Burattini C, Battistini G, Tamagnini F, Aicardi G. Low-frequency stimulation evokes serotonin release in the nucleus accumbens and induces long-term depression via production of endocannabinoid. J Neurophysiol 111: 1104–1105, 2014. First published December 11, 2013; doi:10.1152/jn.00498.2013.—The nucleus accumbens (NAc), a major component of the mesolimbic system, is involved in the mediation of reinforcing and addictive properties of many dependence-producing drugs. Glutamatergic synapses within the NAc can express plasticity, including a form of endocannabinoid (eCB)-long-term depression (LTD). Recent evidence demonstrates cross talk between eCB signaling pathways and those of other receptor systems, including serotonin (5-HT); the extensive colocalization of CB1 and 5-HT receptors within the NAc suggests the potential for interplay between them. In the present study, we found that 20-min low-frequency (4 Hz) stimulation (LFS-4Hz) of glutamatergic afferences in rat brain slices induces a novel form of eCB-LTD in the NAc core, which requires 5-HT2 and CB1 receptor activation and L-type voltage-gated Ca2+ channel opening. Moreover, we found that exogenous 5-HT application (5 µM, 20 min) induces an analogous long-term (5-HT-LTD) at the same synapses, requiring the activation of the same receptors and the opening of the same Ca2+ channels; LFS-4Hz-LTD and 5-HT-LTD were mutually occlusive. Present results suggest that LFS-4Hz induces the release of 5-HT, which acts at 5-HT2 postsynaptic receptors, increasing Ca2+ influx through L-type voltage-gated channels and 2-arachidonoylglycerol production and release; the eCB travels retrogradely and binds to presynaptic CB1 receptors, causing a long-lasting decrease of glutamate release, resulting in LTD. These observations might be helpful to understand the neurophysiological mechanisms underlying drug addiction, major depression, and other psychiatric disorders characterized by dysfunction of 5-HT neurotransmission in the NAc.

long-term depression; serotonin; endocannabinoids; nucleus accumbens

THE NUCLEUS ACCUMBENS (NAc), the ventral extension of the striatal complex, is a chief component of the mesolimbic dopamine (DA) system, receiving DAergic inputs from the ventral tegmental area (Le Moal and Simon 1991) and glutamatergic afferents from cortical and sub cortical limbic structures, such as the prefrontal cortex (PFC) (Christie et al. 1985), the amygdala, and the hippocampus (Brog et al. 1993; Pennartz et al. 1994), catecholaminergic afferents from the locus coeruleus, and a dense serotoninergic innervation from dorsal raphe nucleus neurons (Van Bockstaele and Pickel 1993). Behavioral, anatomic, and physiological studies have shown that the NAc is critically involved in reward and in the translation of motivation to action (Koob and Bloom 1988; Marty and Spigelman 2012; Mogenson et al. 1980), as well as in other functions, including feeding and pain, and in pathologies such as depression, schizophrenia, and attention-deficit and motor disorders (Altier and Stewart 1999; Belujon et al. 2012; Ohno et al. 2012; Salamone et al. 2005; Shirayama and Chaki 2006; Wickelgren 1997).

As observed in other brain regions, glutamatergic synapses within the NAc can express plasticity: both long-term potentiation (LTP) and long-term depression (LTD) can be reliably elicited at these synapses (Ji and Martin 2012; Kombian and Malenka 1994; Pennartz et al. 1993). Recently, more attention has been focused on LTD in the NAc, due to experimental evidence of its possible role as a cellular correlate of complex behaviors, such as sensitization and addiction (see Marty and Spigelman 2012). Distinct forms of LTD at glutamatergic synapses have been observed, including endocannabinoid (eCB) receptor 1 (CB1)-mediated LTD induced by different stimulation protocols: 13 Hz/10 min (Robbe et al. 2002b), 5 Hz/10 min (Hoffman et al. 2003), or 10 Hz/5 min (Grueter et al. 2010). Anatomic and electrophysiological studies have shown that CB1 receptors are located presynaptically on glutamatergic neurons arising in the PFC and projecting to the NAc, and that they can participate in the regulation of transmitter release (Robbe et al. 2001). Induction of eCB-LTD by 13-Hz/10-min or 10-Hz/5-min stimulation requires the activation of postsynaptic metabotropic glutamate receptor (mGluR) 5, which triggers the release of Ca2+ from the intracellular stores, finally resulting in the release of eCBs; eCBs are known to mainly act at presynaptic CB1 receptors, inhibiting the release of glutamate (Grueter et al. 2010; Robbe et al. 2002b). Data obtained with the 10-Hz/5-min protocol suggest that the eCB mainly involved in this process is anandamide (AEA), which also triggers the endocytosis of postsynaptic AMPA receptors via the activation of transient receptor potential vanilloid 1 (TRPV1) channels (Grueter et al. 2010).

