Voltage drives diverse endocannabinoid signals to mediate striatal microcircuit-specific plasticity

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The dorsolateral striatum and cannabinoid type 1 receptor (CB1) signaling mediate habitual action learning, which is thought to require a balance of activity in the direct and indirect striatal output pathways. However, very little is known about how the high CB1—expressing striatal inhibitory microcircuitry might contribute to long-term plasticity capable of sculpting direct and indirect pathway output. Using optogenetic and molecular interrogation of striatal GABAergic microcircuits, we examined voltage-dependent long-term depression of inhibitory synapses (iLTD) onto mouse and rat medium spiny projection neurons (MSNs). The observed iLTD involved recruitment of different endocannabinoid types and showed both presynaptic and postsynaptic selectivity for MSN subtypes, ultimately resulting in a powerful disinhibition of direct pathway MSNs. These results suggest a new role for voltage states in gating circuit-specific forms of synaptic plasticity and illuminate possible circuit dynamics underlying action control.

Action learning involves the acquisition and improvement, or in extreme cases habitization, of particular motor repertoires. Habitual action learning requires the dorsolateral striatum (DLS) and is dependent on CB1 (refs. 1, 2). Cortical input to the striatum synapses on GABAergic MSNs, which in turn project to downstream basal ganglia sites via the direct (striatonigral) and indirect (striatopallidal) pathways. Current models of basal ganglia function propose that action reinforcement is encoded by the striatum as a bias toward direct pathway activity relative to indirect pathway output, a concept referred to as a ‘go’ signal, or, alternatively, by coordinated activity in ensembles of neurons in both pathways that alter the ‘signal-to-noise’ ratio of desired versus undesired actions. Regardless of the precise mechanism, the balance between direct and indirect pathway activity is crucial for proper action production and sequencing.

Given that CB1 has been implicated in habit learning and is strongly expressed at striatal inhibitory presynaptic terminals, we hypothesized that endocannabinoid (eCB)-mediated iLTD generates output pathway bias. However, CB1—expressing inhibitory inputs onto MSNs arise from two sources: MSN dendritic arbor—synapsing MSN recurrent collaterals, and somatic and proximal dendrite—synapsing fast-spiking interneurons (FSIs). We used slice electrophysiology and optogenetics to examine the differential contribution of these presynaptic inputs to iLTD induced at the ‘up’ (≈−60 mV) and ‘down’ (≈−80 mV) state membrane potentials achieved by MSNs in vivo and in vitro. We found that MSNs exhibited two distinct, voltage-specific forms of iLTD operating through differential eCB production and/or release mechanisms that were presynaptic input selective. These findings suggest a previously unknown eCB-mediated mechanism that allows for the differential control of inhibitory inputs to specific striatal output pathways, which could ultimately influence actions.

RESULTS
Membrane voltage-dependent iLTD
Delivery of a low-frequency electrical stimulus (LFS) protocol while voltage-clamping MSNs at −60 mV (near “up state”) elicited iLTD that was CB1—dependent and was recapitulated with application of an L-type voltage-gated calcium channel (VGCC) activator. Using an LFS protocol of 1 Hz for 80 s delivered locally (Supplementary Fig. 1a), we reproduced this iLTD in MSNs held at −60 mV (inhibitory postsynaptic current (IPSC) amplitude = 65.9 ± 4.2% of baseline, n = 6 cells, P < 0.01, t = 8, df = 10; Fig. 1a). Hypothesizing that this iLTD depends on L-type VGCC activation, we reasoned that if we shifted the holding potential to −80 mV, closer to the down state and sufficiently hyperpolarized to prevent channel activation, we would not observe the L-type VGCC-dependent iLTD. Delivery of LFS while voltage—clamping the MSN at −80 mV resulted in iLTD (IPSC amplitude = 77.9 ± 8.3% of baseline, n = 6 cells, P = 0.02, t = 2.7, df = 10; Fig. 1a). Neither form of iLTD resulted from a change in series resistance (Fig. 1b).

The iLTD induced at the −60-mV holding potential (up-state iLTD) is metabotropic glutamate receptor (mGluR) and putatively calcium dependent, and we hypothesized that iLTD induced at −80 mV (down-state iLTD) is a distinct form. Activation of mGluRs combined with calcium signaling can stimulate production of the eCB 2-arachidonoylglycerol (2-AG), and we reasoned that an inhibitor of the 2-AG synthetic enzyme DAG lipase might discriminate between the two iLTD forms. Indeed, pre-incubation with tetrahydrodipstatin (THL, 1 μM) for at least 2 h, followed by superfusion of tetrahydrodipstatin (THL, 10 μM), blocked up-state iLTD (IPSC amplitude = 99.6 ± 8.3% of baseline, n = 6 cells, P = 0.8, t = 0.2, df = 10), but not down-state iLTD (IPSC amplitude = 66.8 ± 7.1% of baseline, n = 7 cells, P = 0.001, t = 4.5, df = 12; Fig. 1c). Delivery of

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high-frequency stimulation (HFS, 100 Hz for 10 s) also induced up-state iLTD (Supplementary Fig. 1b).

Next, we examined the L-type VGCC role in up-state iLTD by applying the channel blocker nifedipine (30 μM), which failed to block up-state iLTD (IPSC amplitude = 75 ± 6.8% of baseline, n = 11 cells, P = 0.002, t = 3.7, df = 20; Fig. 1d). Notably, the proportion of cells exhibiting iLTD following LFS delivery while holding MSNs at −60 mV without nifedipine (11 of 11 cells) versus −60 mV in the presence of nifedipine (6 of 11 cells) was significantly different (Fisher’s exact test, P = 0.04). In addition, we found that iLTD induced while holding MSNs at −60 mV in the presence of nifedipine was insensitive to THL (IPSC amplitude = 70.3 ± 5.5% of baseline, n = 6 cells, P = 0.0003, t = 5.4, df = 10) and that this nifedipine-enabled, THL-insensitive iLTD was still blocked by the CB1 antagonist AM251 (5 μM; IPSC amplitude = 104.5 ± 9.8% of baseline, n = 6 cells, P = 0.7, t = 0.5, df = 10; Fig. 1d). Thus, delivery of LFS while holding MSNs at −60 mV in the presence of nifedipine unmasked the THL-insensitive, down-state form of iLTD. Indeed, we found that down-state iLTD was not blocked by nifedipine (IPSC amplitude = 70.3 ± 5.5% of baseline, n = 6 cells, P = 0.0003, t = 5.4, df = 10), but was prevented by AM251 application (IPSC amplitude = 98 ± 6.8% of baseline, n = 10 cells, P = 0.6, t = 0.5, df = 18; Fig. 1e), and similar results were found using HFS induction of iLTD (Supplementary Fig. 1c). Postsynaptic loading with the calcium chelator BAPTA (20 mM) failed to block either LFS-induced up-state or down-state iLTD (Supplementary Fig. 1d).

