Regulation of dendritic calcium release in striatal spiny projection neurons

Abbreviated Title: Regulation of dendritic calcium release


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Abstract

The induction of corticostriatal long-term depression (LTD) in striatal spiny projection neurons (SPNs) requires co-activation of group I metabotropic glutamate receptors (mGluRs) and L-type Ca\(^{2+}\) channels. This combination leads to the postsynaptic production of endocannabinoids that act presynaptically to reduce glutamate release. Although the necessity of co-activation is agreed upon, why it is necessary in physiologically meaningful settings is not. The studies described here attempt to answer this question using two photon laser scanning microscopy and patch clamp electrophysiology to interrogate the dendritic synapses of SPNs in ex vivo brain slices from transgenic mice. These experiments revealed that postsynaptic action potentials induce robust ryanodine receptor (RYR)-dependent Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (CICR) in SPN dendritic spines. Depolarization-induced opening of voltage gated Ca\(^{2+}\) channels was necessary for CICR. CICR was more robust in indirect pathway SPNs, than in direct pathway SPNs, particularly in distal dendrites. Although it did not increase intracellular Ca\(^{2+}\) concentration alone, group I mGluR-activation enhanced CICR and slowed Ca\(^{2+}\) clearance, extending the activity-evoked intraspine transient. The mGluR-modulation of CICR was sensitive to antagonism of inositol trisphosphate receptors, RYRs, src kinase and Cav1.3 L-type Ca\(^{2+}\) channels. Uncaging glutamate at individual spines effectively activated mGluRs and facilitated CICR induced by back-propagating action potentials. Disrupting CICR by antagonizing RYRs prevented the induction of corticostriatal LTD using spike-timing protocols. In contrast, mGluRs had no effect on the induction of long-term potentiation. Taken together, these results make clearer how co-activation of mGluRs and L-type Ca\(^{2+}\) channels promote the induction of activity-dependent LTD in SPNs.
Introduction

The striatum plays an important role in learning context appropriate actions (Yin and Knowlton, 2006; Gerfen and Surmeier, 2011). This learning is thought to depend upon long-term changes in the strength of corticostriatal synapses formed on principal striatal spiny projection neurons (SPNs) (Lovinger, 2010; Gerfen and Surmeier, 2011). These synapses are capable of both long-term potentiation (LTP) and depression (LTD) (Fino et al., 2005; Pawlak and Kerr, 2008; Shen et al., 2008; Lovinger, 2010).

LTD is the best studied of these forms of plasticity (Calabresi et al., 1992a; Lovinger et al., 2003; Surmeier et al., 2009). Endocannabinoid (eCB)-mediated corticostriatal LTD is induced postsynaptically and expressed presynaptically. Induction requires postsynaptic depolarization, co-activation of group I metabotropic glutamate receptors (mGluRs) and L-type Ca²⁺ channels with a Cav1.3 pore-forming subunit, and an elevation of cytosolic Ca²⁺ (Calabresi et al., 1994; Kreitzer and Malenka, 2005; Adermark and Lovinger, 2007; Lovinger, 2010; Shindou et al., 2011). This combination leads to the production of endocannabinoids (eCBs) that act presynaptically to reduce glutamate release (Lovinger, 2010).

Although these processes are agreed upon, the nature of their interaction is not. Typically, LTD induction is achieved by combining strong somatic depolarization with high-frequency stimulation (HFS) of afferent fibers (Calabresi et al., 1992a; 1994; Kreitzer and Malenka, 2005; Wang et al., 2006; Adermark and Lovinger, 2007). While this combination is unlikely to be achieved normally in SPNs, it reflects the need for dendritic depolarization to achieve the conditions necessary for LTD induction. In vivo, this might be accomplished by convergent synaptic input that triggers state transitions in dendrites (Wilson and Kawaguchi, 1996; Plotkin et al., 2011) or by the temporal convergence of synaptic input and back-propagating action potentials (bAPs). In SPNs, like in other neuron types (Christie et al., 1996; Magee and
Johnston, 1997; Nevian and Sakmann, 2006), bAPs decrementally back-propagate into dendrites, providing sufficient depolarization to open voltage-dependent Ca\(^{2+}\) channels at synapses in the proximal portion of the SPN dendritic tree (Carter and Sabatini, 2004; Day et al., 2008). When paired with trailing synaptic stimulation, repetition of short bAP bursts at theta frequencies induces LTD in SPNs (Shen et al., 2008). This spike-timing-dependent plasticity (STDP)-LTD has all the pharmacological properties of conventional HFS-LTD, suggesting the underlying mechanisms are the same (Lovinger et al., 2003; Kreitzer and Malenka, 2008; Shen et al., 2008; Surmeier et al., 2009).

In pyramidal neurons, opening of voltage-dependent Ca\(^{2+}\) channels is thought to facilitate STDP-LTD induction by increasing mGluR-stimulation of phospholipase C (PLC) (Nevian and Sakmann, 2006). However, it also is possible that by increasing cytosolic inositol trisphosphate (IP3) or activating src kinase, mGluRs promote Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) triggered by opening of voltage-dependent channels (Berridge, 1998; Nakamura et al., 1999; 2000; Nishiyama et al., 2000; Lerner and Kreitzer, 2012). In this scenario, mGluR signaling and activity-dependent Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels would work in concert to elevate postsynaptic Ca\(^{2+}\) concentration into a range necessary to drive eCB generation. Because of its reciprocity, this model suggests that repetition, rather than precise timing of pre- and postsynaptic activity, is critical to LTD induction.

To explore these ideas, two-photon laser scanning microscopy (2PLSM) was used in conjunction with patch clamp techniques in ex vivo brain slices from transgenic mice to monitor Ca\(^{2+}\) concentration in proximal and distal spines of direct pathway SPNs (dSPNs) and indirect pathway SPNs (iSPNs) in situations similar to those necessary for STDP-LTD.
Materials and Methods

Brain slice preparation: Para-sagittal brain slices (275 µm) were obtained from 19-23 day old male and female hemizygous BAC D, or BAC D2 transgenic mice (Gong et al., 2003; Day et al., 2008) following procedures approved by the Northwestern University Animal Care and Use Committee. The mice were anesthetized with a mixture of ketamine (50 mg kg⁻¹) and xylazine (4.5 mg kg⁻¹) and perfused transcardially with 5–10 ml ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1 CaCl₂, 1.5 MgCl₂, 26 NaHCO₃, 1 NaH₂PO₄, and 16.66 glucose, continuously bubbled with carbogen (95% O₂ and 5% CO₂). The slices were then transferred to a holding chamber where they were incubated in ACSF containing (in mM) 2 CaCl₂, 1 MgCl₂, at 35°C for 60 min, after which they were stored at room temperature until recording.

Electrophysiology: Patch pipettes were pulled from thick-walled borosilicate glass on a Sutter P-97 puller. Pipette resistance was typically 3–4 MΩ when filled with recording solution. The internal recording solution contained (in mM): 135 KMeSO₄, 5 KCl, 0.16 CaCl₂, 10 HEPES, 2 ATP-Mg²⁺, 0.5 GTP-Na, 5 phosphocreatine-Tris; 5 phosphocreatine-Na, 0.1 spermine; pH was adjusted to 7.25 with NaOH and osmolarity to 270–280 mOsm⁻¹. For some experiments the internal recording solution lacked CaCl₂, as indicated in the text. For Ca²⁺ imaging experiments, the recording solution also contained 200 µM Fluo-4 pentapotassium salt and 50 µM Alexa Fluor 568 hydrazide Na salt (Invitrogen), unless otherwise indicated. Slices were continuously perfused with carbogen-bubbled ACSF. For experiments in which cadmium was locally puffed onto dendrites, the puffer solution contained HEPES-buffered ACSF, 0 mM CaCl₂ and 200 µM cadmium. Slices were transferred to a submersion-style recording chamber mounted on an Olympus BX51 upright, fixed-stage microscope. Electrophysiological recordings were obtained with a Multiclamp 700B amplifier. Stimulation and display were obtained as previously
described (Day et al., 2008) using the custom-written shareware package WinFluor (John Dempster, Strathclyde University, Glasgow, Scotland, UK), which automates and synchronizes the two-photon imaging and electrophysiological protocols. The amplifier bridge circuit was adjusted to compensate for serial resistance and continuously monitored during recordings.