In recent years, compelling evidence has demonstrated cross talk between CB signaling pathways and those of other receptor systems, such as the DA D2 (Blume et al. 2013), δ-opioid and serotonin (5-HT) 2 receptors (Best and Regehr 2008). The extensive colocalization of CB1 and 5-HT receptors within the NAc suggests the potential for interplay between the signaling cascades activated by these two receptors, which belong to the 7-transmembrane G protein-coupled receptors superfamily (Barnes and Sharp 1999; Pertwee 1997). Interestingly, 5-HT2 receptor activation strongly suppresses excitatory postsynaptic currents in the inferior olive by releasing eCBs that suppress glutamate release through presynaptic CB1 receptor activation (Best and Regehr 2008).
In the present study, performed in a rat slice preparation containing the NAc, we found that prolonged (20 min) low-frequency (4 Hz) stimulation (LFS-4Hz) of glutamatergic affereces induces a novel form of eCB-LTD in the NAc core, which requires 5-HT₃ and CB1 receptor activation and L-type voltage-gated Ca²⁺ channel (VGCC) opening.

MATERIALS AND METHODS

Animals. Young (21- to 38-day-old) male Sprague-Dawley CD IGS rats (Charles River, Calco, Italy) were used in this study. Animals were treated in accordance with the European Community guidelines on animal care, and the experimental protocols were approved by the Ethical Committee of the University of Bologna. Rats were individually housed under controlled conditions (temperature: 24 ± 1°C; humidity: 50 ± 5%), maintained on a 12:12-h light-dark cycle, and fed ad libitum.

Slice preparation. The experiments were carried out in parasaggital brain slices including the NAc. The slices were prepared as previously described by Li and Kauer (2004). Briefly, rats were deeply anaesthetized using halothane (Sigma-Aldrich, Milan, Italy) and quickly decapitated. Their brains were rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 1.3 MgSO₄, 2.5 CaCl₂, 10 glucose, and 1 kynurenic acid, saturated with 95% O₂-5% CO₂ (295 mOsm/l, pH 7.4). Parasaggital midbrain slices (400 μm thick) were obtained from the blocked brain with the use of a vibrating microtome and then transferred to a tissue bath containing aCSF without kynurenic acid at room temperature, saturated with 95% O₂-5% CO₂, and perfused at the rate of 2 ml/min with aCSF without kynurenic acid.

Electrophysiological recordings. After recovering for at least 60 min, a single slice was transferred to a submersion recording chamber and perfused at the rate of 2 ml/min with aCSF without kynurenic acid maintained at room temperature, saturated with 95% O₂-5% CO₂, and added with 50 μM picrotoxin (Sigma-Aldrich) to block GABA_A receptors. Recordings started 30 min after the slice was placed into the chamber. Field excitatory postsynaptic potentials (fEPSPs) evoked by glutamatergic afferent pathway stimulation were recorded in the NAc core with an extracellular microelectrode (glass micropipette filled with aCSF, 2–8 MΩ) connected to a DC amplifier by an Ag-AgCl electrode. Constant-current square pulses (0.2 ms, 40–120 nA) were applied using the stimulus isolation unit to a concentric bipolar electrode (70–80 kΩ) positioned at the rostral edge of the NAc, bordering the cortex, just below the anterior commissure at −400–600 μm from the recording electrode in the rostral direction. Stimulus intensity was adjusted to induce ∼50% of the maximal synaptic response; after this adjustment, the fEPSP average size was 0.6 ± 0.06 mV. After stable baseline recording, LTD was induced by LFS-4Hz, consisting of a single train of 4,800 pulses delivered at 4 Hz in 20 min at test stimulus intensity. A weaker stimulation protocol was also used, consisting of 2,400 pulses at 4 Hz (LFS-weak) delivered in 10 min at test stimulus intensity.