Consistent with up-state iLTD depending on mGluR activation, we recapitulated up-state iLTD by photolyzing MNI-1-glutamate (400 μM) at 1 Hz for 40 s using 5-ms flashes of 380-nm light while holding MSNs at −60 mV induced a depression of eIPSC amplitude (black circles) that was blocked in the presence of THL (red circles). Scale bars represent 300 pA (vertical) and 100 ms (horizontal). Inset current traces show IPSCs before (dark) and 20 min after (light) the iLTD induction protocol. All error bars represent s.e.m.

Figure 1  State-dependent endocannabinoid (eCB)-mediated forms of inhibitory long-term depression (iLTD) at striatal inhibitory synapses. (a) A LFS train (1 Hz for 80 s) delivered while holding rat MSN membrane potential at the up state (−60-mV holding potential VHOLD, black circles) or the down state (−80-mV VHOLD, red circles) induced lasting depression of IPSC amplitude. (b) Examples of LFS-induced decreases in IPSC amplitude at the up and down states (iLTD) from individual MSNs (top), and measurement of series resistance (Rs) from these neurons showing no change associated with iLTD (bottom). (c) The DAG lipase inhibitor THL (10 μM) failed to block either iLTD induced while holding MSNs at −60 mV (black circles), but not at −80 mV (red circles) during LFS. (d) The L-type VGCC antagonist nifedipine (30 μM) failed to prevent depression of IPSC amplitude following LFS delivery while holding MSNs at −60 mV (black circles), but rendered this form of plasticity THL insensitive (red circles).

Supplementary Fig. 1a). Photolysing MNI-l-glutamate (400 μM) at 1 Hz for 40 s using 5-ms flashes of 380-nm light while holding MSNs at −60 mV induced a depression of eIPSC amplitude (black circles) that was insensitive to THL (IPSC amplitude = 91.1 ± 6.4% of baseline, n = 11 cells, P = 0.2, t = 1.4, df = 10; Fig. 1f). This iLTD was sensitive to THL (IPSC amplitude = 91.1 ± 6.4% of baseline, n = 6 cells, P < 0.0001, t = 7.8, df = 10; Fig. 1f). This glutamate uncaging-induced up-state iLTD was nifedipine insensitive, but, similar to up-state iLTD induced by LFS, nifedipine application rendered this iLTD insensitive to THL (Supplementary Fig. 2a). Glutamate uncaging in NBQX and AP5 did not evoke detectable ion current, indicating that up-state iLTD does not depend on GABAA-mediated depolarization-activating L-type VGCCs during induction, which could occur with the LFS protocol in chloride-loaded MSNs. In recordings performed with a physiological internal chloride concentration18,19, LFS delivery with the −60 mV holding potential also resulted in a THL-sensitive iLTD (Supplementary Fig. 2b).

If 2-AG mediates up-state iLTD, then inhibition of 2-AG metabolism should enhance up-state, but not down-state, iLTD. Delivery of LFS at 0.5 Hz for 80 s did not induce iLTD in untreated slices at −60 mV, and we designate this as a subthreshold induction protocol (SIP; Fig. 2a). However, SIP-induced iLTD at −60 mV during bath application of JZL184 (2 μM), an inhibitor of the 2-AG–degrading enzyme...
Wild type (FAAH) (npg © 2013 Nature America, Inc. All rights reserved.) from baseline (to SIP alone −80 mV), which itself was not significantly different than at −80 mV during SIP (−80 mV, 85.8 ± 4.5% of baseline, n = 8 cells, P = 0.004, t = 3.5, df = 14, comparing iLTD at −60 and −80 mV). Notably, in control (no FAAH) cells held at −60 mV during SIP, the change in IPSC amplitude was not significantly different from that observed in FAAH knockout cells held at −80 mV during SIP with JZL184 (−60-mV control, 91.1 ± 4.1% of baseline, n = 7 cells, P = 0.4, t = 0.9, df = 12, compared with −80 mV JZL184; Fig. 2a). Likewise, the change in IPSC amplitude following SIP at −80 mV in JZL184 did not differ from that observed at −80 mV in the absence of drug (−80 mV control, 91.4 ± 4.8% of baseline, n = 8 cells; −80 mV JZL184, P = 0.4, t = 0.8, df = 14; by t test, P = 0.4, F = 0.7, df = 1 by two-way ANOVA compared to SIP alone −80 mV), which itself was not significantly different from baseline (P = 0.1, t = 1.7, df = 14; Fig. 2a.b). JZL184 application alone did not alter IPSC amplitude in response to baseline-frequency stimulation (−60 mV, 97.3 ± 11.8% of baseline, n = 5 cells, P = 0.8, t = 2.3, df = 8). Thus, down-state iLTD may be mediated by a different eCB, possibly anandamide (AEA).