**2-photon laser scanning microscopy (2PLSM) and Ca\(^{2+}\) imaging:** D\(_1\) receptor expressing dSPNs or D\(_2\) receptor expressing iSPNs were identified by somatic eGFP two-photon excited fluorescence using an Ultima Laser Scanning Microscope system (Prairie Technologies). A DODT contrast detector system was used to provide a bright-field transmission image in registration with the fluorescent images. The green GFP signals (490–560 nm) were acquired using 810 nm excitation (Verdi/Mira laser). SPNs were patched using video microscopy with a Hitachi CCD camera and an Olympus 60X/0.9 NA lens. Alexa 568 fluorescence was used for visualization of cell bodies, dendrites, and spines. Following patch rupture, the internal solution was allowed to equilibrate for 15–20 minutes before imaging.

Whole cell maximum projection images of the soma and dendrites were acquired with 0.36 µm\(^2\) pixels with 10 µs pixel dwell time; ~80 images taken with 1 µm focal steps. High magnification maximum projection images of dendrites were acquired with 0.072 µm\(^2\) pixels with 10 µm pixel dwell time; ~20 images taken with 0.5 µm focal steps.

Single bAPs were generated by injecting current pulses (2 nA, 2 ms) in a theta burst pattern: 5 bursts, each burst containing 3 bAPs with a 20 msec inter-event interval (50 Hz); bursts were delivered at 5 Hz. Drugs were either bath applied by dissolving them in the external ACSF or focally applied using pressure ejection through a micropipette, unless otherwise stated. All Ca\(^{2+}\) imaging recordings were performed in a cocktail of synaptic blockers (5 µM NBQX, 50 µM AP-5, 10 µM SR-95531, 1 µM CGP-55845, 1 µM MPEP and 50 µM CPCCOET, all from Tocris) to isolate postsynaptic components of the mechanisms being examined. For
experiments involving DHPG application MPEP and CPCCOET were omitted. Experiments testing the involvement of protein kinase C, phospholipase A2 and phosphatidylinositol-4,5-biphosphate (PIP2) depletion were performed by loading the cell with 1 µM calphostin, 20 µM N-(p-amylcinnamoyl)anthranilic acid (ACA) or 200 nM PIP2, respectively. Dendritic changes in Ca²⁺ were measured using Fluo-4 as previously described (Day et al., 2008). Ca²⁺ transients were expressed as a ratio of green/red (G/R) fluorescence. As this measure is sensitive to photomultiplier tube settings, only data collected under identical settings were compared. In some instances, where drugs were locally puffed, transients are expressed as ΔF/Fo. This is because the puffer solution contained Alexa 568, to confirm the local application of drug, and thus altered the red fluorescence channel. Locally puffing drugs had two benefits: 1) Alterations of the somatic waveform and bAP propagation to the dendritic point of measurement were minimal and 2) before and after Ca²⁺ measurements could be performed in the same continuous line scan. Green fluorescence line-scan signals were acquired at 6 ms per line and 512 pixels per line with 0.08 µm pixels and 10 µs pixel dwell time. The laser-scanned images were acquired with 810 nm light pulsed at 90 MHz (~250 fs pulse duration). Power attenuation was achieved with two Pockels cells (electro-optic modulators; models 350–80 and 350–50, Con Optics, Danbury, CT). The two cells were aligned in series to provide an enhanced modulation range for fine control of the excitation dose (0.1% steps over four decades). The line scan was started 200 ms before the stimulation protocol and continued 4 s after the stimulation to obtain the background fluorescence and to record the decay of the optical signal after stimulation. To reduce photo-damage and photo-bleaching, the laser was fully attenuated using the second Pockels cell at all times during the scan except for the period directly flanking the bAP burst.

Glutamate uncaging: 2-photon uncaging (2PU) of MNI-glutamate was performed
simultaneously with Ca\(^{2+}\) imaging using a Chameleon-XR laser system (Coherent Laser Group, Santa Clara, CA) as previously described (Plotkin et al., 2011). MNI-glutamate (Tocris Cookson, Ellisville, MO) was superfused at 5 mM using a system of syringe pumps (WPI, Sarasota, FL) and a multi-barreled perfusion manifold fitted with a small-volume mixing tip that allowed rapid switching between solutions (Cell MicroControls, Norfolk, VA). MNI-glutamate was uncaged using 1ms pulses of 720 nm light independently controlled by a third Pockels cell modulator (model 302, Con Optics, Danbury, CT). Experiments involved uncaging on a single spine to evoke a somatic EPSP measuring 0.8-4 mV. The uncaging pulses were typically about 10 mW in strength measured at the sample plane. Photolysis power was adjusted to closely mimic spontaneously occurring excitatory postsynaptic potentials (EPSPs) and tuned to achieve the predetermined somatic EPSP amplitude. Simultaneous photolysis and line-scan images were made from shafts/spines. As described above, the custom-written software package WinFluor automated and synchronized the Ca\(^{2+}\) imaging with the electrophysiological stimulation and the photolysis.

**Synaptic plasticity:** STDP was induced as previously described (Shen et al., 2008). Briefly, iSPNs were recorded in current clamp mode using perforated patch recordings. The internal recording solution contained (in mM): 126 KMeSO\(_4\), 14 KCl, 3 MgCl\(_2\), 0.5 CaCl\(_2\), 5 EGTA, 10 HEPES and 180 µg/ml amphotericin B. The external recording solution contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1 MgCl\(_2\), 26 NaHCO\(_3\) and 14 glucose, bubbled with 95% O\(_2\), and 5% CO\(_2\). A theta glass stimulating electrode was placed near a dendrite visualized under IR-DIC optics approximately 100 µm from the soma. STDP was induced by pairing a theta burst pattern of somatically-induced APs (as described above) with timed synaptically evoked EPSPs. For the negative timing protocol, EPSPs followed each AP burst by 10 ms. For the positive timing protocol, EPSPs preceded every AP by 5 ms. Stimulus intensity was
adjusted to evoke baseline single component EPSPs. Postsynaptic cells were depolarized to -70 mV, and GABA<sub>A</sub> receptors were blocked with gabazine (10 µM). The induction protocol was repeated 15 times at 0.1 Hz. Capacitance current was continuously monitored during perforation by applying a 5-10 mV pulse from a holding potential of -70 mV, and input resistance was continuously monitored during recordings. For whole cell current-clamp LTD induction, the internal recording solution contained (in mM): 135 KMeSO<sub>4</sub>, 5 KCl, 5 HEPES, 0.25 EGTA, 10 phosphocreatine-Na, 2 ATP-Mg and 0.5 GTP-Na. Recordings were performed at 30-32°C and constantly perfused with 10 µM SR 95531 hydrobromide and 10 µM (R)-CPP. A concentric bipolar stimulating electrode was placed ~100 µm from the soma and was calibrated to evoke ~10 mV somatic EPSP every 10 s. Baseline was recorded for 10 minutes before induction. The induction protocol consisted of a 3 second 100 Hz train of electrical stimulation, with APs (evoked by 2ms, 1nA current injections) preceding each stimulation by 4 ms. The induction protocol was repeated at 20 s intervals 3 times. Baseline was calculated as the average amplitude for the 5 minutes preceding LTD induction and LTD amplitude.

