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

Running title: Inhibition of sAHP restores STDP rule

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The induction of long-term potentiation (LTP) and long-term depression (LTD) of excitatory postsynaptic currents (EPSCs) was investigated in proximal synapses of layer 2/3 pyramidal cells of the rat medial prefrontal cortex. The spike-timing dependent plasticity (STDP) induction protocol of negative timing with postsynaptic leading presynaptic stimulation of action potentials induced LTD as expected from the classical STDP rule. However, the positive STDP protocol of presynaptic leading postsynaptic stimulation of action potentials (APs), predominantly induced a presynaptically expressed LTD rather than the expected postsynaptically expressed LTP. Thus, the induction of plasticity in layer 2/3 pyramidal cells does not obey the classical STDP rule for positive timing. This unusual STDP switched to a classical timing rule if the slow Ca$^{2+}$-dependent K$^+$-mediated afterhyperpolarization (sAHP) was inhibited by the selective blocker UCL2077, by the β-adrenergic receptor agonist isoproterenol, or by 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.

**Keywords:** β-adrenergic receptor; cannabinoid receptors type 1; excitatory postsynaptic currents; long-term depression; long-term potentiation.
Introduction

Various methods have been demonstrated for inducing long-term potentiation (LTP) and long-term depression (LTD) in the neocortex. LTP and LTD are induced by classical high frequency stimulation (HFS) and low frequency stimulation, respectively (Kirkwood et al. 1993). In addition, long-term plasticity in the neocortex has recently been found to be evoked by spike-timing dependent plasticity (STDP) that appears to be closer to natural physiological conditions. A pairing of pre-leading postsynaptic action potentials (APs) (positive timing) induced spike-timing dependent LTP (tLTP); while post-leading presynaptic APs (negative timing) induced spike-timing dependent LTD (tLTD). Both occurred within narrow timing windows: ~15 ms for the induction of tLTP and up to 100 ms for the induction of tLTD (Markram et al. 1997b; reviewed Sjostrom et al. 2008; Sjostrom et al. 2001).

In its simplest form, the STDP protocol consists of pairing single 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 post-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; Nevian and Sakmann 2006; Sjostrom et al. 2008; Sjostrom et al. 2001; 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 NMDAR activation and the timing of APs that 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 Ca$^{2+}$-dependent K$^{+}$-mediated afterhyperpolarization
(sAHP), which is strongly inhibited by neuromodulatory inputs (McCormick et al. 1993; Nicoll 1988; Satake et al. 2008), 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 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 UCL2077 or by activation of neuromodulatory transmitter receptors by the β-adrenergic receptor agonist, isoproterenol, or the cholinergic agonist, carbachol, enables STDP rules. Therefore, many neuromodulatory systems that inhibit sAHP can effectively control synaptic plasticity in PFC through this mechanism.

Methods

Slice preparation

Male Wistar albino rats (14-17 postnatal days) were deeply anesthetized with chloroform and decapitated in accordance with local and national regulations. The brain was rapidly removed and immersed in ice-cold pre-oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (ACSF) and 350-μm-thick coronal slices were cut with a vibratome (Intracell, 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...
Patch-clamp electrophysiological recordings

Whole-cell patch-clamp recordings were made in pyramidal neurons in layers 2/3 of medial prefrontal cortex. Cells were identified visually by the triangular shape of cell bodies using infrared transmitted illumination fixed-stage upright microscope (model BX51WI) (Olympus UK LTD, London, U.K.) equipped with differential interference contrast optics and CF 8/4 NIR Kappa video camera (Kappa opto-electronics 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, 10 phosphocreatine, 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 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 constantly monitored and remained relatively stable during the experiments (≤ 30% changes).

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

Synaptic plasticity was induced by either STDP or HFS protocols. Two STDP protocols with positive intervals were employed, either a single presynaptic spike preceding 3 postsynaptic
spikes (50-100 Hz) by 10 ms, repeated 50 times at a frequency of 0.1 Hz, or 5 presynaptic spikes
preceeding 5 postsynaptic spikes both at 50 Hz and presynaptic APs leading postsynaptic APs by 5
ms (Fig. 1B-C). STDP protocol with negative timing consisted of 3 postsynaptic spikes (50 Hz)
preceeding 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 ten trains of eight stimuli at 100 Hz, at in intertrain interval of 0.5 sec, repeated three
times (1/10 sec). Both protocols were applied under current-clamp condition. Changes in EPSC
amplitude more than 10% of test values were considered to indicate the induction of synaptic
plasticity.

Pharmacology

D-AP5 (50 µM), NBQX (10 µM), AM251 (1 µM), UCL2077 (10 µM), isoproterenol (10
µM), and carbachol (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 20th and 30th min after induction
protocols to the average control EPSC amplitude. Paired-pulse ratios (PPRs) were measured at a
100-ms interval between EPSCs. PPRs were defined as being second EPSC amplitude/first EPSC
amplitude.