Drug application. Drugs were applied by switching from the control solution to one that differed only for the presence of the added drug. The following pharmacological compounds were applied in this study: the GABA_A receptors blocker picrotoxin (50 μM), the L-type VGCC blocker nifedipine hydrochloride (10 μM), and the ionotropic glutamate receptor antagonist kynurenic acid (1 mM) were purchased from Sigma-Aldrich; the N-methyl-d-aspartate receptor (NMDAR) antagonist t-amino-5-phosphonovoric acid (t-APV), the CB1 receptor antagonists N-piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 2 μM) and 4-[[6-methoxy-2-(4-methoxyphenyl)-3-benzofuran-2-carbonyl]benzonitrile (LY-320135; 10 μM), the mGlurR1 antagonist ((S)+)-α-amino-4-carboxy-2-methylbenzenecacetic acid (LY-367385), the mGlurR5 antagonist 2-methyl-6-(phenylethyl)pyrindine (MPEP), the mGlurR2/3 antagonist 2-amino-2-(2-carboxycycloprop-1-yl)-3-(dibenzo[4-yl)propanic acid (LY-341495), 5-hydroxytryptamine hydrobromide (5-HT; 5 μM), the 5-HT₃ receptor antagonist ketanserin tartrate (10 μM), the 5-HT₇ receptor antagonist pindolol (10 μM), the TRPV1 antagonist capsazepine (10 μM), the D1 receptor antagonist SCH-23390 (25 μM), the D2 receptor antagonist sulpiride (25 μM), the diacylglycero lipase α (DGL) inhibitor tetrahydroxystatin (THL; 10 μM), the fatty acid amide hydrolase (FAAH) inhibitor JNJ-1661010 (1 μM), the selective 5-HT4 reuptake inhibitor (SSRI) citalopram hydrobromide (4 μM), the 5-HT₂ selective agonist α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT; 20 μM), and the selective monoacylglycerol lipase (MAGL) inhibitor 4-[(1,3-benzoxodioxol-5-yl)-hydromethyl]-1-piperidinacarbocyclic acid 4-nitrophenyl ester (JZL-184; 50 μM) were purchased from Tocris Cookson (Bristol, UK). Drugs were made up in dimethyl sulfoxide (DMSO) or bidistilled water stock solutions and diluted 1,000 times into the superfusing aCSF, yielding their final concentrations. In a subset of control experiments (4 slices), 0.1% DMSO was also added to the slice-perfusing aCSF; LFS-4Hz elicited normal LTD in each of these slices, and therefore data obtained were pooled with those obtained in the control slices in all following experiments and defined as “control.” With the exception of kynurecine, 5-HT, and α-methyl-5-HT, none of the drugs used affected basal synaptic transmission.

Data analysis. Sweeps of 1,000 ms comprising the artifact, the nonsynaptic components, and the fEPSP were digitally acquired using the software Axoscope 9.0 (Axon Instruments, Downington, PA) and analyzed off-line using the software Clampfit (Axon Instruments); the peak amplitude values (mV) were exported in an Excel file. Each amplitude value was normalized to the baseline (calculated as the mean of the fEPSP amplitudes recorded in the last 10 min before application of LFS-4Hz). All data are plotted as means ± SE. In each experimental condition, statistical comparison was made between normalized (%) amplitudes (means ± SE) of fEPSPs recorded during the last 5 min of baseline and in the time points of interest (e.g., mean 55–60 min after the end of LFS-4Hz); paired Student’s t-test was used for binary comparison (e.g., baseline vs. follow-up), whereas one-way repeated-measures analysis of variance (ANOVA) with Bonferroni post hoc analysis was used for statistical comparison between normalized (%) amplitude (means ± SE) of fEPSP recorded 55–60 min after the end of LFS-4Hz in control conditions and in the presence of different drug treatments. Differences were considered significant for P < 0.05.