**FACS sorting and gene expression profiling:** Quantitative polymerase chain reaction (qPCR) was used to quantify transcripts of interest with procedures similar to that described previously (Chan et al., 2012). In brief, striata were microdissected, dSPNs and iSPNs separated using fluorescence assisted cell sorting (FACS) based on GFP expression, and total mRNA was isolated using RNase micro kit (Qiagen). cDNA was synthesized using qScript cDNA Supermix (Quanta Biosciences). Real-time PCR was performed using Fast SYBR Mastermix (Applied Biosystems) on a StepOnePlus thermocycler (Applied Biosystems). The thermal cycling conditions comprised an initial denaturing step at 95°C for 20 s, and 40 cycles at 95°C for 3 s, 60°C for 30 s. The PCR cycle threshold (CT) values were measured within the exponential phase of the PCR reaction using StepOnePlus software version 2.1 (Applied
A correction was performed using a passive reference dye (Rox) present in the PCR master mix. Reactions with any evidence of nonspecificity (i.e. low melting temperatures or multiple peaks in melting point analysis) were excluded from the analysis. A relative quantification method (ΔΔCT method) was used to quantify differences in gene expression level.

To increase accuracy of gene expression analysis, a panel of reference genes (Atp5b, Cyc1, Gapdh, H2afz, Hmbs, Uchl1) was included. Weighted CTs based on the stability of each reference gene were calculated. Experiments for each gene of interest were run in triplicate.

Desalted primers were custom synthesized (Invitrogen) and intron-spanning whenever possible. No-template and no-reverse- transcriptase control assays produced negligible signals, suggesting that primer dimer formation and genomic DNA contamination effects were small.

The mRNA levels in each subgroup of samples were characterized by their median values. Results were presented as fold differences between cell types.

**Statistical analysis:** Differences in dendritic spines or shafts were examined using the Mann-Whitney U nonparametric test of significance in most cases. In cases where comparisons were made between the same spines/shafts before and after experimental manipulation statistical significance was tested with the Wilcoxin signed rank test (a non-parametric test). Differences were considered statistically significant if p<0.05.
Results

CICR contributes to bAP induced Ca\(^{2+}\) transients

Recent work has pointed to the involvement of ryanodine receptors (RyRs) and CICR in corticostriatal LTD induction (Lerner and Kreitzer, 2012), but whether or not APs engage dendritically localized RYRs (Martone et al., 1997; Verkhratsky, 2002), where LTD induction occurs, is unknown. To answer this question, SPNs were studied in brain slices using patch clamp techniques and 2PLSM Ca\(^{2+}\) imaging. SPNs were identified in slices from transgenic mice expressing enhanced green fluorescent protein (eGFP) under control of either the D₁ dopamine receptor promoter (for dSPNs) or the D₂ dopamine receptor promoter (for iSPNs) (Fig. 1a). Ca\(^{2+}\) transients evoked by somatically generated AP theta bursts (APs were evoked by 2 nA 2 ms current pulses, 3 pulses per burst (50 Hz), bursts delivered at 5 Hz, 5 bursts total; Figure 1) were measured in proximal (40-60 µm from soma) and distal (100-120 µm from soma) dendritic spines. The theta burst bAP train evoked reliable elevations in intraspine Ca\(^{2+}\), as measured with the high affinity Ca\(^{2+}\) dye Fluo-4, at both proximal and distal sites, although the magnitude of the Ca\(^{2+}\) transient at distal sites was consistently smaller. Because bAPs produce progressively smaller fluorescent signals as they propagate from the soma, a relatively high concentration of Fluo-4 (200 µM) was used to ensure reliable detection of Ca\(^{2+}\) in distal dendrites (Fig. 1b), with minimal affects on decay kinetics (Fig. 1c). This protocol induced dendritic Ca\(^{2+}\) signals well below dye saturation, as confirmed by enhancing bAP invasion into dendrites with millimolar concentrations of the Kv1/4 K⁺ channel antagonist 4-aminopyridine (4-AP) (Fig. 1d). The Ca\(^{2+}\) transients rose rapidly but decayed slowly (rise tau = 24.9 ± 3.2 ms; decay tau = 192.5 ± 22.7 ms; 11 spines), leading to summation of intracellular Ca\(^{2+}\) when bAP bursts were separated by 200 msec.

In the presence of a high concentration (75 µM) of ryanodine in the patch pipette to
antagonize RYRs, the theta burst train generated significantly smaller Ca\textsuperscript{2+} transients in proximal spines of both SPN populations (Fig. 1e-f, Fig. 2a). Ryanodine had no discernible effect on the somatic AP waveform, suggesting that the effect was mediated by an action at the spine (Fig. 1e-f). Ryanodine also reduced bAP-induced Ca\textsuperscript{2+} transients in distal dendritic spines of iSPNs, but not dSPNs (Fig. 1e-f, Fig. 2a). The lack of ryanodine action in distal dSPN spines was not due to the higher concentration of Ca\textsuperscript{2+} indicator used. Rather, it is likely attributable to the more rapid attenuation of bAP amplitude in dSPN dendrites (Day et al., 2008), although differential RYR subunit expression can not be ruled out (see Fig. 3 below). RYR-mediated CICR required repetitive activity, as it was not evoked in iSPN dendritic spines by a single bAP (Fig. 2b). The higher Ca\textsuperscript{2+} indicator concentration used to enhance signal detection did not interfere with the processes examined here, as the effect of ryanodine was robustly observed using a lower Fluo-4 concentration (Fig. 2c).

**Group I mGluR activation enhances bAP-induced dendritic Ca\textsuperscript{2+} transients**

In hippocampal pyramidal neurons, bAP-induced dendritic CICR is enhanced by activation of group I mGluRs (Nakamura et al., 1999; 2000). To determine whether similar mechanisms are engaged by SPNs, the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 50 µM) was rapidly applied to distal SPN dendrites with a puffer pipette, while measuring basal dendritic Ca\textsuperscript{2+} concentration. In the absence of somatic APs, DHPG had no detectable effect on intraspine Ca\textsuperscript{2+} concentration in either type of SPN, regardless of holding potential (Fig. 3a). In contrast, bath application of DHPG significantly increased both the proximal and distal iSPN intraspine Ca\textsuperscript{2+} signal evoked by a theta burst bAP train (Fig. 3b,c). In dSPNs, DHPG only significantly enhanced the bAP Ca\textsuperscript{2+} signal in distal dendrites (Fig. 3c). As with ryanodine, DHPG had no discernible effect on somatic APs (data not shown), suggesting that the effect was dendritically mediated. These studies were performed in SPNs from 3 week old mice to allow
ready imaging of dendrites. However, this reliance raises the possibility that the phenomenon is developmentally regulated, as these mice have not fully matured (Tepper et al., 1998; Uryu et al., 1999; Plotkin et al., 2005). To test this possibility, the effect of DHPG was examined in ex vivo slices from 2 month old mice. The effect at this age was the same as that seen in the younger neurons (Fig. 3d), suggesting that CICR was not just a feature of immature SPNs.

To better understand the differences between dSPNs and iSPNs in the response to DHPG, qPCR approaches were used to characterize the expression of CICR related genes. SPNs were separated using fluorescence-activated cell sorting (FACS) of cells acutely dissociated from brain slices taken from D₁ and D₂ receptor labeled BAC transgenic mice. The relative abundance of mRNAs encoding mGluR1 and mGluR5, as well as those encoding RYR2, RYR3, Cav1.2 and Cav2.3, was higher in iSPNs than dSPNs (Fig. 3e). Expression of mRNA for IP3 receptors (IP3Rs) and Homer 1 and 3 proteins was similar in the two SPN types (Fig. 3e). These differences in gene expression provide a potential explanation for the more robust CICR in iSPNs and the ability of mGluR activation to enhance this response.

As expected, dialysis with ryanodine (75 µM) blocked the enhancement of CICR by DHPG (Fig. 4a). Dialysis with the IP3R antagonist xestospongin C (5 µM) also blocked the DHPG effect (Fig. 4b). Recent work has suggested that mGluR activation of src kinase promotes RYR-dependent CICR in the induction of corticostriatal LTD (Lerner and Kreitzer, 2012). To test this hypothesis, the effect of DHPG on bAP evoked Ca²⁺ transients was examined in the presence of the src inhibitor PP-2 (10 µM in bath). Indeed, PP-2 completely blocked the DHPG-induced enhancement of bAP-evoked Ca²⁺ transients (Fig. 4c). Though group I mGluR activation enhances SPN dendritic CICR via both IP3Rs and RYRs, enhanced RYR engagement may be sufficient for LTD induced by high frequency stimulation induction protocols (Lerner and Kreitzer, 2012), as xestospongin C (IP3R antagonist) did not prevent
HFS-induced LTD in iSPNs (3 X 3 s 100 Hz trains of activity; depression = 64.5 ± 11.6% in control vs 62.9 ± 7.6% in 5 µM xestospongic C; n=4).