Because multiple biosynthetic pathways exist for AEA, we targeted AEA degradation by postynaptically loading URB597 or JNJ1661010, inhibitors of the AEA-degrading enzyme fatty acid amide hydrolase (FAAH). In cells held at −80 mV during SIP, postynaptic loading with URB597 (50 nM) or JNJ1661010 (100 nM) resulted in a significant depression of IPSC amplitude when compared with FAAH inhibitor-loaded MSNs held at −60 mV during SIP (−60 mV URB597, 84.6 ± 5.9% of baseline, n = 6 cells; −80 mV URB597, 54.9 ± 10.3% of baseline, n = 6 cells; P = 0.03, t = 2.5, df = 10; −60 mV URB597, 100.6 ± 8.2% of baseline, n = 5 cells; −80 mV URB597, 73.1 ± 5.8% of baseline, n = 5; P = 0.046, t = 2.4, df = 8; Fig. 2b and Supplementary Fig. 2c). Notably, the SIP-induced down-state iLTD enabled by postynaptic URB597 loading was blocked by AM251 (5 μM; Supplementary Fig. 2d). Postynaptic loading with URB597 or JNJ1661010 alone had no significant effect on IPSC amplitude with stimulation at the baseline rate (−60 mV URB597, 86.8 ± 6.0% of baseline, n = 5 cells, P = 0.06, t = 2.2, df = 8; −60 mV JNJ1661010, 90.4 ± 6.4% of baseline, n = 5 cells, P = 0.2, t = 1.5, df = 8). Finally, immunoreactivity for N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), an enzyme involved in a known AEA biosynthetic pathway, was robust and pervasive in striatal neuron (Supplementary Fig. 3). Thus, several lines of evidence support a role for AEA in down-state iLTD.

We next established that these same forms of iLTD occur in mouse (Supplementary Fig. 4a). We found that delivery of a SIP of 0.2 Hz for 90 s while holding MSNs at −60 mV resulted in no change in IPSC amplitude in either wild-type (IPSC amplitude = 96.6 ± 4.5% of baseline, n = 5 cells, P = 0.3, t = 1.1, df = 8) or FAAH knockout mouse slices (IPSC amplitude = 96 ± 15.6% of baseline, n = 5 cells, P = 0.8, t = 0.3, df = 8) (Fig. 2c). However, following delivery of the same SIP (0.2 Hz for 90 s) in MSNs held at −80 mV, iLTD was observed in FAAH knockout mouse slices (IPSC amplitude = 73 ± 6.6, n = 10 cells, P = 0.003, t = 3.4, df = 18), but not in wild-type mouse slices (IPSC amplitude = 103.3 ± 14.9% of baseline, n = 7 cells, P = 0.8, t = 0.2, df = 12) (Fig. 2d). These data further implicate AEA signaling in down-state iLTD. Notably, manipulating many receptors and channels failed to block down-state iLTD induced by LFS (Supplementary Table 1), including blockade of D1-like (SCH 23390, 10 μM) or D2-like (surpilride 10 μM) dopamine receptor subtypes and knockout of the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Supplementary Fig. 4b). D1- and D2-receptor blockade also failed to inhibit up-state iLTD (SCH 23390, IPSC amplitude = 54.9 ± 9% of baseline, n = 5 cells, P = 0.001, t = 5, df = 8; surpilride, 70.1 ± 7.2% of baseline, n = 5 cells, P = 0.003, t = 4, df = 8).

Voltage-driven cellular specificity of iLTD

We observed that up-state iLTD occurred in every rat MSN (50 of 50 cells; Supplementary Fig. 5a), whereas down-state iLTD occurred in roughly half of all recorded MSNs (28 of 50 cells; Supplementary Fig. 5b). The difference in the proportion of cells exhibiting IPSC amplitude depression following LFS delivery while holding cells at −60 mV versus −80 mV was statistically significant (Fisher’s exact test, P = 0.001). We therefore suspected that down-state iLTD might be output pathway...
specific, and we recorded from striatal slices from D1 GFP BAC transgenic mice. LFS delivery while holding MSNs at −60 mV induced IPSC amplitude depression in GFP-positive cells (D1-expressing, 73.5 ± 4.0% of baseline, n = 6 cells from 4 mice, P = 0.0002, t = 4.9, df = 10) and GFP-negative cells (putative D2-expressing, indirect pathway MSNs; 66.4 ± 7.3% of baseline, n = 6 cells from 3 mice, P = 0.001, t = 6.8, df = 10) (Fig. 3a). In contrast, delivering LFS at −80 mV led to iLTD in GFP-positive MSNs (58.6 ± 7.0% of baseline, n = 6 cells from 4 mice, P = 0.0002, t = 5.9, df = 10), but not GFP-negative MSNs (90.6 ± 8.2% of baseline, n = 6 cells from 3 mice, P = 0.3, t = 1.1, df = 10) (Fig. 3b).

Thus far, up- and down-state forms of iLTD appeared to be mechanistically distinct, functioning through the differential release of

**Figure 3** Up-state iLTD is output pathway nonspecific, whereas down-state iLTD is specific to direct pathway MSNs. (a) LFS delivery while holding D1 dopamine receptor–GFP (D1-GFP)-positive (gray circles) and D1-GFP-negative MSNs (putative D2 receptor-positive MSNs, black circles) at −60 mV induced a lasting depression of IPSC amplitude in both cell populations. (b) LFS delivery during postsynaptic voltage clamping at −80 mV induced a depression of IPSC amplitude in D1-GFP MSNs (gray circles), but not D1-GFP-negative MSNs (black circles). Scale bars represent 300 pA (vertical) and 100 ms (horizontal). Inset current traces show IPSCs before (dark) and 20 min after (light) the iLTD induction protocol. All error bars represent s.e.m.