Values are given as mean ± s.e.m. and error bars in figures also indicate s.e.m. unless
otherwise indicated.
Results

All recordings were carried out in layer 2/3 pyramidal cells in the rat medial prefrontal cortex. In order 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 ~ 6 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 action potentials induces a form of synaptic plasticity that depends on the temporal order of the pre/post pairing. Pre-leading post spike pairing induces tLTP, while 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 (1 EPSP plus 3 APs protocol), as used by Nevian and Sakmann (2006) in layer 2/3 of the somatosensory cortex. The second protocol involved a pairing of a train of five EPSPs with 5 postsynaptic spikes, both at 50 Hz and presynaptic APs leading postsynaptic APs by 5 ms (5 EPSPs plus 5 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 tLTP in the somatosensory...
and visual cortex (Nevian and Sakmann 2006; Sjostrom et al. 2001). 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. 2A-B).

The protocols with a post-leading pre spike pairing typically induced tLTD (Sjostrom et al., 2001; Sjostrom et al., 2003; Rodriguez-Moreno & Paulsen, 2008). A reversed 3 APs + 1 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. 2C-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. 2B, 2D).

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 recently revealed 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 very unlikely because the stimulation electrode was placed in layer 2/3 in close proximity (about 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 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=n.s.).
**High frequency stimulation protocol induced LTP**

Although the STDP protocol was applied just 8-12 minutes 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 an HFS induction protocol was performed. The HFS protocol was applied with a similar delay following membrane rupture and consisted of ten trains of eight stimuli at 100 Hz at an intertrain interval of 0.5 sec, repeated three times every 10 seconds. 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 slow AHP 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 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. 3B-C). We also tested whether sAHP inhibition would affect plasticity when induced with a post- leading pre spike pairing (3 post AP + 1 pre EPSP protocol). We found that a tLTD of 0.85±0.06 (n=7, t-test=2.45, p<0.05) was induced (Fig. 3D). Fig. 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 (muscarinic, β-adrenergic, serotoninergic, H₂-histaminergic, and glutamate metabotropic) have been shown to inhibit the sAHP, acting via distinct second messenger systems; for example, activation of β-adrenoceptors acting via adenylyl 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 sAHP current, and also observed that application of carbachol produced a slow afterdepolarization current instead of an sAHP current (Fig. 4A-B). Previously slow afterdepolarization induction was suggested to occur by muscarinic receptor activation and to be mediated by a nonselective cation current through TRPC channels (Yan et al. 2009). 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 completely reversed 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. 4C-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. 4E-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 (n=9, t-test=3.1, p<0.05) (Fig. 4G-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 (n=18, paired sample t-test=5.28, p<0.001) following the STDP protocols with positive timing (Fig. 5A). These results suggest a decrease in probability of release in cells with tLTD. 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, n=10, paired sample t-test=0.06, p=n.s.) (Fig. 5B). The lack of change in the PPR suggests that synaptic modifications after HFS protocol in these cells did not affect 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 NMDA receptor-dependent, experiments were carried out in the presence of the NMDAR antagonist D-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. 6A, 6E) and 0.97±0.05 after the HFS protocol (n=10) (Fig. 6B, 6F).

It was of note that, the inhibition of NMDARs did not affect the time course of the EPSCs (tau decay in control = 6.1±0.3 ms, n=46, vs. 5.7±0.3 ms in AP5, n=20, t-test=0.76, n.s.), which indicates a very low activation of postsynaptic NMDA receptors by single-pulse stimulation at the holding potential of -70 mV. A small, but significant, increase of the paired pulse ratio (PPR) occurred in the presence D-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 the 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 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 for review Heifets 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, a selective CB1R antagonist. Application of AM251 inhibited the induction of tLTD by the STDP protocol (1 EPSP plus 3APs) and unmasked tLTP (Fig. 6C, 6E). 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; n=12, paired t-test=0.16, p=n.s.), suggesting that the postsynaptic site was predominantly involved in the plasticity processes.

AM251 did not alter LTP induced by the HFS protocol (Fig. 6D, 6F). In all 10 cells, 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, n.s.) were detected following induction of LTP in AM251, showing that LTP was postsynaptically expressed, 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 prefrontal cortex. 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, an STDP protocol with positive timing induces tLTP, while a protocol with negative timing still produces tLTD following suppression of the sAHP.