DHPG also slowed the decay of intraspine Ca\(^{2+}\) concentration following the bAP-evoked transient in iSPNs, but not dSPNs (Fig. 4d). Because the effects of mGluR stimulation were most robust in iSPNs, subsequent studies focused on them. The decay kinetics reflect a variety of processes, including diffusion and intracellular sequestration, but plasma membrane Ca\(^{2+}\) pumps are thought to be the principal determinants of Ca\(^{2+}\) clearance in dendritic spines (Sabatini et al., 2002). A prominently expressed Ca\(^{2+}\) pump in the striatum is the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (Canitano et al., 2002). Profiling SPNs using qPCR revealed that mRNAs for NCX1-3 were all detectable, with NCX1 being the most abundant in iSPNs (Fig. 4e). In agreement with the inferred role of NCXs, the NCX antagonist SN-6 (10 µM) slowed the decay of the bAP-evoked Ca\(^{2+}\) transient, much like the slowing produced by DHPG (Fig. 4f).

Antagonizing NCX pumps with SN-6 diminished but did not fully occlude the DHPG effect on the Ca\(^{2+}\) decay kinetics (Fig. 4g,h), suggesting that the effects of mGluR stimulation might be mediated in part by modulating NCX pumps. How mGluR signaling might be affecting NCX kinetics and Ca\(^{2+}\) clearance is unclear. NCX has been shown to be modulated by signaling through G\(_{\text{q}}\)-linked G-protein coupled receptors (GPCRs), like group I mGluRs (Annunziato et al., 2004; Katanosaka et al., 2005). However, inhibition of the obvious intermediaries (protein kinase C, phospholipase A2, or membrane phosphatidylinositol 4,5-bisphosphate – see Materials and Methods) had no effect on the DHPG-induced slowing of decay kinetics (Table 1). Another possibility is that there is a direct interaction between NCX and mGluRs (Kim et al., 2007), but this was not tested. The observation that DHPG increased dendritic spine Ca\(^{2+}\) amplitude in both iSPNs and dSPNs, but only significantly slowed decay kinetics in iSPNs, argues that group I mGluRs enhanced CICR and slowed Ca\(^{2+}\) decay kinetics through two independent, dissociable
Cav1.3 L-type Ca\textsuperscript{2+} channels are necessary for mGluR effects

In skeletal muscle, strong depolarization of the plasma membrane is capable of inducing ER Ca\textsuperscript{2+} release without Ca\textsuperscript{2+} entry from the extracellular space by bringing about a conformational change in L-type channels that are physically coupled to RYRs (Dulhunty et al., 2002). However, in SPNs, Ca\textsuperscript{2+} influx was necessary for CICR, as blocking plasma membrane Ca\textsuperscript{2+} channels by locally puffing on Cd\textsuperscript{2+} virtually eliminated the dendritic Ca\textsuperscript{2+} elevation triggered by bAP bursts (Fig. 5a,b). Moreover, in the presence of Cd\textsuperscript{2+}, DHPG had no effect on the bAP-evoked Ca\textsuperscript{2+} signal (Fig. 5b,c).

A variety of voltage-dependent Ca\textsuperscript{2+} channels contribute to bAP-evoked transients in SPN dendrites, including L-type Ca\textsuperscript{2+} channels with a Cav1.3 pore-forming subunit (Carter and Sabatini, 2004; Day et al., 2008; Plotkin et al., 2011). In cardiac muscle and cerebellar granule cells, membrane depolarization and Ca\textsuperscript{2+} entry specifically through L-type channels triggers CICR (Chavis et al., 1996; Dulhunty et al., 2002). The effect of DHPG on bAP-evoked Ca\textsuperscript{2+} transients in distal iSPN dendritic spines was examined in the presence of the L-type Ca\textsuperscript{2+} channel antagonist isradipine (5 µM). Isradipine fully blocked the effect of DHPG (Fig. 5d). To determine which subtype of L-type channels mediates this effect, mice lacking the Cacna1d gene, which codes for the Cav1.3 subunit, were examined (Platzer et al., 2000). In iSPNs from these mice, bAPs evoked an elevation in dendritic Ca\textsuperscript{2+} concentration, as expected from previous work showing the engagement of other Ca\textsuperscript{2+} channels by bAPs. However, DHPG had no effect on the dendritic Ca\textsuperscript{2+} transients in Cacna1d\textsuperscript{-/-} mice (Fig. 5e).

One of the ways in which deletion of Cacna1d might have blunted the effects of DHPG is if mGluR signaling facilitated Cav1.3 channel opening (Topolnik et al., 2009; Lerner and Kreitzer, 2012). To address this possibility, Cav1.3 channel opening was enhanced with the
dihydropyridine agonist BAYK 8644 (Adermark and Lovinger, 2007) in wild type mice and the bAP-evoked Ca\(^{2+}\) transient measured. BayK 8644 increased the amplitude of the Ca\(^{2+}\) transient. However, unlike the effect of DHPG, the response to the first burst of APs was enhanced the most, diminishing with repetition (Fig. 5f,g). These results are not consistent with the proposition that mGluRs positively modulate Cav1.3 channel opening. Rather, these results suggest that mGluRs enhance CICR, which is triggered by repetitive opening of Cav1.3 channels.

Although mGluR-mediated enhancement of CICR is dependent upon Cav1.3 Ca\(^{2+}\) channels, CICR itself was not dependent upon these channels. Antagonism of Cav1.3 L-type Ca\(^{2+}\) channels with BPN 4689 (1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-trione, also referred to as compound 8) at saturating concentrations (20 µM) (Kang et al., 2012) reduced, but did not fully eliminate the effect of ryanodine distal iSPN dendritic spines (Fig. 5h). The effects of ryanodine on CICR were significant in the presence of BPN 4689, even at depolarized membrane potentials where Cav3 channels should be largely inactivated (Fig. 5h), suggesting that Cav1.2 and/or Cav2.3 Ca\(^{2+}\) channels also were capable of promoting CICR in response to bAP bursts (Carter and Sabatini, 2004; Plotkin et al., 2011).

In the experiments described thus far, mGluRs were stimulated by bath application of DHPG. To determine whether precisely timed transient activation of mGluRs would engage the same CICR-mediated pathways, glutamate was uncaged at distal iSPN dendritic spines 10 ms after each bAP burst in a train (Fig. 6a-c). The intensity of the uncaging laser pulse was adjusted 1) to produce an uncaging EPSP (uEPSP) that was similar in amplitude to spontaneously occurring EPSPs, and 2) to produce a dendritic Ca\(^{2+}\) transient that was restricted to the targeted spine (Fig. 6a-c). Uncaging glutamate significantly increased the maximum Ca\(^{2+}\) transient evoked by bAP bursts (Fig. 6c,d). The group I mGluR antagonists MPEP (1 µM) +...
CPCCOET (50 µM) significantly attenuated the Ca\(^{2+}\) responses to paired and unpaired bAP trains, but not uncaging alone, as did the combined antagonism of RYRs and IP3Rs with ryanodine and xestospongin C (Fig. 6d). Somatic EPSP amplitudes were unaffected by antagonizing mGluRs or disrupting CICR (data not shown). Normalization revealed that post-pre pairing led to a progressive enhancement of the bAP-evoked Ca\(^{2+}\) transient, which was similar to what was observed with DHPG. This progressive enhancement was prevented by MPEP + CPCCOEt and by the combination of ryanodine and xestospongin C (Fig. 6e).