RESULTS

We recorded fEPSPs evoked in the NAc core by the stimulation of glutamatergic affereces. We found that LFS-4Hz (20-min stimulation at 4 Hz) induces a robust LTD (n = 8; Fig. 1, A and D; Table 1); when the recording was protracted for 180 min after the end of LFS-4Hz stimulation, the mean of fEPSP amplitudes recorded between 175 and 180 min was 74.87%, a value still significantly (P < 0.05) different from baseline (n = 3; data not shown).

We first tested whether this novel form of LTD required activation of the glutamate NMDAR, as previously demonstrated in other forms of LTD in the NAc (Grueter et al. 2010; Thomas et al. 2000). Thus the competitive NMDAR antagonist D-APS (50 μM) was added to the slice-perfusing medium 20 min before and during LFS-4Hz stimulation: this treatment did not affect LTD (n = 6; Fig. 1D; Table 1). To evaluate the involvement of postsynaptic ionotropic glutamate receptors, the slices were perfused with the non-subtype-selective ionotropic glutamate receptor antagonist kynureate (1 mM) 20 min before and during LFS-4Hz stimulation. As expected, kynure-
ate completely blocked fEPSP but did not prevent LTD induction (n = 8; Fig. 1, B and D; Table 1). Control recordings indicate that the depressive effect of kynureate is fully reversed after washout when LFS-4Hz stimulation was not delivered (n = 6; Fig. 1B). Previous studies have shown that in addition to NMDAR, transient activation of mGluR2/3 (with either synaptically released glutamate or a specific exogenous agonist) triggers a presynaptic form of LTD at excitatory synapses in both the NAc (Robbe et al. 2002a) and the hippocampus (Tzounopoulos et al. 1998). Therefore, we next tested whether LFS-4Hz also would induce this form of LTD in our experimental conditions by applying the mGlu2/3 antagonist LY-341495 (0.2 μM; n = 6). Antagonism at mGluR2/3 had no effect on LTD induction (n = 6; Fig. 1, C and D; Table 1).

In addition to NMDA- and mGlu2/3-dependent LTD, NAc synapses have been shown to display eCB-LTD, which depends on the production of eCBs acting at CB1 receptors (Grueter et al. 2010; Hoffman et al. 2003; Robbe et al. 2002b). Therefore, we tested the involvement of the eCB system in LFS-4Hz in the presence of the CB1 receptor antagonists AM251 (2 μM) or LY-320135 (10 μM) (Yu et al. 2013). As shown in Fig. 2, A and C, both antagonists completely prevented LTD induction (n = 6 and 6; Table 1). The two main eCBs produced upon neuronal stimulation in the brain are AEA and 2-arachidonoylglycerol (2-AG) (Piomelli 2003). We first attempted to evaluate the involvement of 2-AG in LFS-4Hz; slices were treated with THL (10 μM), an inhibitor of the 2-AG-producing enzyme DGL. As shown in Fig. 2, A and C, preincubation of the slices (2 h) with THL completely prevented LTD induction (n = 9; Table 1). We next examined whether AEA was involved in LFS-4Hz-induced LTD. To test this possibility, we used a weaker stimulation protocol (10 min instead of 20 min at 4 Hz; LFS-weak), subthreshold to LTD induction in control conditions (n = 5; Fig. 2, B and C; Table 2). Endogenous AEA production was then increased by blocking the activity of the FAAH, the enzyme responsible for AEA degradation, by adding JNJ-1661010. As shown in Fig. 2, B and C, LFS-weak failed to induce LTD in the presence of JNJ-1661010 (1 μM) (n = 5; Table 2), suggesting that AEA is
not involved in this form of LTD. To further test the involvement of 2-AG in LFS-4Hz-induced LTD, slices were incubated (>2 h) with JZL-184 (50 μM) (Puente et al., 2011), an inhibitor of MAGL, the enzyme responsible for 2-AG degradation, and LFS-weak was applied. As shown in Fig. 2, B and C, elevation of 2-AG levels rescued LTD, confirming the involvement of 2-AG in LFS-4Hz-induced LTD (n = 6; Table 2).

A recent study suggested that LTD induced in the NAc core by 10-Hz/5-min stimulation involves TRPV1 activation (Grueter et al. 2010); therefore, we performed a series of experiments in the presence of the TRPV1 antagonist capsazepine (10 μM) and found that capsazepine did not affect LTD induced by LFS-4Hz (n = 6; Fig. 2, A and C; Table 1). Moreover, we performed a series of experiments using the 10-Hz/5-min stimulation protocol and found that, in our experimental conditions, this protocol does not induce LTD (mean fEPSP was 106.52 ± 4.67% at 55–60 min after 10-Hz/5-min LFS; n = 6; data not shown).