**Figure 4** Optogenetic targeting of RGS9-expressing MSNs or striatal PV-expressing FSIs. (a) Striatal slice of an Rgs9-cre; CAG-mRFP-GFP mouse showing immunohistochemical (IHC) detection of Cre-mediated GFP expression in MSNs (green) and IHC detection of PV (red) in cells of striatum and overlying cortex. In striatum, red PV-immunoreactive cells did not colabel with GFP-positive MSNs (inset, top right). (b) IHC enhancement of Cre-mediated GFP expression in FSIs (left) and IHC detection of PV (red, middle) revealed a high incidence of colabeling (merge, right) in a Pvalb-cre; CAG-mRFP-GFP striatal section. (c) Cre-mediated expression of GFP in MSNs of an Rgs9-cre; CAG-mRFP-GFP striatal section (left). Right, mCherry expression at the site of injection of AAV2-DIO-ChR2-mCherry. (d) Current injection into an mCherry-positive cell in an Rgs9-cre mouse striatal slice infected with AAV2-DIO-ChR2-mCherry revealed a maximum firing rate of ~100 Hz (top right). Millisecond-scale blue light exposure (blue bar) induced a single action potential following recording from mCherry-positive MSNs in current clamp (lower right). Following recording from mCherry-positive MSNs in voltage-clamp mode in the presence of TTX (1 µM), AP5 (50 µM), NBQX (50 µM), and GABAzine (10 µM), blue light exposure induced an inward, ChR2-mediated current (lower left). (e) Blue light exposure induced an IPSC that was blocked by TTX (top) and bicuculline (bottom). Scale bars represent 300 pA (vertical) and 100 ms (horizontal) in voltage-clamp recordings, 25 mV (vertical) and 25 ms (horizontal) in current-clamp recordings, 250 µm (a), 20 µm (inset), 50 µm (b) and 0.5 mm (c,f).
Figure 5 The MSN-MSN synapse contributes to both up- and down-state iLTD, whereas the FSI-MSN synapse solely contributes to down-state iLTD. (a) Experimental setup for the optogenetic interrogation of striatal microcircuits. A bipolar concentric stimulating electrode was situated in the AAV2-DIO-ChR2-mCherry infection zone while the recording electrode was placed just outside of this area, in an uninfected zone. Blue light was delivered to the slice to elicit ChR2-mediated activation of elements presynaptic to the recorded MSN (top). (b,c) Rgs9-cre mediated expression of mCherry and ChR2 in MSNs allowing for MSN-MSN synapse interrogation. LFS delivery via the stimulating electrode while holding MSNs at −60 mV depressed MSN-MSN oIPSC amplitude and stimulated eIPSC amplitude (b). Delivering LFS via the stimulating electrode while holding MSNs at −80 mV depressed both oIPSC (blue circles) and eIPSC (black circles) amplitudes (c). (d,e) Pvalb-cre mediated expression of mCherry and ChR2 in FSI allowing for FSI-MSN synapse interrogation. Delivery of LFS via the stimulating electrode while holding MSNs at −60 mV depressed eIPSC amplitude (black circles), but not FSI-MSN-mediated oIPSC amplitude (blue circles) (d). LFS delivery via the stimulating electrode while holding MSNs at −80 mV induced depression of both oIPSC (blue circles) and eIPSC (black circles) amplitudes (e). Scale bars represent 300 pA (vertical) and 100 ms (horizontal). Inset current traces show IPSCs before (dark) and 20 min after (light) the iLTD induction protocol. All error bars represent s.e.m.

To assess the contribution of MSN-MSN or FSI-MSN synapses to up- and down-state iLTD, we selectively interrogated MSN or FSI inputs to postsynaptic MSNs by expressing channelrhodopsin2 (ChR2) and mCherry using a Cre-sensitive viral vector (AAV2-DIO-ChR2-mCherry) in either MSNs, using the Rgs9-cre transgenic mouse, or FSI, using the parvalbumin (Pvalb)-cre transgenic mouse (Fig. 4). Crossing Rgs9-cre mice with a reporter line that expresses GFP in the presence of Cre (the CAG-mRFP-GFP mouse) revealed a GFP expression pattern consistent with MSNs (Fig. 4a): highly abundant throughout the striatum alone. Crossing the Pvalb-cre mouse with the CAG-mRFP-GFP mouse resulted in a GFP expression pattern consistent with FSI distribution: sparser than MSNs and more populous in the dorsolateral than in the dorsomedial striatum (Fig. 4b). Immunostaining tissue from Rgs9-cre; CAG-mRFP-GFP mice for GFP and parvalbumin (PV) revealed no double-labeled cells (replicated across three mice; Fig. 4a). However, co-immunostaining for GFP and PV in Pvalb-cre; CAG-mRFP-GFP mice revealed a high incidence of double labeling (replicated across four mice; Fig. 4b).

We injected AAV2-DIO-Chr2-mCherry into the striatum of adult Rgs9-cre (Fig. 4c) or Pvalb-cre (Fig. 4f) mice. Performing current-injection steps in mCherry-positive cells in Rgs9-cre striatal slices revealed an average peak firing rate of ~100 Hz (Fig. 4d,g) and a peak firing rate of 250 Hz in mCherry-positive cells in Pvalb-cre striatal slices (Fig. 4e), consistent with previous findings of FSI firing capabilities. A brief single blue light flash induced a single action potential, recorded in current clamp, in mCherry-positive neurons in Rgs9-cre (n = 5 slices) or Pvalb-cre (n = 5 slices) mouse slices (Fig. 4d,g). In voltage-clamp mode recordings from mCherry-positive cells while blocking NMDA receptors (AP5, 50 µM), AMPA receptors (NBQX, 5 µM), sodium channels (tetrodotoxin (TTX), 1 µM) and GABA_A receptors (GABA_A, 10 µM), a blue light flash induced a ChR2-mediated, inward current in both neuronal subtypes (n = 4) (Fig. 4d,g). Recording from uninfected MSNs, we observed a single IPSC induced by blue light flashes (oIPSC) (Fig. 4e.h). Light-induced IPSCs (oIPSCs) recorded from MSNs in slices taken from Rgs9-cre and Pvalb-cre mice were sensitive to blockade of sodium channels (TTX, 1 µM) and GABA_A receptors (bicuculline, 10 µM) (n = 5; Fig. 4e.h).