**Induction and expression mechanisms of plasticity in L2/3 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 post stimulation 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. 2008; Sjostrom et al. 2001; Zilberter et al. 2009). These bursts of APs are required to evoke a critical level of maintained postsynaptic depolarization, especially in order to activate postsynaptic NMDARs as single backpropagating 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. 2008; Sjostrom et al. 2001; Zilberter et al. 2009). These bursts of APs are required to evoke a critical level of maintained postsynaptic depolarization, especially in order to activate postsynaptic NMDARs as single backpropagating brief duration APs do not provide sufficient depolarization to activate postsynaptic NMDARs (Lisman et al. 2005).
2006; Markram et al. 1997b; Nevian and Sakmann 2006; Sjostrom et al. 2008; Sjostrom et al. 2001). For this reason, we used burst STDP protocols in the present study. However, both of these protocols, 1 pre leading 3 post, and 5 pre leading 5 post at 50 Hz and at +5-10 ms timing difference 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 prefrontal cortex 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 an STDP protocol with positive timing had a slow onset time, attaining a peak amplitude after ~20 min and was dependent on NMDARs and endocannabinoid receptors. This form of tLTD is very similar to that evoked by the negative timing of the post before pre-stimulation STDP protocol shown in previous studies (Bender et al. 2006; Nevian and Sakmann 2006; Rodriguez-Moreno and Paulsen 2008; Sjostrom et al. 2003). The presynaptic tLTD does not depend on the activation of postsynaptic NMDA receptors; for example, presynaptic tLTD at L5 neocortical synapses requires presynaptic coincidence detection of endocannabinoid (eCB) release and presynaptic NMDA receptor activation (Duguid and Sjostrom 2006; Sjostrom et al. 2003). Release of eCB might be triggered by phospholipase C activation through activation of postsynaptic mGluRs and Ca\(^{2+}\) influx through voltage-dependent calcium channels (Caporale and Dan 2008; Heifets and Castillo 2009). The tLTD therefore 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 CB1R 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 cannabinoid concentration in these layers of the PFC area than other areas. It has been shown that CB1R 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 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 NMDAR 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 spike-timing dependent plasticity in the PFC, the exact mechanism remains to be further investigated. Slow AHP current requires a rise in cytosolic calcium for activation and is voltage insensitive. Though 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 co-localized with L-type channel (Lima and Marrion, 2007) or on the dendritic tree (Andreasen and Lambert, 1995) (Bekkers...
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 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 slow AHP current which can then shunt EPSPs and consequently reduce the postsynaptic NMDAR activity. Moreover, the sAHP may down-regulate the cellular excitability and action potential backpropagation (Gulledge et al. 2005; Sah 1996). Both these factors strongly decrease postsynaptic tLTP, which is completely masked 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\textsuperscript{2+}-activated K\textsuperscript{+}-type 2 channels (SK2), 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\textsuperscript{2+} influx to limit the size of EPSPs and spine Ca\textsuperscript{2+} transients (Lin et al. 2008; Lin et al. 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, serotoninergic, H\textsubscript{2}-histaminergic, and glutamate metabotropic receptors...
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 carbachol strongly reduced the sAHP and restored the STDP rule. It is well known that neuromodulators, such as norepinephrine and acetylcholine (ACh), 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 increases 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 NMDA receptors. Multiple receptors coupled to adenylyl cyclase (i.e., β-adrenergic) and phospholipase C (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 STDP protocol with positive timing, while in experiments with negative timing we still observed LTD, indicating that a critical level of maintained postsynaptic depolarization is required in order 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 connecting 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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References:


Captions to figures

Figure 1. Illustration of the recording configuration for the synaptic plasticity experiments and the induction protocols. A. Diagram illustrating the typical location of stimulating electrode and whole-cell recording electrode in layer 2-3 of a cortical slice. Stimulating electrode was usually located 50-75 µm in the horizontal direction from the recorded cell. B-C. Different types of induction protocols which were used in this study. STDP (1+3) protocol is depicted on B. An EPSP evoked by extracellular stimulation (pre) was paired with a short burst of three APs at 50 Hz elicited by current injections into the postsynaptic cell (post). The first AP followed the onset of the EPSP by 10 ms. Pairings were repeated 50 times at 0.1 Hz stimulation. C. STDP (5+5) protocol consisting of pairing five EPSPs (pre) and APs (post) at 50 Hz with pre-leading before post by 5 ms. Pairings were repeated 20 times at 0.1 Hz stimulation.

Figure 2. Induction of LTD and LTP by STDP and 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. (A) A representative example of STDP experiment showing LTD. Each point denotes EPSC amplitude for each sweep of the experiment. Dashed line shows mean EPSC at the baseline period. Series resistance and input resistance was constantly monitored 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 an STDP negative timing protocol. (C) A representative example of 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 prefrontal cortex. The graph shows the pooled and normalized EPSC amplitudes for all cells.