Because it has been shown that eCB-LTD in the NAc requires activation of mGluR5 (Robbe et al. 2002b), we also tested its involvement in the LTD obtained in our experimental conditions: application of the mGluR5 antagonist MPEP (10 μM) during LFS-4Hz did not affect LTD induction (n = 4; Fig. 1, C and D; Table 1). We further investigated the involvement of group I mGluRs (which comprise both mGluR1 and mGluR5) by applying the mGluR1 antagonist LY-367385 (0.1 mM). Figure 1, C and D, shows that LFS-4Hz still elicited LTD (n = 4; Table 1).

To further clarify the mechanisms underlying LFS-4Hz-induced LTD, we measured the effect of the L-type VGCC blocker nifedipine (10 μM) on LFS-4Hz-LTD. As depicted in Fig. 3, A and E, nifedipine completely prevented LTD induction. According to the observation that the dense afferent DA innervation in the NAc regulates the activity of the medium spiny neurons (MSN), which comprise the great majority of NAc neurons, as well as their ability to pursue plastic changes (Kreitzer and Malenka 2005; Schotanus and Chergui 2008; Thomas et al. 2001), we next examined whether DA is also involved in LFS-4Hz-induced LTD. To this aim, the DA D1 and D2 receptor antagonists SCH-23390 (25 μM) and sulpiride (25 μM) were added to the slice-perfusing medium 20 min before and during LFS-4Hz stimulation. We found that blockade of both D1 and D2 DA receptors did not prevent LTD induction (n = 6; data not shown).

Because CB1 and 5-HT2 receptors are both expressed in the NAc (Barnes and Sharp 1999; Pertwee 1997), and activation of 5-HT2 receptors has been shown to cause production and release of eCBs leading to LTD in the inferior olive (Best and Regehr 2008), we investigated the involvement of 5-HT2 receptors in LFS-4Hz-induced LTD. As shown in Fig. 3, A and E, the 5-HT2 receptor blocker ketanserin (10 μM) completely blocked LTD. Data obtained in the presence of the 5-HT1 receptor antagonist pindolol (10 μM) suggest that this receptor subtype does not contribute to LFS-4Hz-induced LTD (n = 4; Fig. 1, A and E; Table 1).

To confirm the involvement of 5-HT in this form of eCB-LTD, in the next set of experiments LFS-4Hz was replaced by bath application of 5-HT (5 μM) for 20 min. Consistent with previous observations at corticostriatal synapses (Mathur et al. 2011), 5-HT added to the slice-perfusing medium (5 μM, 20 min) produced an initial, large depression and a significant (P < 0.05) and stable LTD following washout (n = 4; Fig. 3, B and E; Table 3). To test if the effect of 5-HT-LTD was mediated by 5-HT2 receptors, slices were perfused with the specific 5-HT2 receptor agonist α-methyl 5-HT (20 μM). As shown in Fig. 3, B and E, the agonist mimicked the effect of 5-HT (n = 6) to induce LTD. A comparison of the amplitude of the LTD obtained in the presence of 5-HT vs. α-methyl 5-HT revealed that the magnitude of LTD was significantly increased by the latter treatment. To further test the involvement of 5-HT2 receptors in 5-HT-LTD, slices were perfused with ketanserin (10 μM) for at least 20 min before and during application of 5-HT (5 μM). As depicted in Fig. 3, C and E, ketanserin completely prevented the effect of 5-HT on fEPSP amplitude (n = 6; Table 3). We then tested whether the effects of 5-HT on synaptic transmission was mediated by the CB1 receptors: coapplication of 5-HT and the CB1 receptors antagonist AM251 (2 μM) completely prevented 5-HT-LTD (n = 4; Fig. 3, C and E; Table 3). We next examined the involvement of the L-type VGCC in 5-HT-LTD by applying 5-HT (5 μM) along with the specific antagonist nifedipine (10 μM). As depicted in Fig. 3, C and E, nifedipine completely prevented 5-HT-LTD (n = 4; Table 3). To test whether endogenous 5-HT in the acute slice preparation would facilitate the induction of LTD, the subthreshold stimulation protocol LFS-weak was applied in the presence of the SSRI citalopram (4 μM). Figure 3, D and E, shows that this treatment enabled LFS-weak to induce LTD (n = 6; Table 2), indicating the involvement of endogenous 5-HT in this process.