Examing the contribution of the MSN-MSN synapse in slices from Rgs9-cre mice, we held uninfected postsynaptic MSNs at −60 mV during LFS, which was delivered via the electrical stimulating electrode as in the previous experiments (NBQX and AP5 still present in the bath; Fig. 5a). LFS depressed electrically evoked IPSC (eIPSC) amplitude (72.2 ± 4.0% of baseline, n = 6 cells, P = 0.0001, t = 9.2, df = 10), used as a positive control, and light-induced IPSC (oIPSC) amplitude (56.2 ± 9.0% of baseline, n = 6 cells, P = 0.001, t = 5.1, df = 10) (Fig. 5b). Similarly, LFS delivered while holding MSNs at −80 mV depressed eIPSC (67.0 ± 5.1% of baseline, n = 7 cells, P < 0.0001, t = 6.7, df = 12) and oIPSC (58.2 ± 8.1% of baseline, n = 7 cells, P = 0.002, t = 4, df = 12) amplitudes (Fig. 5c). It is possible that the observed depression of MSN-MSN IPSC amplitude during down-state iLTD partially, or fully, results from induction of the 2-AG–dependent up-state iLTD that may be occurring in cells that are insufficiently clamped at −80 mV in their distal dendrites. Thus, we applied THL to isolate the contribution of down-state iLTD, which we originally hypothesized to not reach these receptors, but that they are activated during the putatively AEA-mediated down-state iLTD. These hypotheses predicted that down-state iLTD should not be occluded by up-state iLTD, whereas the FSI-MSN synapse solely contributes to down-state iLTD. In the presence of THL, delivery of LFS while holding MSNs at −80 mV still depressed both...
In _Pvalb-cre_ mouse slices, MSNs held at −60 mV during LFS (again delivered with the electrical stimulating electrode) depressed eIPSC amplitude (71.4 ± 4.3% of baseline, _n_ = 8 cells, _P_ < 0.0001, _t_ = 6.3, _df_ = 14), but did not oIPSC amplitude (101.5 ± 14.5% of baseline, _n_ = 8 cells, _P_ = 0.9, _t_ = 0.1, _df_ = 14) (Fig. 5d), indicating that depression of FSI-MSN synapses does not contribute to up-state iLTD. However, holding MSNs at −80 mV during LFS revealed depression of both eIPSC (70.7 ± 4.2% of baseline, _n_ = 8 cells, _P_ < 0.0001, _t_ = 6.3, _df_ = 14) and oIPSC (71.2 ± 5.4% of baseline, _n_ = 8 cells, _P_ = 0.0002, _t_ = 5, _df_ = 14) amplitudes (Fig. 5e), indicating that FSI-MSN synapses contribute to down-state iLTD.

Applying LFS selectively to either MSNs or FSIs using a 1-Hz, 80-s light pulse protocol failed to induce CB1-mediated iLTD in uninjected neurons held at either −60 mV or −80 mV. This further supports the heterosynaptic nature of these forms of plasticity (Supplementary Fig. 7).

To confirm the findings that depression of MSN-MSN synapses contributes to both up- and down-state iLTD, but depression of FSI-MSN synapses contributes to down-state iLTD only, we genetically ablated CB1 from MSNs or FSIs by crossing a _loxP/-_flanked CB1 transgenic mouse with _Rgs9-cre_ (Cnr1<sup>loxP/loxP</sup>; _Rgs9-cre_ or _Pvalb-cre_ mice (Cnr1<sup>loxP/loxP</sup>; _Pvalb-cre_). Using the same _AAV2-DIO-ChR2-mCherry_ infection and slice recording configuration, we found that oIPSCs were readily induced with light exposure parameters similar to those used in previous experiments, and that oIPSCs exhibited properties that did not differ between _Rgs9-cre_ and _Cnr1<sup>loxP/loxP</sup>; _Rgs9-cre_ mice, or between _Pvalb-cre_ and _Cnr1<sup>loxP/loxP</sup>; _Pvalb-cre_ mice (Supplementary Table 2).

Exchanging plasticity, we recorded from uninjected MSNs at −60 mV during LFS in _Cnr1<sup>loxP/loxP</sup>; _Rgs9-cre_ striatal slices, allowing for the assessment of CB1 null MSN-MSN synapses, and failed to observe iLTD of eIPSCs (103.4 ± 3.9% of baseline, _n_ = 6 cells, _P_ = 0.4, _t_ = 0.5, _df_ = 10) or oIPSCs (96.4 ± 6.8% of baseline, _n_ = 6 cells, _P_ = 0.6, _t_ = 0.5, _df_ = 10) (Fig. 6a). However, LFS delivery while holding MSNs at −80 mV resulted in depression of eIPSC amplitude (70.7 ± 4.3% of baseline, _n_ = 8 cells, _P_ < 0.0001, _t_ = 6.6, _df_ = 14), but not oIPSC amplitude (95.3 ± 2.9% of baseline, _n_ = 8, _P_ = 0.3, _t_ = 1.5, _df_ = 14) (Fig. 6b).

These data are consistent with the idea that MSN-MSN synapses contribute to both up- and down-state forms of iLTD and that CB1 receptors on another non-MSN presynaptic population also contribute to down-state iLTD.

We next recorded from slices taken from _Cnr1<sup>loxP/loxP</sup>; _Pvalb-cre_ mice infected with _AAV2-DIO-ChR2-mCherry_ to assess FSI-MSN synapses that lack CB1 on FSIs. LFS delivery while holding MSNs at −60 mV resulted in depression of eIPSC amplitude (65.1 ± 10.7% of baseline, _n_ = 6 cells, _P_ = 0.006, _t_ = 3.4, _df_ = 10), but not oIPSC amplitude (92.2 ± 9.3% of baseline, _n_ = 6 cells, _P_ = 0.4, _t_ = 0.8, _df_ = 10) (Fig. 6c), further suggesting that FSI-MSN synapses do not contribute to this form of plasticity. Finally, we delivered LFS while holding MSNs at −80 mV and again observed a modest decrease in eIPSC amplitude (80.9 ± 2.8% of baseline, _n_ = 9 cells, _P_ < 0.0001, _t_ = 6.4, _df_ = 16), but no change in oIPSC amplitude (98.5 ± 6.5% of baseline, _n_ = 9 cells, _P_ = 0.8, _t_ = 0.2, _df_ = 16) (Fig. 6d), suggesting that depression of FSI-MSN synapses contributes to down-state iLTD.