Figure 3. Selective sAHP blocker UCL2077 restores the classical STDP rule in layer 2-3 pyramidal cell synapses. A. Top. The AHP evoked by a 500 msec, depolarizing 300 pA current injection shows two kinetically distinguishable components, medium (mAHP) and slow (sAHP). Below.
Representative example showing inhibition of I_{sAHP} by UCL2077. The outward current underlying the AHP was evoked by a 250 ms, +50 mV depolarizing voltage step from a holding potential of -60 mV and was recorded at -60 mV (black trace – control, gray trace – after application of UCL2077). The amplitude of sAHP current was measured 700 ms after end of voltage step, this time point indicated with dashed gray line. Inset. Summary bar diagrams illustrating effects of UCL2077 on amplitude of I_{sAHP} (n=5). B and C. STDP protocol with positive timing induced a robust tLTP. (B) A representative example of STDP experiment showing LTP. Each point denotes EPSC amplitude for each sweep of the experiment. Dashed line shows mean EPSC at the baseline period. Series resistance and input resistance was constantly monitored and remained relatively stable during the experiments. (C) The graph shows the pooled and normalized EPSC amplitudes for all cells. D. STDP protocol with negative timing induced a robust tLTD. The graph shows the pooled and normalized EPSC amplitudes for all cells (n=7). E. Summary bar diagrams illustrating effects of UCL2077 on synaptic plasticity induced by STDP with positive and negative timing.

Figure 4. Effect of the β-adrenergic receptor agonist isoproterenol (10 µM) and the cholinergic agonist carbachol (10 µM) on STDP induced plasticity. A-B. Representative traces showing effect of isoproterenol (A) and carbachol (B) on I_{sAHP}. Isoproterenol reduces the amplitude of I_{sAHP}, while carbachol not only inhibits I_{sAHP} but induces depolarizing current. C, E. Representative examples of STDP experiments in which isoproterenol (C) or carbachol (E) were bath applied. Each point denotes EPSC amplitude for each sweep of the experiment. Dashed line, mean EPSC at the baseline period. Series resistance and input resistance was constantly monitored and remained relatively stable during the experiments. D, F. STDP protocol with positive timing induced a robust tLTP if isoproterenol (D) or carbachol (F) were bath applied. The graphs show the pooled and normalized EPSC amplitudes for all cells. G. STDP protocol with negative timing induced LTD in presence of isoproterenol. The graph shows the pooled and normalized EPSC amplitudes for all cells. H.
Summary bar diagrams illustrating effects of isoproterenol and carbachol on synaptic plasticity induced by STDP with positive and negative timing.

**Figure 5.** Site of expression of STDP and HFS-induced plasticity. **A.** PPR increased after LTD induction indicating 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 LTP induced by the HFS protocol, indicating a postsynaptic site of LTP expression. B1. Representative example of recordings before (top trace) and after HFS protocol (bottom trace). B2. Pooled data from 10 HFS experiments resulted in LTP.

**Figure 6.** Pharmacological properties of synaptic plasticity induced by STDP and HFS protocols. **A-B.** Plasticity induced by both STDP and HFS protocols was dependent on activation of NMDARs. Pooled and normalized EPSC amplitudes showing that the STDP (A) and HFS (B) protocols are unable to produce long-term plasticity in the presence of AP5. **C-D.** Application of AM251, a selective CB1R antagonist switched the sign of plasticity induced by STDP protocol (C), but did not affect plasticity induced by HFS protocol (D). **E-F.** Summary bar diagrams illustrating effects of NMDAR and CB1R antagonists on synaptic plasticity induced by STDP (E) and HFS (F) protocols.
STDP(1+3) 50 times at 0.1 Hz
pre post
10 ms 20 ms

STDP(5+5)
20 times at 0.1 Hz
pre post
5 ms 20 ms

L 2-3

Bath

45 mV

A

B

C

Stimulator

+ -

Bath

50 mm

L 2-3
A. The figure shows a comparison of sAHP and mAHP in control and UCL2077 conditions. The sAHP and mAHP are plotted over time with 15 pA and 1 s, respectively.

B. There are three panels showing the effect of STDP (5 mV, 15 ms) on the amplitude of the sAHP and mAHP. The amplitude is measured in pA with a scale of 0-200.

C. The graph illustrates the normalized EPSC over time after STDP (min) for UCL-2077 (10 μM) with control conditions. The data points are plotted with error bars, and the sample size (N) is indicated as 14.

D. Similar to C, but for UCL-2077 (10 μM) with control conditions. The sample size (N) is indicated as 7.

E. The bar graph compares the normalized EPSC for positive and negative spike timing, showing the effect of UCL2077 with control conditions. The sample sizes (n) are 24, 14, 10, and 7 for positive control, positive UCL2077, negative control, and negative UCL2077, respectively.
Before After

A1

STDP

100 pA

50 ms

HFS

100 pA

50 ms

B1

B2

p<0.001

p=n.s.