Presynaptic CB1 receptors have been shown to inhibit glutamate release at the prelimbic cortex-NAc synapses (Robbe et al. 2001); therefore, if 5-HT receptor activation and LFS-4Hz share common mechanisms to inhibit excitatory synaptic transmission, LFS-4Hz-LTD and 5-HT-LTD may be mutually exclusive. We first verified whether repetition of LFS-4Hz caused saturation of LTD. As shown in Fig. 4A, a second LFS-4Hz applied during LTD consolidation failed to induce a further depression: fEPSP amplitude was 74.02 ± 5.44% (n = 5; P < 0.05 NS).
0.05 vs. baseline) between 25 and 30 min after the end of the first LFS-4Hz stimulation, and 73.74 ± 3.78% between 25 and 30 min after the end of the second LFS-4Hz stimulation, a value similar to the one recorded after the first LFS-4Hz [n = 5; no significance (NS) vs. first LFS-4Hz-LTD]. As shown in Fig. 4B, to verify whether 5-HT-LTD also occludes LFS-4Hz-LTD, we first perfused the slices with 5-HT (5 μM) and then applied LFS-4Hz: we found that fEPSP amplitude was 78.99 ± 2.96% (n = 5; P < 0.05 vs. baseline) between 25 and 30 min after the end of 5-HT application, and 76.20 ± 1.34% between 25 and 30 min after the end of LFS-4Hz stimulation (n = 5; NS vs. 5-HT-induced LTD). Conversely, 5-HT (5 μM) had no significant effect when applied after LFS-4Hz-LTD was already induced: fEPSP amplitude was 70.52 ± 4.46% of baseline (n = 7; P < 0.05 vs. baseline) between 25 and 30 min after LFS-4Hz, and 72.98 ± 4.41% between 25 and 30 min after 5-HT application (n = 7, NS vs. LFS-4Hz-LTD; Fig. 4C).

### DISCUSSION

In recent years, LTD in the NAc has been investigated in several studies (Grueter et al. 2010; Hoffman et al. 2003; Ji and Martin 2012; Kahn et al. 2001; Kombian and Malenka 1994; Pennartz et al. 1993; Thomas et al. 2000, 2001; Robbe et al. 2002a, 2002b), but the present work appears to provide the first evidence of an eCB-dependent LTD requiring 5-HT2 and CB1 receptor activation and increase in Ca2+ entry through L-type VGCC. Thus at least two distinct forms of eCB-dependent LTD can be induced in the NAc depending on the induction protocol used, because previous studies have shown that stimulations at higher frequencies induce LTDs requiring mGluR5...
and CB1 receptor activation, a rise in postsynaptic Ca\(^{2+}\) mediated by intracellular stores (Grueter et al. 2010; Robbe et al. 2002b), and TRPV1 channel activation that triggers AMPA receptor endocytosis (Grueter et al. 2010).

eCBs are arachidonic acid-derived lipid signaling molecules with potent actions at CB receptors, namely CB1, widely expressed throughout the central nervous system (Herkenham et al. 1991), and CB2, mainly present in peripheral tissues and