MSN membrane potentials oscillate between up and down states _in vivo_. Thus, we examined whether state-dependent iLTD is inducible by timing electrical stimulation with transitions to and achievement of up- or down-state plateaus reached during postsynaptic membrane potential oscillations induced by postsynaptic current
injection. Given that in vivo up states are thought to be induced by excitatory synaptic barrage onto MSNs\(^1\), NBQX and AP5 were excluded from the bath. We evoked oIPSC test pulses in slices from Rgs9-cre mice, again infected with AAV2-DIO-ChR2-mCherry, to assess the MSN-MSN synapses shown to be depressed in both up- and down-state iLTD. Timing 30 pulses of electrical stimulation at 30 Hz with the transition to and during up-state potential plateaus (−60 mV), repeated 120 times, induced iLTD (oIPSC amplitude = 65.4 ± 9% of baseline, \(n = 6\) cells, \(P = 0.003\), \(t = 3.8\), df = 10) that was blocked by THL (oIPSC amplitude = 95.5 ± 8.7% of baseline, \(n = 5\) cells, \(P = 0.6\), \(t = 0.5\), df = 8) (Fig. 7a). Timing 30 pulses of electrical stimulation at 30 Hz with transition to and during down-state potential plateaus (−80 mV) resulted in iLTD (oIPSC amplitude = 78.1 ± 8.6% of baseline, \(n = 8\) cells, \(P = 0.02\), \(t = 2.6\), df = 14) that was insensitive to THL (oIPSC amplitude, 75.4 ± 7.9% of baseline, \(n = 5\) cells, \(P = 0.01\), \(t = 3.1\), df = 8), but was blocked by AM251 (oIPSC amplitude = 97.4 ± 5.8% of baseline, \(n = 7\) cells, \(P = 0.7\), \(t = 0.4\), df = 12) (Fig. 7b). Thus, up- and down-state iLTD induction can occur with two-state membrane oscillations in MSNs, such as those observed in vivo.

**DISCUSSION**

Our findings show the existence of two distinct forms of iLTD that are induced at up and down states, implicating voltage state transitions in neural circuit sculpting (Supplementary Fig. 8). We conclude that up-state iLTD is 2-AG mediated and L-type VGCC dependent, although we found that, in the presence of nifedipine, iLTD was induced when MSNs were voltage clamped at −60 mV. We reason that nifedipine application unmasking the (putatively) AEA-mediated iLTD form normally seen only in the down state, as it was THL insensitive, CB1-dependent and occurred in approximately half of all MSNs. Nifedipine unmasking of the down-state form of iLTD was observed across various induction protocols, including LFS, HFS and glutamate uncaging, and supports the finding that postsynaptic calcium chelation failed to completely block up-state iLTD. Our findings with nifedipine indicate that down-state iLTD is not state-specific per se, as it could occur at any membrane voltage (−60 mV or −80 mV) if the L-type VGCC was not active, which would most often be a hyperpolarized, down-state potential under physiological conditions. In contrast, up-state iLTD conformed to the definition of state specific as induction required activation of a VGCC. Notably, neither iLTD form could be induced by activation of MSN-MSN or FSI-MSN synapses alone.

Given the direct pathway specificity of 2-AG–independent iLTD, it is perhaps surprising that the magnitude of depression revealed in the presence of nifedipine at −60 mV was similar to that of the 2-AG–mediated up-state iLTD (Fig. 1d). However, the magnitude of depression could be quite strong in subsets of rat MSNs and in mouse direct pathway MSNs (Fig. 3 and Supplementary Fig. 5). It is unlikely that this magnitude variability results from insufficient voltage clamp at distal dendrites, as down-state iLTD was only observed in GFP-positive MSNs from D1-GFP slices. This indicates that distal dendrites were clamped at least at potentials more hyperpolarized than that necessary for up-state iLTD induction. Moreover, both forms of iLTD had similar properties when evoked with LFS in high or low intracellular chloride conditions or when induced by glutamate uncaging under conditions that produced no detectable postsynaptic current. Thus, the balance of direct and indirect pathway MSNs sampled during experiments on rat tissue affect the magnitude of IPSC amplitude depression observed following down-state iLTD induction.

Combined with the finding that L-type VGCCs in MSNs are activated at voltages near the up state\(^26\), our data provide evidence that
voltage states allow direct pathway MSNs to control which presynaptic inhibitory microcircuit populations are selected for iLTD. The conclusion that differential eCB release from MSNs may contribute to input- and output-selective control of MSNs is supported by the loss of depolarization-induced suppression of inhibition (DSI) in striatal MSNs in DAG lipase alpha knockout mice\(^8\)\(^{27}\), the NAPE-PLD expression in striatum as evidenced here and elsewhere\(^28\), the expression of FAAH in striatum\(^29\)–\(^{34}\), and the evidence for 2-AG and AEA co-release from single neurons, including MSNs\(^35\)\(^{36}\). Based on our experiments using postsynaptic loading of cell-impermeant BAPTA, it is reasonable to conclude that 2-AG is liberated from postsynaptic MSNs during up-state iLTD, whereas the results of the URB597 and [\(\text{N}11661010\)] internal solution experiments suggest that the down-state iLTD signaling molecule (putatively AEA) is also liberated from the recorded MSN. It is possible that FAAH inhibitors diffuse to other possible sources of AEA release, such as glia or presynaptic terminals. However, as up- and down-state iLTD is governed by postsynaptic membrane voltage alone, any recruitment of extra-postsynaptic sources of AEA is likely to be achieved through a mechanism secondary to a postsynaptic voltage transition.

The MSN-MSN synapse specificity of up-state iLTD is likely explained by the subcellular expression pattern of DAG lipase, which is restricted to MSN dendritic arbors\(^2\)–\(^7\). The restriction of FSI terminals onto MSN somata and nearby dendrites\(^8\)\(^{28}\)–\(^{34}\) appears to explain why the 2-AG–dependent, up-state form of iLTD does not affect FSI-MSN synapses. However, it is known that FSI-MSN inputs can be depressed by eCB action following a DSI protocol\(^38\), and that striatal DSI (single neuron recordings) is lost in DAG lipase alpha knockout mice\(^8\). This suggests that FSI-MSN DSI is 2-AG mediated\(^27\). This would seem contradictory to our finding that the 2-AG–mediated up-state iLTD does not occur at FSI-MSN synapses. However, the strong depolarization in the DSI protocol likely contributes to a much greater activation of calcium channels than does LFS, which involves membrane voltage maintenance at the L-type VGCC activation potential range. Thus, DSI induction could promote excessive spread of 2-AG, possibly beyond the DAG lipase–localized MSN dendritic compartment.