As both LTD and LTP depend upon an elevation in post-synaptic Ca\(^{2+}\) concentration (Lovinger, 2010), activation of group I mGluRs might promote both forms of plasticity. To address this question, LTP was induced by pairing the bAP theta burst with interleaved positively-timed (+5 ms between synaptic stimulation and bAP) presynaptic electrical stimulation to induce STDP LTP (Shen et al., 2008). Surprisingly, antagonizing group I mGluRs had no effect on the magnitude of LTP (Fig. 6f). Examination of postsynaptic spine Ca\(^{2+}\) transients induced by STDP LTD (post-pre) and LTP (pre-post) pairing protocols offered a clue about how this result should be interpreted. Brief post-pre pairing (1 burst, below the threshold necessary for mGluR-enhancement of CICR) induced linear cytosolic Ca\(^{2+}\) summation (calculated as the response to paired stimuli divided by the arithmetic means of pre- and post- synaptic stimuli), whereas pre-post pairing, consistent with previous reports (Carter and Sabatini, 2004), produced supralinear Ca\(^{2+}\) summation (Fig. 6g). Antagonizing NMDARs with AP-5 eliminated the supralinearity (Fig. 6g). These results suggest that Ca\(^{2+}\) influx through NMDARs, not simply an elevation in intraspine Ca\(^{2+}\), is critical to LTP induction in SPNs and that mGluRs are not necessary for this to occur.

STDP-LTD is dependent upon CICR

Both HFS-LTD and STDP-LTD are dependent upon group I mGluR stimulation and an
Recent work by Kreitzer and colleagues (Lerner and Kreitzer, 2012) has shown that HFS-LTD is dependent upon RYRs, clearly implicating CICR in the phenomenon. To determine if CICR also plays a central role in STDP-LTD, iSPNs were subjected to a post-pre timing protocol that has been shown to induce LTD (Shen et al., 2008) in the presence or absence of ryanodine (75 µM). Blocking RYR-dependent CICR significantly diminished STDP-LTD (Fig. 7a), suggesting that both HFS and STDP LTD engaged similar mechanisms.
Discussion

The data presented demonstrate four key features of activity-dependent regulation of intracellular Ca\(^{2+}\) concentration in the dendrites of SPNs. First, the opening of low-threshold, voltage-dependent Ca\(^{2+}\) channels by bAPs triggered RYR-dependent CICR in dendritic spines in both iSPNs and dSPNs. Second, activation of group I mGluRs enhanced bAP-evoked dendritic CICR; this modulation required Ca\(^{2+}\) entry through Cav1.3 L-type channels and both src kinase and IP3/RYRs. Third, mGluR activation slowed Ca\(^{2+}\) clearance from dendritic spines, broadening the activity-dependent Ca\(^{2+}\) signal. Fourth, CICR was necessary for the induction of STDP-LTD. These studies provide a mechanistic footing for the interaction between postsynaptic spiking and presynaptic glutamate release in the induction of synaptic plasticity.

bAPs evoked CICR in SPNs

A heterogeneous group of voltage-dependent Ca\(^{2+}\) channels contribute to bAP-evoked cytosolic Ca\(^{2+}\) transients in SPN dendrites (Carter and Sabatini, 2004; Day et al., 2008; Plotkin et al., 2011). However, this Ca\(^{2+}\) transient is not solely attributable to extracellular Ca\(^{2+}\) entering the cytoplasm through these channels. Antagonism of RYRs significantly diminished the dendritic Ca\(^{2+}\) transient evoked by short trains of bAPs, demonstrating that a part of it was attributable to release of Ca\(^{2+}\) from intracellular stores triggered by opening of plasma membrane Ca\(^{2+}\) channels – so-called CICR (Armisén et al., 1996; Berridge, 1998; Verkhratsky, 2002). Our finding that both iSPNs and dSPNs express mRNA for all three RYRs (RYR1-3) and previous work showing RYR protein in SPN dendritic spines (Martone et al., 1997; Verkhratsky, 2002) buttress the conclusion.

Somatic CICR in neurons is well documented (Lipscombe et al., 1988; Alford et al., 1993; Shmigol et al., 1995; Cohen et al., 1997; Jacobs and Meyer, 1997; Usachev and Thayer, 1997; Berridge, 1998; Tully and Treistman, 2004; Richter et al., 2005). However, there are only a
handful of examples of dendritic CICR (Emptage et al., 1999; Nakamura et al., 1999; 2000; Rose and Konnerth, 2001; Carter et al., 2002; Goussakov et al., 2010). In our experiments, bAPs evoked CICR in the proximal dendritic spines of both iSPNs and dSPNs; bAPs did not evoke CICR in distal dendrites of dSPNs, in contrast to iSPNs. Although this difference could be attributable in part to the more robust expression of mGluR1/5s or RYR2/3s in iSPNs, it also could be due to the relatively poor invasion of distal dendrites by bAPs in dSPNs (in comparison to iSPNs) (Day et al., 2008). This feature of dSPNs would limit bAP opening of voltage-dependent Ca\(^{2+}\) channels necessary to trigger CICR. As basal dopamine levels are minimal in superfused ex vivo brain slices (Day et al., 2008), mGluR1/5 enhancement of dendritic CICR occurred in the absence of either D1 or D2 receptor signaling. Thus, the D2 receptor dependence of LTD induction lies elsewhere, as shown by recent work implicating regulation of RGS4 (Lerner and Kreitzer, 2012).

In SPNs, RYR-dependent CICR can be triggered by Ca\(^{2+}\) entry through both Cav1 (L-type) and non-Cav1 channels. This lack of specificity is similar to that found in hippocampal pyramidal neurons (Nakamura et al., 2000) and dis-similar to that reported in other cell types where Cav1 channels play a dominant role (Chavis et al., 1996; Dulhunty et al., 2002). This promiscuous coupling should allow CICR to be engaged in both up-states, where depolarization evoked Ca\(^{2+}\) transients are dominated by Cav1 Ca\(^{2+}\) channels, and in down-states, where Cav3 Ca\(^{2+}\) channels makes a larger contribution (Carter and Sabatini, 2004; Plotkin et al., 2011).

**mGluR activation enhanced CICR**

Both pharmacological and synaptic activation of group I mGluRs led to a progressive enhancement in dendritic CICR evoked by bAPs. This enhancement was observed in both dSPNs and iSPNs, albeit more robust in iSPNs. Consistent with this observation, mRNA for group I mGluR receptors (mGluR1, mGluR5) was found in both types of SPN, in agreement with
inferences drawn from previous studies (Testa et al., 1994). In hippocampal pyramidal neurons, pharmacological activation of mGluRs also enhances bAP-evoked CICR by engaging IP3Rs (Nakamura et al., 1999; 2000). IP3Rs also appeared to contribute to the enhancement of CICR in SPNs. Both types of SPN robustly expressed IP3Rs and the IP3R antagonist xestospongin C reduced the mGluR effect on CICR. However, inhibition of src kinase completely eliminated the effects of mGluR stimulation. Because mGluR stimulation alone had no measurable effect on intracellular Ca\(^{2+}\) concentration, our interpretation of this result is that mGluR activation initiates two parallel signaling cascades: one that is anchored by src kinase which leads to enhanced RYR Ca\(^{2+}\) sensitivity (Fagni et al., 2000; Lerner and Kreitzer, 2012) and another that involves phospholipase C, which leads to an elevation in the sensitivity of IP3Rs to Ca\(^{2+}\) (Nakamura et al., 1999; 2000; Nevian and Sakmann, 2006).

The enhancement of RYR-dependent CICR by IP3R signaling creates a context for understanding discrepancies in the literature about the requirements for LTD induction in SPNs. First, our results are consistent with the recent report that RYRs are necessary for LTD induction (Lerner and Kreitzer, 2012). Using a robust induction protocol involving high frequency stimulation and postsynaptic depolarization which is likely to strongly elevate postsynaptic Ca\(^{2+}\) concentration, there was no dependence upon IP3Rs. Using a less robust STDP protocol, the added engagement of IP3R signaling appears to be necessary to reach the threshold needed for LTD induction (Fino et al., 2010).