Fig. 3. LFS-4Hz-induced LTD of glutamatergic synaptic transmission in the NAc core requires 5-hydroxytryptamine (or serotonin) type 2 (5-HT\(_2\)) receptor activation and L-type Ca\(^{2+}\) channel opening; 5-HT-induced LTD requires 5-HT\(_2\) and CB1 receptor activation and L-type Ca\(^{2+}\) channel opening. Graphs show normalized (%) fEPSP amplitudes (means ± SE) recorded every 2.5 min before and after LFS-4Hz or LFS-weak stimulation or 5-HT application; time courses of LFS-4Hz, drug, and 5-HT applications are indicated by horizontal bars. Representative fEPSP traces recorded before and after LFS-4Hz stimulation were taken at the numbered time points as indicated. A: LFS-4Hz did not induce LTD in the presence of the 5-HT\(_2\) receptor antagonist ketanserin (Ket; 10 \(\mu\)M; \(n = 6\)), but it still induced LTD after pretreatment with the 5-HT\(_1\) receptor antagonist pindolol (Pind; 10 \(\mu\)M; \(n = 6\)). Calibration bars: 0.3 mV, 10 ms. E: summary bar graph of normalized (%) fEPSP amplitudes (means ± SE) during 55–60 min after LTD induction in control conditions (LFS-4Hz; \(n = 8\)) or in the presence of nifedipine (10 \(\mu\)M; \(n = 7\)), pindolol (10 \(\mu\)M; \(n = 3\)), or ketanserin (10 \(\mu\)M; \(n = 4\)); during 55–60 min after the application of 5-HT (5 \(\mu\)M; \(n = 4\), 2 \(\mu\)M; \(n = 5\)), 5-HT and ketanserin (10 \(\mu\)M; \(n = 6\)), or 5-HT and nifedipine (10 \(\mu\)M; \(n = 4\)); and during 55–60 min after a subthreshold 4-Hz/10-min stimulation in control conditions (LFS-weak; \(n = 5\)) or in the presence of citalopram (4 \(\mu\)M; \(n = 6\)). *\(P < 0.05\) compared with control conditions. §\(P < 0.05\) compared with 5-HT (Bonferroni test).
Table 3.

LTD induced by 5-HT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normalized fEPSP Amplitude, %</th>
<th>Significance vs. Baseline</th>
<th>Significance vs. Control</th>
</tr>
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<tr>
<td>Control</td>
<td>80.79 ± 1.96</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Nifedipine</td>
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<td>NS</td>
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<td>Ketanserin</td>
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<td>NS</td>
</tr>
<tr>
<td>AM251</td>
<td>97.84 ± 3.09</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
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Summary of effects of bath application of serotonin (5-HT) on synaptic transmission in NAc slices in control conditions or after preapplication of drug. Normalized fEPSP amplitudes are means ± SE calculated from values recorded between 55 and 60 min after the end of LFS-4Hz. Statistical comparisons were made versus baseline (Student’s paired t-test) and control (1-way ANOVA).

Fig. 4. LFS-4Hz-LTD and 5-HT-LTD of glutamatergic synaptic transmission in the NAc core are mutually occlusive. Graphs show normalized (%) fEPSP amplitudes (means ± SE) recorded every 2.5 min before and after LFS-4Hz stimulation and/or 5-HT application; time courses of LFS-4Hz, drug, and 5-HT application are indicated by horizontal bars. A: LFS-4Hz induced LTD (n = 5; P < 0.05 vs. baseline, first LTD); a second LFS-4Hz stimulation applied during stabilization of fEPSP amplitude failed to cause additive LTD (n = 5; no significance (NS) vs. first LTD). Calibration bars: 0.3 mV, 10 ms. B: 5-HT (5 μM) induced LTD (n = 5; P < 0.05 vs. baseline); a LFS-4Hz stimulation applied during stabilization of 5-HT-LTD failed to cause additive LTD (n = 5; NS vs. 5-HT-LTD). C: LFS-4Hz induced LTD (n = 5; P < 0.05 vs. baseline); 5-HT (5 μM) applied during stabilization of LFS-4Hz-LTD failed to cause additive LTD (n = 5; NS vs. LFS-4Hz-LTD). The Bonferroni test was used for statistical comparisons.
receptor ligand 2-AG, which has no activity at TRPV1 (De Petrocellis et al. 2000). 2-AG is the most abundant eCB in the brain (Mechoulam et al. 1995; Sugiuira et al. 1995); in particular, its concentration in the mesencephalon is 100 times higher than that of AEA (Bisogno et al. 1999). In addition, 2-AG acts as a potent and full agonist at CB1 receptors, whereas AEA is only a partial agonist (Savinainen et al. 2001; Stella et al. 1997; Sugiuira et al. 1995, 1999). Biochemical studies indicate that the major enzymes involved in 2-AG biosynthesis are phospholipase C (PLC) and DGL (Piomelli 2003), although other pathways have also been suggested (Sugiuira et al. 2006). The involvement of 2-AG in LFS-4Hz-LTD was confirmed by the experiments performed in the presence of the DGL inhibitor THL, which was proven to have no direct effect on CB1 receptors (Szabo et al. 2006; Uchigashima et al. 2007); moreover, it is further supported by the evidence that LTD can be induced by a subthreshold stimulation protocol when endogenous 2-AG levels are increased by an inhibitor of MAGL, the 2-AG-hydrolyzing enzyme. This finding is consistent with previous observations in the hippocampus (Ameri and Simmet 2000; Edwards et al. 2006; Hashimotodani et al. 2007; Stella et al. 1997), cerebellum (Maejima et al. 2005; Safo and Regehr 2005), and prefrontal cortex (Melis et al. 2004), supporting the involvement of 2-AG.