The selection of MSN-MSN or FSI-MSN circuits for iLTD likely interacts with the different roles for these synaptic populations in modulating striatal output. A single MSN provides only 1–3 synapses to another MSN dendrite, but an estimated 475 MSNs synapse onto one MSN\(^8\). This suggests that a MSN-MSN synapse, or pattern of synapses, may provide a highly spatially resolved modulatory partner(s) of neighboring cortico-thalamostriatal synapses. Thus, when glutamatergic drive and local dendritic depolarization and activation of L-type VGCCs induce up-state iLTD of a MSN-MSN synapse, a lasting enhancement of local excitatory postsynaptic potentials may ensue. Such dendritic heterogeneity in and across MSNs may contribute to the observed desynchronized firing of MSNs that are locked in the same voltage oscillatory phase, a mechanism that is proposed to increase information storage capacity\(^39\).

Although much fewer in number than MSNs, an estimated 1–4 FSIs synapse onto a given MSN, and each FSI is calculated to form roughly 50–175 synapses onto one MSN soma and proximal dendrites, providing a powerful inhibition of MSN output\(^8\)\(^{40}\)\(^{41}\). This could allow for down-state iLTD, which depresses MSN and FSI inputs onto direct pathway MSNs, to potently generate the go signal that is thought to encode action reinforcement and habit learning, a CB1-dependent complex motor learning behavior\(^2\). Of course, this form of plasticity may also be involved in motor learning that is not associated with habits, \textit{per se}. Given that both types of iLTD are dopamine independent, these forms of plasticity might be involved in late stage motor learning or action refinement, when phasic dopamine firing diminishes\(^42\).

The particular conditions under which down state iLTD may be induced in vivo likely depend on vigilance state. During waking conditions, down-state iLTD induction could occur in inactive direct pathway MSNs dwelling at down state resting potentials. This could, in turn, increase the downstream signaling probability of these neurons. Regular and distinct MSN membrane voltage oscillations are observed during slow-wave sleep in vivo\(^12\). Thus, in light of our findings that up- and down-state iLTDS are inducible by a voltage oscillation and stimulation timing–dependent protocol, iLTD induction during such conditions may be a mechanism underlying slow-wave sleep augmentation of action consolidation\(^43\).

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

B.N.M. and D.M.L. designed the experiments. B.N.M. performed the experiments and analyzed the data. C.T. and N.T. generated Prohl-Cre and CAG-mRFP-GFP mouse lines. B.N.M. and D.M.L. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Male and female Sprague Dawley rats (Charles River) were used for characterization of up- and down-state iLTD and group housed in groups of at least four; 2–4-month-old male and female Rgs9-cre (C57B6j)45 and Pvalb-cre (C57B6j)35 mice were used for viral-mediated expression of ChR2 in MSNs and FSIs, respectively. All mice were group housed in groups of 2–4 on a 12-h light/dark cycle (lights off at 6 p.m.) with food and water available ad libitum. To selectively ablate CB1 from MSNs or FSIs, we crossed homozygous Cnr1loxP/loxP mice (C57B6j)46 with Rgs9-cre and Pvalb-cre mice, respectively. For anatomical characterization of Cre expression, Rgs9-cre and Pvalb-cre mice were crossed with mice expressing mRFP flanked by a loxp stop sequence upstream to a GFP sequence all driven under the chicken 

Brain slice preparation. Following isoflurane anesthesia of P13–20 rats, or P14–50 wild-type or transgenic mice, brains were removed and 250–300-µm-thick coronal sections through the striatum were prepared in carbogen-bubbled, ice-cold modified artificial cerebral spinal fluid (aCSF) containing 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM MgCl2, 26 mM NaHCO3, 1.2 mM NaH2PO4 and 5 mM d-glucose. Sections were equilibrated for 30–60 min at 33 °C in carbogen-bubbled aCSF containing 124 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 26 mM NaHCO3, 1.2 mM NaH2PO4 and 10 mM d-glucose. Sections were subsequently incubated in the same solution at 24 °C until hemisections were transferred to the recording chamber. Hemisections were perfused with a aCSF containing 5 mM NBQX and 50 µM AP5 in all experiments with the exception of the voltage oscillation and stimulation-timing experiments.

Whole-cell voltage and current-clamp recordings. All whole-cell recordings were performed between 29° and 31 °C. To record IPSCs in voltage-clamp mode, recording pipettes of 2–4 MΩ resistance were filled with a CsCl-based internal solution of 300–310 mM CsCl (pH 7.3) containing 150 mM CsCl, 10 mM HEPES, 2 mM MgCl2, 0.3 mM Na-GTP, 5 mM QX-314, 3 mM Mg-ATP and 0.2 mM BAPTA. When indicated, we also used a low-chloride CsMeSO3-based internal solution of 300–310 mM CsCl (pH 7.3) containing 114 mM CsMeSO3, 5 mM NaCl, 1 mM TEA-Cl, 10 mM HEPES, 5 mM QX-314, 1.1 mM Mg-EGTA, 0.3 mM Na-GTP and 4 mM Mg-ATP. To record light-induced action potentials in current-clamp mode, recording pipettes were filled with a potassium-based solution of 300 mM KCl (pH 7.3) containing 126 mM potassium glutonate, 4 mM MgCl2, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM Mg-ATP-Na and 10 mM phosphocreatine. Recordings were filtered at 2 kHz and digitized at 10 kHz. Clampex 10.3 software controlling an isolation unit connected to a concentric bipolar stimulating electrode placed in the dorsolateral striatum was used to deliver pulses of 10–100-µA amplitude and 40–60 µs in duration. Experiments were discarded if series resistance varied by more than 15% or increased over 25 MΩ as previously described49. Up-state iLTD was also induced by photolysis of MN1-t-glutamate (400 µM) using 5-ms pulses of 380-nm light at a frequency of 1 Hz for 40 s while holding MSNs at a −60-mV holding potential throughout the entire experiment. 380-nm light was delivered through the epifluorescence light path using a mercury bulb lamp, a Chroma Technologies filter cube (380 ± 10 nm), a 40× 0.80 NA immersion objective and a Uniblitz shutter system (Vincent Associates). In other experiments, a 1-Hz, 80-s light pulse (5 ms) induction protocol was delivered instead of using the electrical stimulating electrode. In these experiments, baseline and post induction test pulses were optogenetically induced at a frequency of 0.05 Hz. A stimulation and voltage timing–dependent protocol was also used to induce up- or down-state iLTD. Up-state iLTD induction involved postsynaptic current injection of a modified cosine wave function to induce the membrane voltage to oscillate between −60 mV and −80 mV. The modification of the cosine function was a sustained plateau lasting 0.5 s at −60 mV. The period of this modified cosine wave was 1.5 s. Epochs of 30 electrical stimulation pulses at 30 Hz were delivered for (1 s) starting at the transition to −60 mV and terminating at the end of the −60-mV plateau. This process was repeated 120 times. For down-state iLTD, the postsynaptic current injection protocol was inverted (wave period of 1.5 s), resulting in a 0.5-s plateau at −80 mV. Epochs of 30 pulses at 30-Hz electrical stimulation were delivered starting at the beginning of the −80-mV plateaus and ending at the termination of the transition to the −60-mV peaks, repeated 120 times. Clampex 10.3 software was used to program all protocols.

