductance Ca\(^{2+}\)-activated, K\(^+\)-type 2 channels, underlying medium AHP, were shown to increase the threshold for induction of LTP (Ngo-Anh et al. 2005). These channels are expressed in the postsynaptic density of CA1 neurons, where they are activated by synaptically evoked Ca\(^{2+}\) influx to limit the size of EPSPs and spine Ca\(^{2+}\) transients (Lin et al. 2008, 2010). Thus different types of AHP currents may influence the synaptic plasticity. The sAHP channels are targets for several protein kinases and phosphatases, and several neurotransmitters regulate the sAHP amplitude via the activation of these enzymes (Sah 1996). Muscarinic, β-adrenergic, serotonergic, H\(_{3}\)-histaminergic, and mGluRs have been demonstrated to inhibit the sAHP (McCormick et al. 1993; Nicoll 1988; Satake et al. 2008). In the present study, we demonstrated that application of the β-adrenergic agonist isoproterenol and the cholinergic agonist carbamol strongly reduced the sAHP and restored the STDP rule. It is well known that neurotransmitters, such as norepinephrine and acetylcholine, play important roles in experience-dependent neural plasticity (Caporale and Dan 2008). However, it should be mentioned that their effect at the cellular level is not limited to inactivation of sAHP. For example, β-adrenergic and cholinergic receptor activation increase dendritic AP amplitude through the decrease of A-type K\(^+\) currents in dendrites, by
shifting their activation to more positive potentials (Hoffman and Johnston 1999; Johnston et al. 1999). These neuromodulators may also prime the induction of plasticity by affecting steps that are downstream from the activation of NMDARs. Multiple receptors coupled to adenyl cyclase (i.e., β-adrenergic) and PLC (i.e., muscarinic) intracellular cascades, “prime” the induction of associative LTP and LTD accordingly (Seol et al. 2007). Nevertheless, in our experiments, we did not find evidence that isoproterenol affects steps that are downstream from the activation of NMDARs. Its effect on LTP induction was significant only in the STDP protocol with positive timing, whereas in experiments with negative timing, we still observed LTD, indicating that a critical level of maintained, postsynaptic depolarization is required to activate postsynaptic NMDARs.

The strong suppression of the sAHP by isoproterenol, carbachol, and the specific sAHP inhibitor UCL2077 in the present study provides convincing evidence that the restoration of the classical STDP timing in the presence of these agents is due to sAHP suppression. Based on these data, we suggest that the size of the sAHP is a powerful regulatory factor underlying the mechanism of synaptic plasticity in these cells. Because the amplitude of sAHP is under the control of multiple neuromodulatory systems, a linking link exists between the neuromodulatory factors and learning. Our findings, therefore, may shed light on a fundamental question in the field of memory research regarding how environmental and behavioral stimuli influence LTP and thus contribute to the modulation of memory (Malinow and Malenka 2002).

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

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

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