The coordinated facilitation of CICR by mGluR signaling was most prominent in distal dendrites (>100 µm from the soma). The most likely explanation for this regional difference is a ‘ceiling’ effect. In SPN dendrites, bAPs decrementally propagate, leading to progressively weaker activation of voltage-dependent Ca\(^{2+}\) channels and smaller CICR. In the proximal dendrites, where the bAP amplitude is the greatest, the robust opening of voltage-dependent
Ca$^{2+}$ channels might effectively maximize CICR. In the distal dendrites, where bAP amplitudes are considerably smaller and the opening of voltage-dependent channels more restricted, CICR should be sub-maximal and the mGluR enhancement of CICR easier to see.

The mGluR-mediated enhancement of CICR was dependent upon Cav1.3 L-type Ca$^{2+}$ channels. The basis for this exclusivity (in contrast to CICR per se) is not entirely clear, but might depend upon scaffolding of mGluRs and Cav1.3 channels into microdomains near synapses (Olson et al., 2005; Zhang et al., 2005). In hippocampal interneurons, mGluR stimulation of protein kinase C appears to potentiate the opening of L-type Ca$^{2+}$ channels, rather than CICR per se (Topolnik et al., 2009). The most compelling evidence against this interpretation of our results is that direct enhancement of L-type channel opening with BAYK 8644 led to a very different pattern of changes in postsynaptic Ca$^{2+}$ concentration with repetitive stimulation than did mGluR stimulation. It is possible that this difference underlies the contrasting effects of mGluR stimulation in SPNs, where it induces LTD, and in hippocampal interneurons, where it induces LTP (Sung et al., 2001; Topolnik et al., 2009).

**CICR was necessary for STDP-LTD induction**

Our studies show that like HFS LTD (Lerner and Kreitzer, 2012), STDP-LTD also depended upon CICR in SPNs, establishing another mechanistic link between the two induction protocols. Previous work (Lerner and Kreitzer, 2012) also demonstrated that the mGluR effect on LTD induction was dependent upon src kinase. Again, our results are in agreement and extend them by showing that this dependence is likely to be mediated by regulation of RYRs, whose Ca$^{2+}$ sensitivity is increased by src kinase phosphorylation (Zhang et al., 2004). Our studies also provide a partial explanation for the long-standing observation that while corticostriatal LTD can be induced at glutamatergic synapses onto both SPN populations (Wang et al., 2006; Shen et al., 2008), it is more readily inducible in iSPNs (Kreitzer and Malenka,
Clearly, CICR and its enhancement by mGluRs was more robust in iSPNs than dSPNs.

The involvement of CICR in LTD induction creates a reliance upon activity in the recent past, as intracellular Ca\(^{2+}\) stores are ‘leaky integrators’ of past activity (Berridge, 1998). This dependence could be homeostatic, helping to ensure that quiescent SPNs do not undergo changes in synaptic strength that could further lower their activity below a set point.

Our studies also provide insight into the roles of timing and repetition in the induction of STDP. Previous work (Pawlak and Kerr, 2008; Shen et al., 2008; Fino et al., 2010) has shown that in the presence of GABA\(_A\) receptor antagonists repeated pairing of postsynaptic spikes with trailing synaptic stimulation (post-pre pairing) leads to LTD in SPNs, whereas reversing the order of stimulation (pre-post pairing) induces LTP in conditions permissive for second messenger signaling (e.g., perforated patch recording). Our work suggests that the timing dependence of STDP-LTP is largely a consequence of the ability of pre-post protocols to effectively engage NMDARs, leading to supralinear elevations in postsynaptic Ca\(^{2+}\) concentration. NMDARs are also necessary for HFS-LTP (Calabresi et al., 1992b; Lovinger, 2010; Paillé et al., 2010). In the absence of NMDAR engagement, the timing dependence of plasticity should be less stringent. Both pre-post and post-pre pairing should be effective means of producing CICR and STDP-LTD as long as the repetition rate of the induction protocol allows for summation of mGluR-mediated signaling and intracellular Ca\(^{2+}\). This is certainly the case in HFS protocols.

This also implies that neuromodulators that regulate SPN NMDARs could shape the timing dependence of STDP. D\(_2\) dopamine receptors, for example, are likely to promote LTD not only through intracellular signaling cascades that enhance eCB synthesis (Lovinger, 2010; Lerner and Kreitzer, 2012), but also by suppressing Ca\(^{2+}\) entry through NMDARs (Higley and Sabatini, 2010) and weakening timing dependence. M1 muscarinic receptors also shape LTD induction,
as might GABAergic signaling (Calabresi et al., 1998; 1999; Wang et al., 2006; Fino et al., 2010). These factors could contribute to discrepancies in the literature about the timing dependence of STDP-LTD (Pawlak and Kerr, 2008; Shen et al., 2008; Fino et al., 2010; Shindou et al., 2011). Unraveling these factors will require experimental models in which the timing of neuromodulatory and GABAergic input to SPNs is tightly controlled, something that is now achievable with optogenetic approaches.

Summary

Our results show that CICR is a significant component of dendritic integration in SPNs, and critical to corticostriatal STDP-LTD induction. In particular, our studies elucidate the relationship between two obligatory participants in the most common form of corticostriatal LTD: group I mGluRs and Cav1.3 L-type voltage-dependent Ca\(^{2+}\) channels (Lovinger, 2010). Activation of group I mGluRs sensitized IP3Rs and RYRs to Ca\(^{2+}\) entry through Cav1.3 channels in SPN dendrites, promoting CICR. CICR was necessary for the induction of corticostriatal LTD, explaining the requirement for co-activation of mGluRs and Cav1.3 channels. In addition, mGluR signaling slowed the extrusion of Ca\(^{2+}\), broadening the duration of the postsynaptic Ca\(^{2+}\) signaling resulting from CICR.

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**Figure Legends**

**Figure 1.** Intracellular Ca\(^{2+}\) stores contribute to bAP-induced dendritic Ca\(^{2+}\) transients in dSPNs and iSPNs.  (A) Maximum intensity projection images of an iSPN (top) and dSPN (bottom). Patch electrodes are shaded gray.  (B) Comparison of Ca\(^{2+}\) transients in distal (100-120 µm from soma) iSPN dendritic spines loaded with either 100 µM Fluo-4 (n=4 cells, 28 spines) or 200 µM Fluo-4 (n=5 cells, 11 spines), in response to a theta burst pattern of bAPs.  Average Ca\(^{2+}\) transients are fit with summating biexponentials.  The larger amplitude transients afforded by higher Fluo-4 concentration allowed for reliable quantification of pharmacologically decreased events.  (C) Ca\(^{2+}\) transient decay kinetics (measured from the peak of the 5\(^{th}\) burst in a theta burst train of bAPs) were not significantly altered by 200 (n=5 cells, 11 spines) vs 100 µM (n=4 cells, 28 spines) Fluo-4.  (D)Average dendritic spine Ca\(^{2+}\) transients from distal (100-120 µm from soma) iSPNs in response to a theta burst pattern of bAPs in the presence (red; N=8 spines, 3 cells) or absence (black, N=9 spines, 3 cells) of 1 mM 4-AP.  (E) Average dendritic spine Ca\(^{2+}\) transients from proximal (40-60 µm from soma) and distal (100-120 µm from soma) dendritic spines loaded with (red) or without (black) 75 µM ryanodine.  Example images of spines from which line scans were taken are shown (line scan is represented by the yellow line).  Transients are in response to somatically generated theta bursts of bAPs.  Shaded areas indicate S.E.M.s, solid lines are summating biexponential fits of the average G/R transients.  Dashed horizontal lines indicate G/R = 0.  Ca\(^{2+}\) transient areas corresponding to each burst are shown below (temporal boundaries indicated by dashed lines; dots are averages, shaded areas are S.E.M.s).  Ca\(^{2+}\) transient areas are fit with a single exponential.  Average somatic voltage traces and current pulses used for AP initiation are shown in register below in the presence (red) and absence (black) of ryanodine.  Ca\(^{2+}\) transient magnitudes were decreased by ryanodine, indicating a significant contribution of intracellular Ca\(^{2+}\) release to
bAP-evoked dendritic Ca\textsuperscript{2+} transients (proximal: control n=5 cells, 26 spines, ryanodine n=5 cells, 20 spines; distal: control n=5 cells, 13 spines, ryanodine n=5 cells, 13 spines). (F) As in (E), for dSPNs (proximal: control n=4 cells, 24 spines, ryanodine n=4 cells, 32 spines; distal: control n=4 cells, 27 spines, ryanodine n=4 cells, 36 spines). Ca\textsuperscript{2+} transient magnitudes were decreased by ryanodine in distal dSPN spines. * p<0.05, Mann-Whitney nonparametric test.