In addition to eCB, present results point toward the involvement of 5-HT, acting at 5-HT2 receptors, in LFS-4Hz-LTD, as demonstrated by LFS-4Hz experiments performed in the presence of the 5-HT2 receptor blocker and by the evidence that LTD can be induced by the subthreshold stimulation protocol when the endogenous 5-HT levels are elevated by the SSRI citalopram. This evidence is further supported by the observation that LTD can also be elicited by bath application of 5-HT or a specific 5-HT2 receptor agonist.

It was recently shown that 5-HT increases the activity of GABAergic interneurons in the NAc through 5-HT2 receptors, resulting in inhibitory action on MSN (Blomeley and Bracci 2009). One might argue that the 5-HT2-dependent LTD observed in the current study could derive from increased GABAergic signaling; however, this possibility seems unlikely, because the experiments were carried out in the constant presence of the GABA(A) receptor antagonist picrotixin. Rather, present data support the view that 5-HT2 receptor activation might trigger eCB production and release, in accordance with a previous observation in the dorsal principal olive (Best and Regehr 2008).

We also investigated the possible involvement of L-type VGCC in both LFS-4Hz- and 5-HT-induced LTD, because they are particularly important for down- to up-state transition of MSN (Carter and Sabatini 2004). Moreover, in vivo studies have demonstrated that these channels play a critical role in psychostimulant-induced behavioral and neuronal plasticity in various regions of the mesolimbic DA system, including the NAc (Gnegy 2000; Licata and Pierce 2003). We found that nifedipine prevented both LFS-4Hz- and 5-HT-induced LTD, indicating that these channels play a critical role in this process, as previously observed in eCB-induced LTD in dorsal striatum MSN (Kreitzer and Malenka 2005), as well as in other forms of synaptic plasticity in various brain areas, including the hippocampus (Bolsakov and Siegelbaum 1994; Grover and Teyler 1992; Morgan and Teyler 1999), lateral amygdala (Weisskopf et al. 1999), and perirhinal cortex (Seoane et al. 2009). It has been shown that large increases in intracellular Ca2+ (10–50 μM) are needed to activate phospholipase D and AEA biosynthesis, whereas lower intracellular Ca2+ elevations can activate PLC and DAG, yielding 2-AG production (Brenowitz and Regehr 2003; Hashimotodani et al. 2005). It seems reasonable to suppose that 5-HT2 activation by 5-HT (either endogenously released in response to LFS-4Hz stimulation or exogenously applied) might stimulate 2-AG production by increasing both DGL activity and L-type VGCC permeability, resulting in intracellular Ca2+ level elevation compatible with 2-AG production and release.

Altogether, present results suggest that LFS-4Hz stimulation of glutamatergic afferences induces a novel form of eCB-LTD in the NAc core involving the release of 5-HT at 5-HT2 receptors, increasing Ca2+ influx through L-type VGCC and leading to 2-AG production and release; the endocannabinoid travels retrogradely and binds to presynaptic CB1 receptors, causing a long-lasting decrease of glutamate release, resulting in LTD. Glu, glutamate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor; NMDA, N-methyl-D-aspartate receptor; G, G protein; PI3, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PLC, phospholipase C.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.B. and G.A. conception and design of research; C.B. and G.B. performed experiments; C.B., G.B., and F.T. analyzed data; C.B. and G.A. interpreted results of experiments; C.B. and G.B. prepared figures; C.B. drafted manuscript; C.B., G.B., and F.T. edited and revised manuscript; C.B., G.B., F.T., and G.A. approved final version of manuscript.

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