Optogenetics. Rgs9-cre or Pvalb-cre mice were anesthetized with isoflurane and stereotaxically injected with 300 nl per striatal side with an AAV vector containing a loxp-flanked, inverted open reading frame for ChR2 and mCherry driven by an EF-1α promoter (Uтенп Vector Core). Striatal coordinates relative to bregma were anterior-posterior: +0.6 mm; medial-lateral: ± 2.3 mm; dorsal-ventral: −3.2 mm. A survival period of at least 14 d was allowed before brains were dissected for electrophysiological recordings. A CrystalLaser CL series 473-nm blue laser (<500 mW) was coupled to a fiber optic cable mounted on a micromanipulator with the tip placed within 1 mm of the slice or blue light was again delivered through the epifluorescence light path using a mercury bulb lamp, a Chroma Technologies filter cube (470 ± 20 nm), a 40× 0.80 NA immersion objective and a Uniblitz shutter system to activate ChR2. Regardless of light application method, the slice was always field illuminated. The exposure time of blue light used to generate IPSCs ranged from 500 µs to 5 ms. The order of test pulse eIPSCs and oIPSCs was alternated with each recorded neuron and iLTD was always induced electrically using the stimulating electrode. The eIPSC amplitude was always set higher than the oIPSC amplitude to better insure that electrical stimulation was recruiting the same presynaptic terminals that were activated by blue light. Test pulses were delivered at a frequency of 0.05 Hz with a 500-µs inter-pulse interval. To assure that recordings were not performed in MSNs expressing ChR2 (particularly for Rgs9-cre mice), we placed the stimulating electrode in the mCherry-positive expression zone in the striatum and situated the recording electrode outside of this area (Fig. 5a). To further reduce the possibility of recording from ChR2-expressing MSNs, biccuculline was superfused onto the slice after the experiment was finished. If the observed current was not completely blocked (indicating presence of ChR2-mediated current), the data were discarded.

Immunohistochemistry. Mice were perfused with 24 °C phosphate-buffered saline (pH 7.3) followed by ice-cold 4% paraformaldehyde (wt/vol) in phosphate-buffered saline. Brains were removed and placed in ice-cold paraformaldehyde overnight. Coronal sections through the striatum were cut at a thickness of 45 µm on a vibratome. CAG-mRFP-GFP mice were used as reporter mice to demonstrate the distribution of Cre expression in either the Rgs9-cre or Pvalb-cre lines. For double labeling using these reporter mice, heat denaturation (90 °C) in phosphate-buffered saline (pH 6.2) for 5 min was used to fully denature the constitutive mRFP signal. Rabbit antibody to PV (1:3,000, SWANT, catalog number PV 25), rabbit antibody to NAPE-PLD (1:100, Sigma, catalog number SAR2103842) and chicken antibody to GFP (1:3,000, Abcam, catalog number ab13970) primary antibodies were used, followed by cyanine (Cy3)-conjugated donkey antibody to rabbit IgG (1:1,000, Jackson Immunoresearch, catalog number 711-165-152) or Alexa Fluor 488–conjugated donkey antibody to chicken IgG (1:1,000, Jackson Immunoresearch, catalog number 703-545-155) secondary antibodies according to protocols previously used48. © 2013 Nature America, Inc. All rights reserved.
fluorescence microscope equipped with 5× 0.12 NA, 10× 0.3 NA and 20× 0.8 NA objectives was used to image immunostained tissue. An Olympus MVX10 fluorescence dissection microscope equipped with a 0.63× 0.15 NA lens was used for assessing viral infection in adjacent slices.

**Statistics.** Mean amplitudes of eIPSCs and oIPSCs were measured using cursors in Clampfit 10.3 and the data were analyzed in GraphPad Prism (GraphPad Software). eIPSC and oIPSC amplitudes were averaged per minute and expressed as a percentage of average baseline amplitude. All data sets met normal distribution criteria and statistical tests were used accordingly. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications.[14] With the exception of genotype, animals were selected at random for all experiments. In all experiments, with the exception of the D1-GFP MSN versus D1-GFP–negative MSN experiments, MSNs were randomly sampled. For electrophysiology experiments, n = cells, the ratio of cells per slice = 1, and the ratio of cells per animal ≤ 2. For all experiments, IPSC amplitudes at t = 25–30 min were used for comparison against baseline (t = 0–5 min), unless otherwise indicated, and variances between groups were not statistically different. The experimenter was not blinded to outcome. All replicates were biological. For all IPSC amplitude data set comparisons, a two-tailed unpaired Student’s t test was performed to analyze data sets. When indicated, a two-way ANOVA was used for multiple IPSC data set comparisons or a two-tailed Fisher’s exact test was used for assessing the likelihood of observing LTD (>15% of baseline IPSC amplitude depression) or no LTD (<15% of baseline IPSC amplitude depression).

**Drugs.** QX-314 was purchased from Sigma-Aldrich and URB597 was purchased from Enzo Life Sciences. Cell-impermeant BAPTA tetracesium salt (m.w. 1004.03) was purchased from Invitrogen. All other pharmacological agents were purchased from Tocris Bioscience.

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