**Figure 2.** bAP-mediated intracellular Ca\textsuperscript{2+} release requires repetitive activity. (A) Box plots showing percent block of the entire calcium transient (sum of bursts 1-5) by ryanodine in proximal and distal iSPNs and dSPNs (from Fig. 1e,f). No effect of ryanodine was seen in distal dSPN spines using a lower concentration (100 µM) of Fluo-4 (control n=3 cells, 32 spines, ryanodine n=3 cells, 27 spines), though the variance was large due to the small Ca\textsuperscript{2+} signal. (B) Average Ca\textsuperscript{2+} transients in distal iSPN spines induced by a single bAP in the presence (red; n=5 cells, 30 spines,) and absence (black; n=3 cells, 19 spines,) of ryanodine. (C) Comparison of Ca\textsuperscript{2+} transients in proximal (40-60 µm from soma) iSPN dendritic spines loaded with 100 µM Fluo-4 with (red, n=4 cells, 32 spines) and without (n=4 cells, 26 spines) 75 µM ryanodine in the electrode. Areas of Ca\textsuperscript{2+} transients corresponding to each burst are shown below. Ca\textsuperscript{2+} transient areas are fit with a single exponential. Ryanodine significantly reduces bAP-evoked Ca\textsuperscript{2+} transients in lower (100 µM Fluo-4) Ca\textsuperscript{2+} dye concentration conditions. * p<0.05, Mann-Whitney nonparametric test.

**Figure 3.** Activation of group I mGluRs enhances bAP-evoked Ca\textsuperscript{2+} transients in iSPN and dSPN dendritic spines. (A) Box plots showing basal intracellular Ca\textsuperscript{2+} concentrations in distal (100-120 µm from soma) spines (sp) and shafts (sh) of iSPNs and dSPNs, before and after bath application of DHPG (50 µM; n=5 cells, 8-10 spines, 7-9 shafts). DHPG itself had no
significant effect on dendritic basal $\text{Ca}^{2+}$.  (B) Average theta burst bAP-evoked $\text{Ca}^{2+}$ transients in iSPN dendritic spines before and after bath application of DHPG (50 µM).  Shaded areas indicate S.E.M.s, solid lines are summating biexponential fits of the average G/R transients.  Dashed horizontal lines indicate G/R = 0.  $\text{Ca}^{2+}$ transient areas corresponding to each burst are shown below (temporal boundaries indicated by dashed lines; dots are averages, shaded areas are S.E.M.s).  $\text{Ca}^{2+}$ transient areas are fit with a single exponential.  DHPG significantly increased $\text{Ca}^{2+}$ transients in iSPN spines (proximal: n=5 cells, 10 spines; distal: n=5 cells, 11 spines).  (C) As in (B), for dSPNs (proximal: n=4 cells, 6 spines; distal: n=5 cells, 9 spines).  DHPG significantly increased $\text{Ca}^{2+}$ transients in distal dSPN spines.  (D) Average theta burst bAP-evoked $\text{Ca}^{2+}$ transient areas in distal dendritic spines of 2 month old iSPNs (n=4 cells, 24 spines).  DHPG significantly increased $\text{Ca}^{2+}$ transients in mature iSPN spines.  * p<0.05, Wilcoxin Signed Rank Test.  (E) Relative mRNA expression in FACS pooled dSPNs and iSPNs (n=4-17), showing cell-specific differential regulation of membrane, scaffold and ER associated genes.  # p<0.05, Two-tailed t-test.

**Figure 4.** DHPG enhances bAP-evoked dendritic $\text{Ca}^{2+}$ transients via CICR and non-CICR mechanisms.  (A-C) Average theta burst bAP-evoked $\text{Ca}^{2+}$ transients in distal iSPN dendritic spines in the presence or absence of DHPG (50 µM).  Traces are from iSPNs (A) loaded with the RYR antagonist ryanodine (75 µM, n=6 cells, 20 spines), (B) loaded with the IP3R receptor antagonist xestospongin C (5 µM, n=5 cells, 14 spines) or (C) incubated in PP-2 (10 µM, bath, n=5 cells, 25 spines).  Example images of spines from which line scans were taken are shown.  Shaded areas are S.E.M.s, and solid lines are fits of the average G/R $\text{Ca}^{2+}$ transients.  $\text{Ca}^{2+}$ transient areas corresponding to each burst are shown in register below.  $\text{Ca}^{2+}$ transient areas are fit with a single exponential.  Ryanodine, xestospongin C and PP-2 all prevented the
DHPG-induced enhancement of Ca$^{2+}$ transients in dendritic spines.  (D) Normalized Ca$^{2+}$ transient amplitudes corresponding to the decaying portion (starting from the peak of the last burst) of the transient.  DHPG significantly slowed the decay in iSPNs but not dSPNs in the presence of ryanodine, xestospongic C and PP-2.  Graphs produced from same data shown in (A-C) and Fig. 3b,c.  The effects of DHPG on decay kinetics is preserved with lower Ca$^{2+}$ indicator concentration  (n=4 cells, 26 spines).   (E) Relative mRNA expression of NCX1-3 in FACS pooled dSPNs and iSPNs (n=4-8).  (F) Averaged fit Ca$^{2+}$ transients in distal iSPN spines in response to the NCX pump antagonist SN-6 (n=5 cells, 11 spines).  Fits are normalized to the peak of the first burst.  Normalized Ca$^{2+}$ transient amplitudes corresponding to the decaying portion of the transient are shown below.  SN-6 significantly slowed the decay kinetics.  (G) Normalized Ca$^{2+}$ transient amplitudes corresponding to the decaying portion of the transient before (black) and after (red) incubation in DHPG in distal dendritic spines of iSPNs in the presence of SN-6 (N=22 spines, 4 cells).  (H) Box plots showing the percent DHPG-induced slowing of the Ca$^{2+}$ transient decay, in the presence of SN-6, taken from data in (G).  Blockade of NCX pumps partially occluded the DHPG-induced slowing of the Ca$^{2+}$ decay.  (I) Correlation of DHPG-induced slowing and DHPG-induced changes in Ca$^{2+}$ transients in distal spines of iSPNs and dSPNs, taken from data in (D).  Lines are linear fits of data points, dashed portion shows extrapolated fit.  * p<0.05, Wilcoxin Signed Rank Test.

**Figure 5.**  DHPG- enhancement of bAP-evoked dendritic Ca$^{2+}$ transients requires Cav1.3-mediated CICR.  (A) Maximum intensity projection image of an iSPN loaded with 50 M Alexa 568.  Inset shows a high magnification image of the region identified by a dashed box.  Puffer electrode used to locally deliver cadmium to the distal dendritic region is shaded pink.  (B) Average theta burst bAP-induced Ca$^{2+}$ transients in distal iSPN dendritic spines.
Recordings were made in the presence of a local dendritic cadmium puff in the absence of DHPG (n=5 cells, 15 spines), or a local dendritic cadmium puff (puffer contained 200 µM cadmium + 50 µM DHPG; n=5 cells, 14 spines) after bath application of DHPG (50 µM). Shaded areas are S.E.M.s, and solid lines are fits of the average ∆F/Fo Ca²⁺ transients. Puffer solution was supplemented with 25 µM Alexa 568 to visually confirm drug delivery. Ca²⁺ transient areas corresponding to each bAP burst are shown below. Ca²⁺ transient areas are fit with a single exponential. Extracellular Ca²⁺ was required for DHPG enhancement of bAP-evoked Ca²⁺ transients. (C) The magnitude of the bAP-evoked Ca²⁺ transient component due to bath application of DHPG in the presence of cadmium was calculated by subtraction. DHPG produced no additional Ca²⁺ component in the presence of cadmium. For reference, the magnitude of the Ca²⁺ transient due to DHPG in the absence of cadmium is shown in black (calculated from data presented in Fig. 1). (D) Average theta burst bAP-evoked Ca²⁺ transients in distal iSPN dendritic spines in the presence of isradipine (5 µM), plus or minus 50 µM DHPG (n=3 cells, 15 spines). L-type VGCCs were necessary for the DHPG-induced enhancement of bAP-evoked Ca²⁺ transients. (E) Average theta burst bAP-evoked Ca²⁺ transients in distal iSPN dendritic spines in the presence or absence of 50 µM DHPG in Cav1.3 null mice (Cav1.3 KO; n=5 cells, 11 spines). Cav1.3 L-type VGCCs were necessary for the DHPG-induced enhancement of bAP-evoked Ca²⁺ transients. (F) Average theta burst bAP-induced Ca²⁺ transients in distal iSPN dendritic spines in the presence or absence of the L-type VGCC positive modulator Bay K 8644 (5 µM, bath applied; n=5 cells, 13 spines). Recordings are from wild type D₂ BAC mice. Increasing L-type VGCC contribution produced a decrementing enhancement of theta burst bAP-evoked dendritic Ca²⁺ transients. (G) The percent change in theta burst bAP-evoked dendritic Ca²⁺ transients caused by application of DHPG in control or Cav1.3 null mice or application of Bay K in control mice. Data for the wild type (WT) DHPG
application group was calculated from Fig. 1 and plotted for comparison. While both DHPG
and Bay K enhanced bAP-evoked dendritic Ca\(^2+\) transients, the kinetics of the enhancement
were opposite. * p<0.05, Wilcoxin Signed Rank Test. (H) Total Ca\(^2+\) transient areas (sum of
bursts 1-5) in response to theta burst bAP trains evoked from resting membrane potential
(hyperpolarized) or subthreshold membrane potential (~60 mV; depolarized) in ACSF, 20 µM
BPN 4689, loaded with 75 µM ryanodine or loaded with 75 µM ryanodine plus 20 µM BPN 4689
(n=2-5 cells, 9-25 spines). In the absence of DHPG, non L-type Ca\(^2+\) channels also contribute
to bAP-mediated CICR. * p<0.05, Mann-Whitney nonparametric test.

**Figure 6.** Paired pre- and post-synaptic activity can modulate CICR in iSPNs. (A)
Example of 2PU of glutamate on a distal SPN spine, showing a Ca\(^2+\) transient restricted to the
targeted spine (middle) and the corresponding somatic uEPSP (bottom). (B) Example of the
spatial restriction of 2PU-induced glutamate spread at a distal SPN spine, as determined by the
uEPSP (bottom) (C) Top: Schematic showing the negative timing of pre synaptic glutamate
uncaging events and postsynaptically triggered bAPs in distal iSPNs. Middle: Average Ca\(^2+\)
transients in distal iSPN dendritic spines in response to uncaging events only (pre; blue), bAPs
only (post; black) or negatively timed uncaging events plus bAPs (paired; red). Shaded areas
are S.E.M.s and solid lines are fits of the average G/R Ca\(^2+\) transients. An example image of a
spine and uncaging location from which a line scan was taken is shown to the right. Bottom:
Example somatic voltage recordings in response to uncaging events, in register with Ca\(^2+\)
transients above. Arrow indicates a spontaneous depolarization. (D) Box plots showing the
integrated areas under the final 400 ms of the measured Ca\(^2+\) transients triggered by glutamate
uncaging alone (UCs), theta burst bAPs alone (bAPs) or glutamate uncaging plus theta burst
bAPs (Pair). Paired and unpaired UC and theta burst bAP recordings were made under control
conditions (n= cells, 14 spines), +2.5 µM MPEP and 125 µM CPCCOEt (bath applied; n=4 cells, 8 spines) or +75 µM ryanodine and 5 µM xestospongin C (loaded via electrode; n=4 cells, 7 spines). Both mGluRs and intracellular Ca\(^{2+}\) stores were activated by this transient stimulation protocol. (E) Areas corresponding to each bAP burst of the paired protocol are compared to the Ca\(^{2+}\) transients calculated by the arithmetic sum of the UC and bAP components (calculated). Plots are normalized to the first burst for comparison. Measured sequential bursts of paired theta burst trains are significantly higher than arithmetically predicted- this observation is prevented by MPEP+CPCCOEt or ryanodine (Ry)+xestospongin C (XC). * p<0.05, Wilcoxin Signed Rank Test. (F) iSPNs were held in current clamp mode under perforated patch conditions and a positively timed STDP protocol known to induce LTP was delivered in the presence or absence of bath applied CPCCOEt + MPEP (n=4), using intrastrialal electrical stimulation. Filled circles are means, error bars are S.E.M.s. Blockade of group I mGluRs had no significant effect on STDP-LTP induction. * p<0.05, Mann-Whitney nonparametric test. (G) Distal dendritic spine Ca\(^{2+}\) transients were measured in iSPNs in response to paired bAPs (3 bAPs, 50 Hz) and glutamate uncaging either in a post-pre configuration (black) or pre-post configuration (red) in the presence (N=15-17 spines, 5-9 cells) or absence (N=9-12 spines, 3-9 cells) of 100 µM AP-5. The linearity index was calculated by dividing the response to paired stimuli by the arithmetic sum of the individual stimuli. This brief pairing scenario induced NMDA receptor-mediated supralinear Ca\(^{2+}\) summation in the pre-post but not post-pre scenario. * p<0.05, Wilcoxin Signed Rank Test.

**Figure 7.** Corticostriatal LTD requires CICR in iSPNs. (A) iSPNs were held in current clamp mode under perforated patch conditions, and the negatively timed STDP protocol was delivered in the presence or absence of bath applied ryanodine (75 µm, pre-incubated for 1
hour; control: n=6; Ry: n=5) using local intrastriatal electrical stimulation. Blockade of RYRs significantly reduced STDP-LTD (p<0.05, Mann-Whitney nonparametric test). (B) Proposed model of corticostriatal LTD induction.  **PLD=phospholipase D, AEA = anandamide**, based on Lerner et al. (Lerner and Kreitzer, 2012).

**Table 1.**  DHPG-mediated slowing of calcium decay kinetics is not mediated by three common PLC-linked pathways.  Normalized calcium transient decays (normalized to peak Ca²⁺ amplitude of the last bAP burst) in response to 50 µM DHPG in the presence and absence of 1 µM calphostin (n = 4 cells, 22 spines), 200 µM PIP2 (n = 4 cells, 24 spines) or 20 µM ACA (n = 4 cells, 16 spines), all in the presence of 75 µM ryanodine.  Comparisons made at 300 ms.  *p<0.05, Wilcoxon Signed Rank Test.
Plotkin et al., Figure 5
Plotkin et al., Figure 6
Plotkin et al., Figure 7

**Figure A:**
- **STDP LTD**
  - Post: Pre
  - Δt = 10 ms
  - Normalized EPSP (%)
  - Time (min)

<table>
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<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
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<tbody>
<tr>
<td>Control</td>
<td>120</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
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<tr>
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<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

  p < 0.05

**Figure B:**
- PRE
- POST
- mGluR1/5
- Cav1.3
- Ca²⁺
- Ca²⁺
- mGluR1/5
- CB1
- PLD
- AEA
- NCX7
- RyR
- IP₃R
- src

- Before: -85 mV
- After: -87 mV

- 100 ms
- 2 mV

**Currents:**
- bAP

**Signs:**
- Pre
- Post
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<tr>
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<td>ACA</td>
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